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National Status and Trends Program
for Marine Environmental Quality

Sampling and Analytical Methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992

Volume II

Comprehensive Descriptions of Complementary Measurements

Silver Spring, Maryland
July, 1993

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Coastal Monitoring and Bioeffects Assessment Division
Office of Ocean Resources Conservation and Assessment
National Ocean Service

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Comprehensive Descriptions of Complementary Measurements

G. G. Lauenstein and A. Y. Cantillo
(Editors)

Silver Spring, Maryland
July, 1993

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PREFACE

The quantification of environmental contaminants and their effects by the National Oceanic and Atmospheric Administration's National Status and Trends Program began in 1984. Polycyclic aromatic hydrocarbons, butyltins, polychlorinated biphenyls, DDTs and other chlorinated pesticides, trace and major elements, and a number of measures of contaminant effects are quantified in estuarine and coastal samples. There are two major monitoring components in this program, the National Benthic Surveillance Project which is responsible for quantification of contamination in fish tissue and sediments, and developing and implementing new methods to define the biological significance of environmental contamination, and the Mussel Watch Project which monitors pollutant concentrations by quantifying contaminants in mollusk bivalves and sediments. Methods are described for sample collection, preparation, and quantification. The evolution of methods, method detection limits, and the Quality Assurance Project are also discussed.

This document is Volume II of the document entitled "National Benthic Surveillance and Mussel Watch Projects Analytical Protocols 1984-1992," and contains detailed descriptions of complementary methods used by cooperating laboratories participating in the NS&T Program for the determination of physical properties of the sampling site such as salinity and tidal horizon; ancillary parameters in sediments and tissues such as total organic carbon and percent dry weight; and histopathological examination of tissues.

G. G. Lauenstein and A. Y. Cantillo
Editors

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Office of Ocean Resources Conservation and Assessment
National Ocean Service

Procedure for Determining Tidal Horizon, 1986-1992

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ABSTRACT

This document describes the method used for estimating tidal horizon during sampling for the NOAA National Status and Trends Program Mussel Watch Project for the years 1986 through 1992.

1. INTRODUCTION

This document describes the method used for estimating tidal horizon during field sampling for the NOAA National Status and Trends Program Mussel Watch Project. Tidal horizon is the vertical height (m) of bivalves above or below mean low water. At each sampling site, tidal horizon was established during the first sampling at a site, and subsequent sample collections were conducted from the same tidal horizon. Bivalve samples were collected at the lowest possible point on the tidal horizon.

2. EQUIPMENT AND MATERIALS

Local tide table
Line level and line
1-m or longer wooden ruler or measuring tape

3. SUMMARY OF PROCEDURES

3.1. Determining mean low water

For the sampling day, the mean low water (MLW) (m) was determined from published local tide tables, and the tidal horizon was measured as close as possible to the time of low water for that day (Figure II.1).

3.2. Determining tidal horizon

3.2.1. Tidal horizon at low water

The vertical height (m) of the bivalve population above or below the existing low water level was measured by using a 1-m wooden ruler and recorded in a sampling log.

The height (m) of the tide on the sampling day with reference to the mean low water (MLW) was obtained from tide tables. The height above or below was added to the measured vertical height.

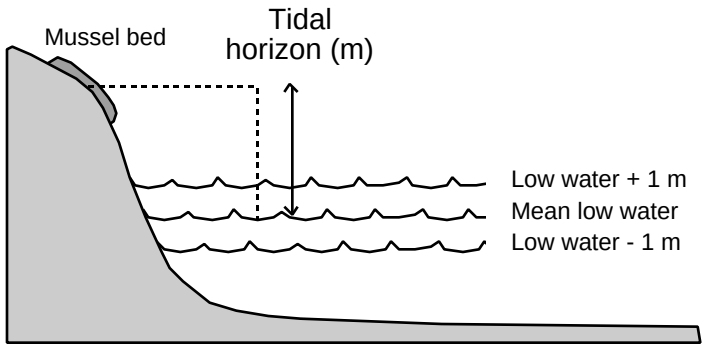

Figure II.1. Determining tidal horizon.

$$H_t = h_m + h_d$$

where H_t is the tidal horizon, h_m is the measured height (m) of bivalves above the observed low water line, and h_d is the height (m) of tide above or below MLW.

Tidal horizon can be a positive or negative number, depending on the tide level.

For example, if the low tide for the sampling day was 0.5 m below the MLW (h_d = -0.5), and bivalves were collected 2 m above the observed low tide (hm = 2 m), then the tidal horizon is calculated as shown below.

$$H_t = h_m + h_d = 2 + (-0.5) = 1.5 \text{ m}$$

Therefore, the tidal horizon for that sampling is 1.5 m.

3.2.2. Tidal horizon at other than low water.

Tidal horizon measurements not made at that day's low water must also take into consideration the height of the tide above that lower level. In that case, H_t was determined by the following equation.

$$H_t = h_e + h_m$$

$$H_t = [(h_h - h_l) \left(\frac{t - t_1}{t_2 - t_1} \right) - h_l] + h_m$$

where h_e is the estimated height (m) above MLW, h_m is the height (m) of bivalves above observed tidal height, h_h is the height (m) of high water, h_l is the height (m) of low water, t is the time (hh:hh) of measurement of vertical height of bivalves, t_1 is the time (hh:mm) of high or low tide preceding time of measurement, and t_2 is the time (hh:mm) of high or low tide following measurement.

For example, bivalves were collected at 1200 hr (t) at a depth of 0.6 m above low tide (h_m). The previous high tide was at 0845 (t_1) that day, and the next high tide was at 1450 hr (t_2). According to the local tide tables for the sampling day, the height of high water above MLW (h_h)

was 1.2 m, and the height of low water above MLW (t_1) was -0.1 m, or 0.1 m below MLW. Therefore, tidal horizon is calculated as shown below.

$$H_t = h_e + h_m = [(h_h - h_l) \left(\frac{t - t_1}{t_2 - t_1} \right) - h_l] + h_m$$

$$H_t = [(1.2 - (-0.1)) \left(\frac{1200-0845}{1450-0845} \right) - (-0.1)] + 0.60 \text{ m}$$

$$H_t = [(1.2 - (-0.1)) \left(\frac{3.75}{6.08} \right) - (-0.1)] + 0.60 \text{ m}$$

$$H_t = [(1.3) \text{ x } (0.61 + 0.1)] + 0.60 \text{ m}$$

$$H_t = 0.92 + 0.60 \text{ m} = 1.52 \text{ m}$$

Therefore, the tidal horizon for that sample collection is 1.52 m.

4. CONCLUSION

By determining tidal horizon during the first sample collection at a site, and ensuring that subsequent sample collections were made at the same tidal horizon, variability caused by differences in sampling conditions (i.e., position of tide relative to the sampled mussel bed) was minimized. The procedure described herein was suitable for determining tidal horizon.

Procedure for Measuring Temperature of Bottom Water

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ABSTRACT

This document describes the field method for measuring bottom water temperature for the NOAA National Status and Trends Mussel Watch Project, 1986 through 1992.

1. INTRODUCTION

This document describes the field method for estimating bottom-water temperature at sampling sites of the NOAA National Status and Trends Program Mussel Watch Project. A portable salinity-conductivity-temperature meter was used to measure the temperature at East Coast sites, and a glass mercury thermometer was used at the West Coast sites.

Bottom water temperature was measured to 0.5°C at each bivalve sampling site. Measurements were typically made at East Coast sites by using a surface-deployed probe of a portable salinity-conductivity-temperature (SCT) meter. At West Coast sites, subsurface temperature was directly measured using a hand-held glass mercury thermometer. In the event the SCT meter failed to function properly, temperature measurements were made from bottom water samples collected with a Niskin or similar bottle using either a portable digital thermometer or a glass mercury thermometer.

2. EQUIPMENT AND MATERIALS

Beaker, 500-mL. Fisher Scientific, Pittsburg, PA.
SCT meter, YSI Model 33 Portable. VWR Scientific Inc., Media, PA.
SCT probe, 50-ft. VWR Scientific Inc., Media, PA.
Thermometer, immersion, ERTCO certified, fractionally graduated, 76 mm, -1° to 51°C, 0.1 div. VWR Scientific Inc., Media, PA.
Thermometer, digital, battery operated. Thomas Scientific Co., Swedesboro, NJ.

3. SUMMARY OF PROCEDURES

3.1. Portable SCT meter

The SCT meter was zeroed and the battery charge checked before each use. The probe was deployed, lowered to 1 ft above the bottom, and allowed to equilibrate with the bottom water. The temperature was recorded to the nearest 0.5°C.

3.2. Digital thermometer

The digital-thermometer probe was placed in a sample of bottom water collected in a sampling bottle (e.g., a Niskin bottle or other suitable water sampler) and allowed to equilibrate with the water for several seconds. The temperature reading was recorded to the nearest 0.5°C.

3.3. Glass mercury thermometer

The bulb end of the glass thermometer was placed in a bottom water sample or directly into the surface water and allowed to equilibrate for several seconds. The temperature reading was recorded to the nearest 0.5°C.

4. QUALITY CONTROL

Semi-annually, or before extended field use, all field instruments used to measure temperature were calibrated using a National Institute of Standards and Technology (NIST)-certified mercury thermometer. The calibrations were performed by using an ice bath (0°C) and a gradually heated water bath.

4.1. Procedural calibrations

In addition to the factory calibration, procedural calibrations and maintenance checks were performed to ensure proper instrument operation. Maintenance checks were performed prior to each measurement. For the SCT meter, the checks involved testing the battery power and zeroing the instrument. The battery charge on the digital thermometer was verified on the display. The thermometer was also visually examined for damage.

The instrument probe or the bulb end of the glass thermometer was placed in a slurry of ice and distilled water (0°C) and allowed to equilibrate, and the temperature measured from both the NIST-certified thermometer and the instrument being calibrated. Next, the water and ice slurry was heated gradually and the temperature measured at approximately 10° intervals using a NIST-certified thermometer and the instrument being calibrated. The temperatures measured by the field instruments were plotted against the temperatures measured by the NIST-certified thermometer, and the correction factor for each instrument was calculated. These correction factors were applied to measurements made after this calibration and before the next calibration. All temperature measurements were made to the nearest 0.5°C.

4.2. Factory calibrations

All instruments were factory calibrated according to NIST specifications. The manufacturer of the portable SCT meter issued a certificate of traceability stating that the instrument was calibrated using standards that are traceable to NIST specifications or natural physical constants.

4.3. Accuracy

The NIST-certified thermometer probe and each of the field instruments were placed in a water bath that had been equilibrated to room temperature. The temperature was recorded to the nearest 0.5°C, and the instruments removed from the water bath. This procedure was repeated five times. The difference was calculated between the NIST-certified thermometer measurement and each respective field instrument measurement. Accuracy was calculated using the following equation:

$$a = (\bar{x}_f - \bar{x}_{NIST})\ 100\%$$

where a is the percent accuracy, $\bar{x}_f$ is the mean of the five field-thermometer measurements, and $\bar{x}_{NIST}$ is the mean of the five NIST-certified thermometer measurements.

4.4. Precision

Precision was calculated by dividing the standard deviation of the five values obtained in Section 4.3 by the mean of the five values, or by using the following equation:

$$P = \frac{SD}{\bar{x}}\ 100\%$$

where P is the precision of the instrument in percent, SD is the standard deviation of the differences in the five measurements, and $\bar{x}$ is the mean of the differences in the five measurements. Acceptable limits for precision was 10%.

5. CONCLUSIONS

The above described procedures were suitable for providing water temperature readings at the Mussel Watch sites at the time of sampling.

Procedure for Measuring Salinity in Bottom Water

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ABSTRACT

Salinity of bottom water was determined at each bivalve sampling site during each sampling. The procedure described herein was followed for the years 1986 through 1992 of the National Oceanographic and Atmospheric Administration's (NOAA) National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

At each East Coast bivalve sampling site, salinity of bottom water was measured in parts per thousand ($^{o}/_{oo}$) by using a surface deployed probe attached to a portable salinity-conductivity-temperature (SCT) meter. A refractometer was used only when the portable meter failed. A refractometer was used to measure salinity at West Coast bivalve sampling sites.

2. EQUIPMENT AND MATERIALS

2.1. Equipment

Bottle, glass salinity. VWR Scientific, Media, PA.
Bottle, Niskin (or comparable) water sampler. VWR Scientific, Media, PA.
Refractometer, salinity. VWR Scientific, Media, PA.
SCT meter, portable, YSI Model 33. VWR Scientific, Media, PA.
SCT probe, 50-ft. VWR Scientific, Media, PA.

2.2. Materials

Standard Sea Water, P103. IAPSO, Ocean Scientific International Ltd., Wormley, Godalming, Surrey, UK.

3. PROCEDURE

Salinity was typically measured by using a portable SCT meter equipped with a probe and a 50-ft probe cable. This document describes the measurement of salinity only. The measurement of temperature has been described separately.

3.1. Portable meter

The probe was deployed, lowered to 1 ft above the bottom, and allowed to reach equilibrium with the bottom water. After determining the water temperature, the salinity of the bottom water was measured. The temperature dial was adjusted to the water temperature (°C). The meter was switched to the correct measurement scale, and the salinity was recorded from the read scale ($0\text{-}40^{o}/_{oo}$). If the meter reading fluctuated by more than $2^{o}/_{oo}$, the probe was

checked for fouling and the salinity measurement repeated. Salinity was measured to the nearest 0.5°/oo.

3.2. Refractometer

At West Coast sampling sites and in the event of a failure of the portable meter during East Coast sampling, a small Niskin or similar sampling bottle was deployed to collect bottom water samples which were subsequently used for salinity determinations. Water was decanted from the Niskin bottle into a glass salinity bottle, which was sealed and stored at ambient temperature until analyzed.

For salinity determinations using the refractometer, 2 to 3 drops of seawater were placed onto the refractometer's prism surface. The refractometer was pointed toward a light source light and the salinity read directly from the light/dark scale seen in the viewfinder.

4. QUALITY CONTROL

4.1. Portable SCT meter calibration

A certificate of traceability was included with the portable SCT meter. The certificate states that the instrument was calibrated during manufacture using standards whose calibrations were traceable to National Institute of Standards and Technology (NIST) specifications or natural physical constants. Before each use, the portable meter was recalibrated, and the battery charge checked.

4.2. Refractometer calibration

The refractometer was calibrated daily prior to use, and at a minimum of once every 30 min thereafter while in use. Several drops of deionized water were placed on the prism surface. While pointing the refractometer toward a bright light, the light/dark boundary was adjusted to read 0.0°/oo. If the reading deviated from 0.0°/oo by more than 0.5°/oo, the instrument was recalibrated following the manufacturer's instructions.

4.3. Accuracy

After calibration of the SCT meter, five measurements were taken using IAPSO Standard Seawater. Using these measurements and the known value of the standard seawater, the accuracy of the portable SCT meter was calculated using the following equation:

$$a = (\bar{x}_a - \bar{x}_m)\ 100\%$$

where a is the percent accuracy, $\bar{x}_a$ is the actual value of IAPSO Standard Seawater (°/oo), and $\bar{x}_m$ is the mean measured value of the standard seawater (°/oo). Acceptable limits for accuracy measurements were 0.5°/oo.

4.4. Precision

Using the five measurements described in Section 4.2, precision was calculated using the following equation:

$$P = \frac{SD}{\bar{x}} \ 100\%$$

where P is the precision of the instrument in percent, SD is the standard deviation of the differences in the five measurements, and $\bar{x}$ is the mean of the differences in the five measurements. Acceptable limits for precision were ±10%.

5. CONCLUSIONS

The above described procedures were suitable for providing salinity readings of the bottom water at the Mussel Watch sites at the time that samples were taken.

Hydrographic Measurements

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ABSTRACT

This procedure describes the technique used to determine surface water temperature and salinity at sites where oyster samples were obtained for the NOAA National Status and Trends Mussel Watch Project along the Gulf of Mexico Coast.

1. INTRODUCTION

Measurements of temperature and salinity are useful in defining the environmental conditions where oyster samples were collected. Surface water temperature and salinity were determined on the day samples were collected.

2. APPARATUS AND MATERIALS

2.1. Equipment

Pipette, glass
Refractometer, temperature compensated, 10419. Cambridge Instruments, Inc., Buffalo, NY.
Thermometer, ±1°C resolution, model 133. Life Guard, El Monte, CA. (A floating thermometer may also be used.)
Vials, glass, 7-drams, with Teflon-lined caps. Lab Products, Houston, TX.

2.2. Reagents

Distilled water
Standard seawater, P94. IAPSO Standard Seawater Service, Institute of Oceanographic Sciences, Surrey, England.

3. SAMPLE COLLECTION, PRESERVATION AND STORAGE

Surface water samples were collected in 7-dram glass vials. The vials were filled with surface seawater and capped. The vials were then transferred to the laboratory after the field activities were completed for that day. Temperature measurements were made by the field crew while on station.

4. PROCEDURE

The vial containing the seawater sample was allowed to equilibrate to ambient temperature prior to measurement of salinity. The refractometer was zeroed using a few drops of distilled water. A few of drops of the sample were pipetted from the sample vial onto the refractometer and the salinity read directly from the refractometer to the nearest part per thousand. After

every 20 readings, a few of drops of a standard seawater were used to verify refractometer operation.

Temperature measurements were made in the field while on station at each sampling site. The floating thermometer was deployed while on station and the temperature read to the nearest degree after it was allowed to equilibrate for approximately 5 min.

5. CONCLUSIONS

Temperature and salinity data collected at each sampling site for the Status and Trends program provide an indication of the spatial and temporal variability of inshore, estuarine, and lagunal regions found along the Gulf Coast. Salinity has been used by Parker (1960) to define molluscan assemblages of the Gulf Coast along with substrate characteristics. Year to year variations in the salinity regime for any one site can result in the replacement of competing oyster species. Surface temperature measurements ranged from approximately 4°C to approximately 25°C from the tip of Florida to South Bay in South Texas. Salinity measurements varied from $1^{o}/_{oo}$ to $40^{o}/_{oo}$ from Florida to Texas.

6. REFERENCE

Parker, R. H. (1960) Ecology and distributional patterns of marine macroinvertebrates, Northern Gulf of Mexico. In: F. P. Shepard, F. B. Phleger, and T. H. van Andel (eds.). Recent Sediment, Northwest Gulf of Mexico: A Symposium Summarizing the Result of Work Carried on in Project 51 of the American Petroleum Institute, 1951-1958. Scripps Institute of Oceanography, University of California, La Jolla, CA.

Sediment Grain-Size Analysis Procedures Followed by Battelle Ocean Sciences, Science Applications International Corporation, and Geo Plan, Inc.

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ABSTRACT

This document describes the sediment grain-size methods used by Battelle Ocean Sciences and its subcontractors, Science Applications International Corporation (SAIC) and Geo Plan, Inc., from 1986 through 1992 during the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

This document summarizes the methods used by Battelle Ocean Sciences and its subcontractors, Science Applications International Corporation (SAIC), and Geo Plan, Inc., for sediment grain-size analysis for the East and West coast portions of the NOAA National Status and Trends Program Mussel Watch Project. Methods based on Folk (1974) were used by Battelle Ocean Sciences and Geo Plan, Inc. Methods used by SAIC were the American Society for Testing and Materials (ASTM) Method D4221-58 (1978 revision) for wet and dry graduated sieving to fractionate gravel, sand, and fines for gravimetric analysis, and Stockhan and Fochman (1977) for the separation of silt and clay fractions for gravimetric analysis by pipetting.

Battelle and SAIC performed sediment grain-size analyses in 1986 and 1987. In 1986, both laboratories performed sediment grain-size analyses as part of an interlaboratory comparison study. No sediment grain-size analysis was performed in 1988. During 1989, Battelle performed the sediment grain-size analysis. During 1990 and 1991, Geo Plan, Inc. performed the sediment grain-size analyses. No sediments were analyzed in 1992.

2. EQUIPMENT AND MATERIALS

2.1. Equipment and supplies

Air pump, aquarium. Aquatic Ecosystems Inc., Apopka, FL.

Aquarium heater. Carolina Biological Supply Co., Burlington, NC.

Aquarium, for the water bath. Carolina Biological Supply Co., Burlington, NC.

Beakers, 50 mL. Carolina Biological Supply Co., Burlington, NC.

Brushes, soft bristled. Carolina Biological Supply Co., Burlington, NC.

Bubbling wand, plastic. J&H Berge Inc., Plainfield, NJ.

Carboy, 20 L. Carolina Biological Supply Co., Burlington, NC.

Electronic balance, Mettler AC100. Fisher Scientific, Pittsburg, PA.

Funnel, metal large enough to accommodate a sieve. Fisher Scientific, Pittsburg, PA.

Graduated cylinders, 1-L, glass. Fisher Scientific, Pittsburg, PA.

Jars, 200-mL, glass, with lids. Fisher Scientific, Pittsburg, PA.

Light source for microscope. Fisher Scientific, Pittsburg, PA.

Microscope, dissecting. Carolina Biological Supply Co., Burlington, NC.

Oven, drying, capable of sustaining 105°C. Fisher Scientific, Pittsburg, PA.

Petri dish, gridded. Carolina Biological Supply Co., Burlington, NC.

Pipettes, 25-mL. Fisher Scientific, Pittsburg, PA.

Sieve Shaker, Tyler Ro-Tap. Fisher Scientific, Pittsburg, PA.

Sieve, 62 µm. Fisher Scientific, Pittsburg, PA.

Sieves, 2.0, 1.0, 0.5, 0.25, 0.125, and 0.062 mm. Fisher Scientific, Pittsburg, PA.

Squirt bottles, plastic. Fisher Scientific, Pittsburg, PA.

Table top orbital shaker. VWR Scientific, Boston, MA.

Thermometer. Fisher Scientific, Pittsburg, PA.

Wash basin, plastic. Cole-Parmer Instrument Co., Chicago, IL.

2.2. Chemicals

Sodium hexametaphosphate [$(NaPO_3)_6$] [10124-56-8] (0.5% solution). Fisher Scientific, Pittsburgh, PA.

Deionized water

3. SUMMARY OF SEDIMENT GRAIN-SIZE PROCEDURES, 1986

3.1. Sample preparation

3.1.1. Pre-determination of beaker tare weights

3.1.1.1. Battelle

Sequentially numbered 50-mL beakers were washed in soapy water, rinsed with tap water, and placed in a 105°C oven overnight. The beakers were removed from the oven and weighed to 0.1 mg after 2, 4, and 8 hr using an electronic balance, accurate to 0.0001 g. The average of the three weights was used as the representative beaker weight during the analysis.

3.1.1.2. SAIC

All beakers were placed in a 105°C oven for approximately 2 hr. Beakers were cooled for approximately 2 hr and weighed.

3.1.2. Preparation of dispersing solution

3.1.2.1. Battelle

A dispersing solution of approximately 0.5% sodium metaphosphate was used to prevent flocculation of clay particles during analysis. The solution was prepared by dissolving approximately 100 g of sodium metaphosphate in 20 L of deionized water. Triplicate 25-mL aliquots were taken from each 20 L carboy prepared to calculate the dispersant weight. Each of the three aliquots was placed in individual 50-mL beakers, dried in a 105°C oven overnight, cooled, and weighed.

Dispersant weights were calculated as follows:

$$\text{Dispersant wt (g/L)} = \frac{\text{Total wt - Beaker wt.}}{25\ \text{mL}} \frac{1000\ \text{mL}}{\text{L}}$$

The dispersant weight was determined by calculating the average weight of the three aliquots.

3.1.2.2. SAIC

A dispersing solution of approximately 0.5% sodium metaphosphate was used to prevent flocculation of clay particles during analysis. The solution was prepared by dissolving 100 g of sodium metaphosphate in 20 L of deionized water. The dispersant weight per 20-mL aliquot was calculated by taking 5 20-mL aliquots, drying the aliquots in a 105°C oven overnight, cooling the aliquots in a desiccator for approximately 1 hr, and weighing the aliquots. The dispersant weight was determined by calculating the average of the 5 aliquots.

3.1.3. Preparation of sediment

3.1.3.1. Battelle

Sediment grain-size samples were generally refrigerated until the initiation of sample processing. The entire sediment sample was homogenized and a 20 to 25-g aliquot was removed for analysis. Each aliquot was placed in a labeled jar containing approximately 200 mL of dispersing solution and allowed to stand overnight.

3.1.3.2. SAIC

The sample was removed from the sample bag and placed into a 16-oz glass jar. The entire sample was homogenized and a 20-25 g aliquot was removed and placed on a 62 µm urn sieve for wet sieving.

3.2. Procedures

3.2.1. Wet-sieving

3.2.1.1. Battelle

The initial separation of coarse and fine fractions was accomplished by wet-sieving. After soaking overnight in dispersant, the sample was shaken for 7 min in a table top orbital shaker. The sample was then poured onto a 62-µm screen sitting in a funnel suspended on the ring of a ring stand so that the funnel opening was directly over the mouth of the 1-L cylinder (Figure II.2). Using dispersant and a soft bristled brush, the sediment was gently brushed and rinsed with dispersant until the liquid passing through the screen appeared clear or until approximately 800 mL were contained in the cylinder. The screen was placed in a collecting basin and the process was continued using tap water until all the silt-clay material had passed through the screen, hence, the clays would be collected and the sand/gravel fraction retained on the screen. The water in the basin was decanted, any sediment remaining in the basin was rinsed into the cylinder with dispersant, and dispersant was added to the cylinder up to the 1-L mark. The sand and gravel fraction, which was retained on the screen, was rinsed with deionized water into a 50-mL labeled beaker and placed into a 105°C oven overnight.

3.2.1.2. SAIC

The sediment was rinsed using dispersant and brushed into a 1-L cylinder using a soft-bristle brush to break up aggregates in a manner similar to what was described above for Battelle's procedure.

3.2.2. Collection of silt-clay fraction

3.2.2.1. Battelle

The cylinder containing the silt-clay fraction of the sediment sample was placed in a 26°C water bath which was monitored daily to ensure the 26°C temperature was maintained. If the temperature of the water bath had cooled the heater thermostat was turned up, and warm water was added to increase the temperature to 26°C. The sediment in the cylinder was mixed thoroughly for 2 min by using a bubbling wand attached to an air pump, and 25-mL aliquots were pipetted at a depth of 20 cm at intervals of 20 sec; 3 min, 22 sec; 12 min, 38 sec; 50 min, 34 sec; 3 hr, 22 min; 5 hr, 24 min; and 21 hr, 34 min after stirring.

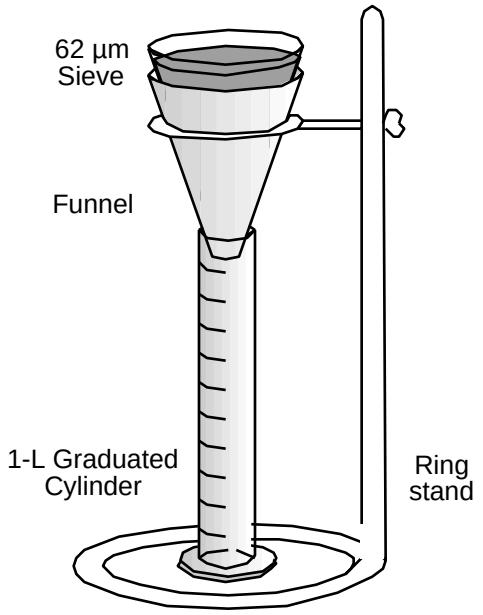

Figure II.2. Ring stand arrangement.

The pipetting intervals were calculated using the equation

$$T = \frac{D}{(1500)\ A\ d^2}$$

where T is time (min); D is the depth of pipette (cm) used for sampling the 25-mL aliquots; A is a constant dependent on the viscosity of the water which is a function of temperature, on the force of gravity, and the density of the particles; and d is the particle diameter (mm). A table showing the values of the constant A for various temperatures was provided by SAIC and is shown below.

Temp. (°C)	Constant A (for clays)
16	3.23
20	3.57
24	3.93
28	4.30
32	4.68

Prior to mixing each sample, the bubbling wand was rinsed in a 1000 L graduated cylinder containing deionized water. After mixing the bubbling wand was removed from the sample and stored in the graduated cylinder containing deionized water. Each aliquot was placed in a 50- mL beaker and dried overnight. After drying, the samples were removed from the oven, allowed to cool, and weighed.

3.2.2.2. SAIC

After wet-sieving into the 1-L cylinder, the fines were homogenized with a slotted plunger and allowed to stand overnight. The fines were re-homogenized in the 1-L cylinder with a slotted plunger, thus providing an even distribution of particle sizes in suspension. After allowing the solution to equilibrate for 20 sec, a 20-mL aliquot was pipetted from a depth of 20 cm which is the mid-point of the cylinder without reagitating the suspension. The 20-mL aliquot was transferred to a pre-weighed weighing pan for gravimetric analysis. One additional 20-mL aliquot was taken at a 10-cm depth at the same time to represent the fines minus the silt

fraction. The time at which the withdrawal was performed was calculated by using the equation in Section 3.2.2.1 above.

The second aliquot was transferred to a pre-weighed weighing pan and was placed, along with the weighing pan containing the first aliquot, in a 105°C oven for a minimum of 8 hr. The tins were cooled in a desiccator for 1 hr and weighed.

3.2.3. Collection of sand and gravel fraction

3.2.3.1. Battelle

The sand and gravel fraction, which was retained on the 62-µm sieve, was transferred to a 50–mL beaker, dried at 105°C in an oven overnight, and cooled to room temperature. It was then placed on the top screen (2.0-mm pore size) of a series of nested screens of 2.0-, 1.0-, 0.5-, 0.25-, 0.125-, and 0.062-mm pore size and shaken on a Ro-Tap Sieve Shaker for 15 min to disaggregate the solidified material. The material retained on each screen was placed in a gridded petri dish and examined under a dissecting microscope. If more than 25% aggregation was observed, the sample was returned to the Ro-Tap Sieve Shaker, reshaken for further disaggregation, and reanalyzed under the microscope. Upon successful disaggregation, the material on each screen was weighed to 0.1 mg on an electronic balance and the weight recorded.

3.2.3.2. SAIC

After the wet-sieving, the coarse fraction retained in the sieve was placed under a heat lamp until completely dry. The coarse fraction was then placed on a clean piece of aluminum foil and lightly brushed to further break up aggregates of dry fines. The sediment that passed through the sieve was transferred to the 1-L cylinder with the rest of the fine fraction. The fines in solution were diluted to 1 L with dispersant solution and re-homogenized. The coarse fraction, retained on the sieve was rinsed briefly with deionized water to remove the dispersant residue, transferred into a pre-weighed 50-mL beaker by rinsing with deionized water, and placed in a 105°C oven overnight to dry. The coarse fraction was cooled in a desiccator for ≥1 hr and weighed to 0.0001 g.

The coarse fraction was then dry sieved through a 2-mm sieve by moving the sieve in a jarring action continuously until no significant additional material passed through the sieve for 1 min. The material retained on the sieve, constituting the gravel fraction of particles greater than 2 mm, was weighed after desiccation. The material which passed through the sieve, the sand fraction smaller than 2 mm and larger than 62 µm, was also redesiccated and weighed.

3.3. Determination of weight percent distributions

3.3.1. Battelle

Grain-size results were presented as percent weight at 1-phi intervals [gravel (≤ -1φ), sand (0 - 4φ), silt (5 - 8φ), and clay (9 - >10φ)] unless specified otherwise in the project protocol. The percent weight was calculated as follows.

1. The sample weight of the fine fraction was calculated by subtracting the pre-determined beaker weight from the beaker and sample weight. This sample weight was based on a 25-mL aliquot and must be corrected to the sample weight per liter.

2. To determine the total weight of the fine fraction, the corrected weight of the first pipetting, which represents the total amount of the silt-clay in the cylinder, was added to the < 62 µm weight fraction from the coarse fraction analysis. The resulting weight, less the dispersant weight of the sample, represents the total weight of the fine fraction.

3. Pipettings for the fine fraction were made in descending order based on phi class size (largest phi class to smallest phi class). The weight fractions for each of the phi classes in the fine fraction were calculated by subtracting the corrected sample weight of one pipetting (smallest phi class) from the corrected weight of the preceding pipetting (largest phi class). The dispersant weight is subtracted from the corrected sample weight of the last pipetting (smallest phi class analyzed) to determine the weight fraction for the last pipetting.

4. The corrected sample weight of each phi class comprising the coarse fraction was determined by subtracting the sample beaker weight from the sample plus beaker weight. The total weight of the coarse fraction was calculated by adding the weight fractions of phi classes -1 through 4. The weight fractions of the phi classes were determined after the sample was shaken on the Ro-Tap Sieve Shaker; each of the -1φ through 4φ classes were weighed (refer to Section 3.2.3.1).

5. The total weight of the sample was calculated by adding the weight fractions of all the phi classes that comprise the coarse and fine fractions.

6. The percent weight for each phi class was calculated by dividing the weight fraction of each phi class by the total sample weight and multiplying by 100. The total percent weights for gravel, sand, silt, and clay fractions were calculated by summing the percent weights for the appropriate phi classes: gravel (≤ 1φ), sand (0 - 4φ), silt (5 - 8φ), and clay (9 - >10φ).

3.3.2. SAIC

A 20-mL aliquot taken 20 sec after homogenization was exactly 1/50 of the total fine fraction contained in a 1-L cylinder,

$$\frac{20\ \text{mL}}{1000\ \text{mL}} = \frac{1}{50}.$$

When the second aliquot was taken, the silt fraction (> 4 µm) settled below the 10 cm depth. At that time, the sample consisted of only the clay fraction. In every case, the sample aliquot weights were corrected for the dispersant weight prior to reporting data.

3.4. Battelle quality control

3.4.1. Interlaboratory calibration

Interlaboratory method comparability was assessed during 1986 by triplicate analyses of splits of prepared sediment samples. Three samples of various particle-size distributions were chosen for triplicate analyses. Acceptable interlaboratory precision was 40% for sand, silt, and clay fractions. Because of variability in gravel fractions, the acceptable level of precision was 100%.

3.4.2. Instrument calibration

Analytical balances used for gravimetric determinations were calibrated prior to the initiation of analyses and thereafter monthly with class S reference weights or equivalent, following the procedure outlined by the manufacturer. Allowable weight ranges were specified on the reference weights and balances were calibrated within those limits.

3.4.3. Documentation of analytical precision

Analytical precision was defined as the coefficient of variation of triplicate analyses of a sample, and was calculated as

$$\%CV = \frac{SD}{\bar{x}} 100\%$$

where CV is the percent coefficient of variation, $\bar{x}$ is the mean weight of each size class, and SD is the standard deviation. Minimally, duplicate analyses were performed with 5% frequency. Acceptable precision was 20% for sand, silt, and clay fractions and 50% for gravel fractions. If these limits were not met, all affected samples were reanalyzed.

4. SUMMARY OF SEDIMENT GRAIN-SIZE PROCEDURES, 1987

The methods used by Battelle and SAIC in 1987 are the same as those used in 1986 with the following modifications to the Battelle method.

4.1. Analysis of silt-clay fraction

Pipettings were taken at only two intervals, 20 sec, and 3 hr and 22 min.

4.2. Analysis of sand and gravel fraction

The dried coarse fraction was not split into phi classes by the Ro-Tap Sieve Shaker. However, the shaker split the coarse fraction into gravel ($\leq -1\varphi$) and sand ($0 - 4\varphi$).

5. SUMMARY OF SEDIMENT GRAIN-SIZE PROCEDURES, 1988

No sediment grain-size analysis was performed.

6. SUMMARY OF SEDIMENT GRAIN-SIZE PROCEDURES, 1989

Sediments from newly established 1989 sampling locations were analyzed for grain size. Grain size determinations of samples collected from some 1988 sites were deferred to 1989. Sediment grain size data was presented as the percentage of gravel, sand, silt, and clay. Samples were analyzed at Battelle by using the procedure outlined in 1986 with modifications incorporated in 1987 (Sections 4.1 and 4.2). SAIC did not perform sediment grain size analyses during 1989.

Precision was calculated by triplicate analyses for 10% of the samples analyzed. Precision was expressed as the coefficient of variation and acceptable limits of precision were 20% for sand,

silt, and clay. Because of the inherent heterogeneity of sediment less than 2 mm in diameter and the small sample size, no limits of precision were assessed for the gravel fraction. If triplicate analyses did not meet the 20% precision criteria, techniques were re-evaluated and, if necessary, affected samples were reanalyzed.

7. SUMMARY OF SEDIMENT GRAIN-SIZE PROCEDURES, 1990 and 1991

Geo Plan, Inc. performed sediment grain-size analyses for 1990 and 1991 samples collected following Battelle procedures used in 1986 with modifications described in Sections 4.1, 4.2, and 6.

8. CONCLUSIONS

The techniques discussed in this document have allowed sediment grain size analyses to be used successfully as a normalizing parameter with respect to contaminant levels in sediments throughout the Mussel Watch Project.

9. REFERENCES

ASTM (1978) A test for dispersive characteristics of clay soil by durable hygrometer. Method D4221-58, 1978 revision. ASTM, Philadelphia, PA.

Folk, R. L. (1974) Petrology of Sedimentary Rocks. Hemphill Publishing Company, Austin, TX. 182 pp.

Stockhan, J., and E. Fochman (1977) Particle Size Analysis.

Sediment Grain Size Analyses

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ABSTRACT

This procedure describes the analytical method used to determine sediment grain size distributions of gravel, sand, silt and clay. This method was used for the NOAA National Status and Trends Mussel Watch Project effort along the Gulf of Mexico Coast.

1. INTRODUCTION

Sediment texture is an important variable in the evaluation of contaminant concentrations and benthic systems. Numerous studies have shown a correlation between contaminant concentration and grain size. In benthic ecosystem studies, cross correlations between stations are often dependent upon substrate characteristics.

Sediment size is based on a grade scale consisting of classes having definite size ranges. The size of each class can be expressed by the particle diameter in units of millimeters or by the dimension expressed as the negative logarithm in base 2 ($\log_2$). This scale using $\log_2$ for the class boundaries is known as the phi (φ) scale, where

$$\varphi = -\log_2 d$$

and d is the particle diameter in millimeters. The particle size distribution is separated into the following classes:

1. Gravel (-5 phi to -2 phi)
2. Sand (-1 phi to +4 phi)
3. Silt (+5 phi to +7 phi)
4. Clay (+8 phi and below)

The most common method for the analysis of silt and clay sized particles is the pipette method (Folk, 1974). It is based on the settling velocity of the particles, usually computed on the basis of Stokes' Law. At given times, small volumes of suspension are withdrawn, evaporated, and the residue weighed.

2. APPARATUS AND MATERIALS

2.1. Equipment and supplies

Bags, Ziploc, gallon size. Dow, Indianapolis, IN.
Balance, analytical, 0.1 mg accuracy. Fisher Scientific, Pittsburgh, PA.
Beakers, 50-mL
Cylinders, graduated, 1-L

Desiccator. Boekel, Philadelphia, PA.
Mason jars, 1 pint, 70610-00518. Kerr Glass Manufacturing Corp., Los Angeles, CA.
Ovens, drying, maintained at 40-50°C and 100-130°C, 1305M. VWR Scientific, Westchester, PA.
Pipette, 20-mL
Rods, stirring, glass
Shaker table, H-4325. Humboldt Mfg. Co., Norridge, IL.
Sieve, 8 inch diameter, 63 mm, ASTME-11 specification. Scientific Products, McGraw Park, IL.
Sieve, size 10, 2000 mm, -1 phi for gravel, ASTME-11 specification. Scientific Products, McGraw Park, IL.
Sieve, size 230, 63 mm, +4 phi for sand, ASTME-11 specification. Scientific Products, McGraw Park, IL.
Timer, 1 sec intervals
Whirl-Paks, 18-oz. NASCO, Ft. Atkinson, WI.

2.2. Reagents

Sodium hexametaphosphate solution [$(NaPO_3)_6$] [10124-56-8], 5.5 g/L distilled water. Mallinckrodt, Paris, KY.
Hydrogen peroxide (H_2O_2) [7722-84-1], 30%. Mallinckrodt, Paris, KY.

3. SAMPLE COLLECTION, PRESERVATION AND STORAGE

Sediment were collected using a hand scoop and were placed in Whirl-Paks or Ziploc plastic bags. At a minimum, 50 g of sample was placed in a plastic Ziploc or Whirl-Pak bag, sealed, and labeled. Samples were refrigerated at 4°C. The samples were never frozen.

4. PROCEDURE

4.1. Preparation of samples for dry sieving and pipette analysis

The volumetric glassware and analytical balances were calibrated prior to use. The sample was homogenized by massaging the sample in the bag. Approximately 15-20 grams of sample was placed in a large, glass jar. This sample size was selected to minimize the interference of grains with each other during settling and the possibility of flocculation. This sample size also maximized the amount of material to be weighed since the error in weighing small samples becomes large with respect to the sample weight. The sample was treated with approximately 50-100 mL of 30% hydrogen peroxide for 12 hr prior to analyses to oxidize organic matter. The volume of hydrogen peroxide varied with amount of organic matter present. The sample was washed with distilled water to remove soluble salts. Four hundred milliliters of the sodium hexametaphosphate solution was added to disperse the sample. The sample was then shaken for 24 hr on the shaker table.

4.2. Size analysis of sand/gravel fraction by dry sieving

The 63-mm screen was placed over a 1-L graduated cylinder. The entire sample containing the dispersed sediment was poured through the screen into the cylinder and washed with dispersant to rinse any fine-grained sediment into the cylinder. This step separated the gravel/sand fraction which remained on the screen from the silt/clay fraction in the cylinder. The gravel/sand fraction was washed from the screen into a preweighed beaker using distilled

water and placed in an oven at 100-130°C to dry for 24 hr. The beaker was removed from the oven and cooled to room temperature in a dessicator. The beaker was weighed to 0.1 mg using an analytical balance to give a sand/gravel weight. If measurements for both sand and gravel were required, this fraction was dry-sieved to separate gravel from sand using the size 10 and size 230 sieves, stacked so that the size 10 sieve was on the top. The sand/gravel fraction was emptied from the beaker onto the sieves. The sieve stack was gently shaken to break up any aggregates and then placed on the shaker table. The sample was shaken for 15 min. The contents of the size 10 sieve was emptied onto a piece of clean paper, and the sample transferred into a preweighed beaker. The beaker was weighed to 0.1 mg on the analytical balance. This weight represented the amount of gravel in the sample. The contents of the size 230 sieve were weighed in a similar manner. The resulting weight represented the amount of sand in the sample.

4.3. Analysis of silt/clay sized material by settling

The graduated cylinder containing the silt/clay material was filled to exactly 1 L with dispersant solution. The sample was stirred vigorously using a glass stirring rod and left to stand for one day. If the sample showed no sign of flocculation, analysis proceeded. If flocculation occurred, the sample was discarded and the analysis repeated.

After standing for one day, the sample was stirred vigorously using a glass stirring rod, starting at the bottom of the graduated cylinder and stirring up to the top until all the sediment was distributed uniformly throughout the cylinder. Long, smooth strokes through the full length of the cylinder were used to stir the sample. The timer was started when the stirring rod emerged from the liquid at the end of the final mixing stroke. A pipette was inserted to a depth of 20 cm, and at 20 sec, exactly 20 mL was withdrawn. The suspension was pipetted into a preweighed beaker. The pipette was rinsed with 20 mL of distilled water and the rinsing water was added to the beaker. This 20 mL aliquot contained the silt and clay fractions, also known as the 4 phi fraction.

After allowing the sample in the graduated cylinder to stand for two hr, 3 min time, a 20-mL aliquot was withdrawn at a depth of 10 cm. The suspension was pipetted into another preweighed beaker and the pipette rinsed with 20 mL of distilled water, which was added to the beaker. The contents of this beaker represented the 8 phi fraction, or the amount of clay present in the sample.

The beakers containing the 4 phi and 8 phi fractions were placed in an oven and the contents evaporated to dryness for at least 24 hr at 100-130°C. After 24 hr, they were removed from the oven and left to cool to room temperature in a dessicator. The beakers were weighed to 0.1 mg with an analytical balance, and the weights recorded on a data sheet.

5. CALCULATIONS

The 4 and 8 phi dry weights included the weight of the added dispersant. It was necessary to subtract the weight contribution of the dispersant from these size fractions. The dispersant weight contribution was determined by measuring three 20 mL samples of dispersant with the pipette and dispensing each into three preweighed beakers. The three beakers were placed in a drying oven at 100-130°C for 24 hr and the contents evaporated to dryness. The beakers were then allowed to cool in a dessicator and weighed to the nearest 0.1 mg. A mean dispersant weight was determined. This total was then multiplied by 50 (1000 mL/20 mL) to yield the weight of the dispersant in the silt and clay fraction.

Three weights were needed to calculate the total dry sample weight:

Total dry sample weight (g) = wt. sand (g) + wt. gravel (g) + wt. of 4 phi residue (g)

$$\% \text{ gravel} = \frac{\text{Wt. gravel fraction (g)}}{\text{Total dry sample wt. (g)}} \ 100\%$$

$$\% \text{ sand} = \frac{\text{Wt. sand fraction (g)}}{\text{Total dry sample wt. (g)}} \ 100\%$$

$$\% \text{ clay} = \frac{\text{Wt. 8 phi residue (g) - Mean dispersant wt. (g)}}{\text{Total dry sample wt. (g)}} \ \frac{TV}{AV_1} \ 100\%$$

where TV is the total volume of silt/clay/dispersant solution and AV_1 is the aliquot volume of clay fraction.

$$\% \text{ silt} = \frac{\text{Wt. 4 phi residue (g) - Mean dispersant wt. (g)}}{\text{Total dry sample wt. (g)}} \ \frac{TV}{AV_2} \ 100\%$$

where AV_2 is the aliquot volume of silt/clay fraction.

6. QUALITY CONTROL

Duplicate samples were run every 20 samples. Duplicate analyses agreed to within 10% relative percent difference or better. The minimum detection is 0.5 phi for each fraction.

7. CONCLUSIONS

The grain size analysis method used by GERG is a standard method that has been used by most laboratories for many years. The method, when employed by trained analysts, provides reproducible results. These grain size data can then be used with confidence to normalize other data produced by the NOAA National Status and Trends Program or other programs.

8. REFERENCES

Folk, R. L. (1974) Petrology of Sedimentary Rocks. Hemphill Publishing Company, Austin, TX. 182 pp.

Sediment Dry Weight Determination Procedures Followed by Battelle Ocean Sciences

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ABSTRACT

This document describes the sediment dry weight determination methods used by Battelle Ocean Sciences for the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends Program Mussel Watch Project for the years 1986 through 1992.

1. INTRODUCTION

This document summarizes the methods used by Battelle Ocean Sciences for sediment dry weight determination analyses for the NOAA Status and Trends Mussel Watch Project. Methods used were based on MacLeod *et al.* (1985). Sediment samples were not analyzed during 1988 and 1992.

2. EQUIPMENT AND MATERIALS

Balance, Mettler AC100 or equivalent. Fisher Scientific, Pittsburgh, PA.
Dessicator. Fisher Scientific, Pittsburgh, PA.
Drying oven, Blue M Model SW-I7TA or equivalent. Fisher Scientific, Pittsburgh, PA.
Spatula, stainless steel. Fisher Scientific, Pittsburgh, PA.
Weighing pans, aluminum. Fisher Scientific, Pittsburgh, PA.

3. SUMMARY OF SEDIMENT DRY WEIGHT DETERMINATION METHOD

3.1. Sediment procedure

A weighing pan, etched with the sample number, was placed on half of a 9-in. strip of aluminum foil. The foil was folded around the pan, but not sealed, and placed in the drying oven overnight. The foil envelope containing the pan was then placed in a dessicator for 30 min. After cooling for 30 min, the pan was removed from the foil envelope and weighed to the nearest 0.01 g. For the calculations, that weight was considered the tare weight. The sediment was homogenized and all pebbles, biota, detritus, etc. were discarded. Approximately 10 ± 0.5 g of sediment were added with a spatula to the pan. The pan was weighed to the nearest 0.01 g. This was the wet weight. The pan was then placed into the unsealed foil envelope and dried in the drying oven for 24 hr at 105°C. The sample was removed from the oven and cooled in a dessicator for 30 min, and weighed to the nearest 0.01 g. This was dry weight #1. The sample was again placed in the oven and dried at 105°C for another 24 hr. It was then cooled in a dessicator for 30 min and reweighed to the nearest 0.01 g. This was dry weight #2. Forceps were used in the handling of the sample pans.

3.2. Dry weight calculation

The percent dry weight was calculated as follows

$$\text{Percent dry wt.} = \frac{\left(\frac{(\text{Dry wt. \#1 - Tare wt.}) + (\text{Dry wt. \#2 - Tare wt.})}{2}\right)}{(\text{Wet wt. - Tare wt.})} 100\%.$$

4. CONCLUSION

Sample dry weight determinations have been made for all samples analyzed as part of the Mussel Watch Project. The techniques described above are suitable for producing data acceptable for use in standardization of analyte concentrations on a dry weight basis.

5. REFERENCE

MacLeod, W. D., D. W. Brown, A. J. Friedman, D. G. Burrows, 0. Maynes, R. W. Pearce, C. A. Wigren, and R. G. Bogar (1985) Standard Analytical Procedures of the NOAA National Analytical Facility, 1985-1986: Extractable Toxic Organic Compounds. NOAA Tech. Memo. NMFS F/NWC-92. 121 pp.

Dry Weight Determination of Sediments

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ABSTRACT

The percent dry weight determination procedure used for the NOAA National Status and Trends Mussel Watch Project sediment samples is described.

1. INTRODUCTION

Dry weight measurements of sediments are necessary when results of sediment analyses are expressed on a dry weight basis. Once the dry weight has been determined, the percent moisture can also be calculated.

2. APPARATUS AND MATERIALS

2.1. Equipment

Balance, analytical, capable of measuring milligram accuracy, Model 8303D. Fisher Scientific, Pittsburgh, PA.
Beakers, glass, 50-mL
Oven, drying, capable of maintaining 40 to 50°C, Model 1305M. VWR Scientific, West Chester, PA.
Spatula

2.2. Reagents

Methanol (CH_3OH) [67-56-1], (pesticide quality or equivalent)
Methylene chloride (C_2H_2) [75-09-2], (pesticide quality or equivalent)

3. PROCEDURE

Sediments were collected in pre-cleaned and/or pre-combusted glass jars and frozen (-20°C). The pre-combusted jars were heated to 400°C prior to use. Sediment samples were thawed and then well-mixed using a solvent rinsed spatula. The spatula was rinsed first in methanol to remove traces of moisture, and then with dichloromethane to remove organic contaminants. The analytical balance was calibrated with calibration weights before use. Approximately 1 g of sample was placed in a tared 50-mL beaker and the weight of the beaker containing wet sample recorded. This was repeated for all samples in the sample set. The beakers were placed in the drying oven at 40-50°C and the samples dried for 24 hr. It was established that 24 hr was sufficient time at 40°C to remove all the water from a 1 g sample. The following day, the beakers containing dried samples were removed from the oven, allowed to cool, and weighed again. For each sample, the balance was allowed to stabilize before weights were recorded.

4. CALCULATIONS

The percentage dry weight was determined by difference as follows:

$$\text{Percent dry weight} = \left[\frac{\text{(Wt. of beaker + dry sample) - (Wt. of empty beaker)}}{\text{(Wt. of beaker + wet sample) - (Wt. of empty beaker)}}\right] 100\%$$

5. QUALITY CONTROL

Duplicate samples were analyzed on approximately 10% of the samples. Duplicate analyses agreed to within a 10% relative percent difference or better.

6. CONCLUSION

Sediment samples are normally 30 to 60% water by weight. Dry weight measurements can be used to compare sediment reported on a dry weight basis to analyses reported on a wet weight basis, when the appropriate conversions are made.

Tissue Dry Weight Determination Procedures Followed by Battelle Ocean Sciences

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ABSTRACT

This document describes the tissue dry weight determination methods used by Battelle Ocean Sciences for the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends Program Mussel Watch Project for the years 1986 through 1992.

1. INTRODUCTION

This document summarizes the methods used by Battelle Ocean Sciences for sediment dry weight determination analyses for the NOAA Status and Trends Mussel Watch Project. Methods used were based on MacLeod *et al.* (1985), and updated in MacLeod *et al.* (this document).

2. EQUIPMENT AND MATERIALS

Balance, Mettler AC100 or equivalent. Fisher Scientific, Pittsburgh, PA.
Dessicator. Fisher Scientific, Pittsburgh, PA.
Drying oven, Blue M Model SW-17TA or equivalent. Fisher Scientific, Pittsburgh, PA.
Spatula, stainless steel. Fisher Scientific, Pittsburgh, PA.
Weighing pans, aluminum. Fisher Scientific, Pittsburgh, PA.

3. SUMMARY OF TISSUE DRY WEIGHT DETERMINATION METHOD

3.1. Tissue procedure

A weighing pan, etched with the sample number, was placed on half of a 9-in strip of aluminum foil. The foil was folded around the pan, but not sealed, and placed in the drying oven overnight. The foil envelope containing the pan was then placed in a dessicator for 30 min. After cooling for 30 min, the pan was removed from the foil envelope and weighed to the nearest 0.1 mg. For the calculations, that weight was considered the tare weight. Using a spatula, approximately 0.5 g of tissue was spread on the pan. The pan was weighed to the nearest 0.1 mg. This was the wet weight. The pan was then placed into the unsealed foil envelope and dried in the drying oven for 24 hr at 105°C. The sample was removed from the oven and cooled in a dessicator for 30 min, and weighed to the nearest 0.1 mg. This was dry weight #1. The sample was again placed in the oven and dried for another 24 hr. It was then cooled in a dessicator for 30 min and reweighed to the nearest 0.1 mg. This was dry weight #2. Forceps were used in the handling of the sample pans.

3.2. Dry weight calculation

The percent dry weight was calculated using the following equation:

$$\text{Percent dry wt.} = \frac{\left(\frac{(\text{Dry wt. \#1 - Tare wt.}) + (\text{Dry wt. \#2 - Tare wt.})}{2}\right)}{(\text{Wet wt. - Tare wt.})} 100\%.$$

4. CONCLUSION

Sample dry weight determinations have been made for all samples analyzed as part of the Mussel Watch Project. The techniques described above are suitable for producing data acceptable for use in standardization of analyte concentrations on a dry weight basis.

5. REFERENCE

MacLeod, W. D., D. W. Brown, A. J. Friedman, D. G. Burrows, 0. Maynes, R. W. Pearce, C. A. Wigren, and R. G. Bogar (1985) Standard Analytical Procedures of the NOAA National Analytical Facility, 1985-1986: Extractable Toxic Organic Compounds. NOAA Tech. Memo. NMFS F/NWC-92. 121 pp.

Total Organic Carbon and Carbonate Analysis Procedures Followed by Battelle Ocean Sciences and Global Geochemistry Corporation

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ABSTRACT

This document describes the total organic carbon (TOC) and carbonate (TIC) analytical methods used by Battelle Ocean Sciences and its subcontractor, Global Geochemistry Corporation, for the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends Program Mussel Watch Project for the years 1986 through 1992.

1. INTRODUCTION

This document summarizes the methods used by Battelle Ocean Sciences and its subcontractor, Global Geochemistry Corporation, for total organic carbon (TOC) and carbonate (TIC) analyses for the NOAA National Status and Trends Program Mussel Watch Project.

The Mussel Watch Project began in 1986. During 1986, 1987, 1989, 1990, and 1991 Global Geochemistry Corporation performed the TOC and TIC analyses. Sediment samples were not analyzed in 1988 and 1992.

2. EQUIPMENT AND MATERIALS

2.1. Equipment

Carbon analyzer, Model 761-100. LECO, St. Joseph, MI.
Crucibles, filtration. LECO, St. Joseph, MI.

2.2. Materials

Hydrochloric acid (HCl) [7647-01-0], reagent grade. Fisher Scientific, Pittsburgh, PA.
Iron and copper chips. LECO, St. Joseph, MI.
Steel rings (for standards). LECO, St. Joseph, MI.

3. SUMMARY OF TOTAL ORGANIC CARBON AND CARBONATE PROCEDURES

3.1. Total organic carbon analyses

A LECO Model 761-100 carbon analyzer was used to determine the TOC contents of solid samples. Before sample analysis, LECO filtration crucibles were pre-combusted for 2 hr at 450°C and allowed to cool. Approximately 170 to 250 mg of dry, finely ground and homogenized sample was weighed to 0.1 mg and placed in a carbon-free filtration crucible supplied by LECO. Each crucible was used only once. The sample was treated with 6N HCl to remove inorganic carbon. One hour after no further reaction was observed, the sample was flushed with deionized water until no acid was left. The sample was then dried overnight. Iron

and copper chips supplied by LECO were added to the sample prior to analysis to accelerate combustion. The principle of operation is the high temperature conversion of all carbon in the sample to CO_2 in the presence of oxygen. The CO_2 was then quantified by thermal conductivity detection.

3.2. Total inorganic carbon analyses

Before sample analysis, LECO filtration crucibles were pre-combusted for 2 hr at 450°C and allowed to cool. TIC was determined by placing 170 - 250 mg weighed to 0.1 mg of dried, ground, and homogenized sample into a pretreated LECO crucible. The sample was heated at 450°C for 2 hr to remove organic carbon. Immediately prior to sample analysis, iron and copper chips were added to accelerate combustion.

3.3. Quality assurance/quality control

3.3.1. Documentation of limits of detection (LOD)

The LOD is defined as the lowest concentration level that can be determined to be statistically different from a blank. Global Geochemistry Corporation determined the LOD for TOC/TIC in sediments during 1987 as the instrument detection limit. The reported LOD was 100 µg/g dry weight of sample. Any deviation from this LOD was reported together with field sample data.

3.3.2. Verification of instrument performance

Instrumental performance was assessed through the daily analysis of standards. Standards, steel rings containing different amounts of carbon, supplied by LECO, were used to prepare daily calibration curves for TOC and TIC. Additionally, after calibration, one standard was analyzed per 10 samples of TOC or TIC to ensure instrument calibration throughout analysis. The calibration range encompassed all expected sample concentrations. If the results of any sample were outside the calibration range, the sample was reanalyzed by using a smaller, appropriately sized aliquot that would yield a result within the calibration range. The method detection limit for these analyses was 0.01% carbon or 100 µg/g.

3.3.3. Monitoring of interference/contamination

One procedural blank was analyzed as part of each analytical sample string of 50 samples. Blank values were subtracted from the respective sample values to give corrected sample data. In addition, blank values were reported with sample data.

3.3.4. Documentation of analytical accuracy

Accuracy was determined by analysis of LECO-supplied standards of known carbon content. Minimally, one standard was analyzed for every ten TOC or TIC samples. Analytical accuracy was calculated using

$$\% \text{ Accuracy} = \frac{\text{Lab value - Standard value}}{\text{Standard value}} \ 100\%.$$

Accuracy was maintained at 5% of true values for all years, except 1991, when it was 10%. If accuracy criteria were not met, any affected samples were reanalyzed. All accuracy data were reported with the respective sample data.

3.3.5. Documentation of analytical precision

Precision was determined for TOC and TIC by replicate analysis of a single homogenized sample. Minimally, one set of duplicate analyses was performed for every 10 carbon or carbonate samples. Precision, defined by the coefficient of variation, was calculated using the following equation:

$$\%CV = \frac{SD}{\bar{x}} (100\%)$$

where %CV is the coefficient of variation (%) between duplicate samples, $\bar{x}$ is the mean weight of either the total carbon or total carbonate, and SD is the standard deviation of the duplicate measurements. Precision was maintained at 10%. If the precision criteria were not met, any affected samples were reanalyzed. All precision data were reported with respective sample data. Several aliquots of a Battelle in-house control material were provided among the samples to act as a blind measure of precision.

4. CONCLUSIONS

Total organic carbon and total inorganic carbon content of sediments are determined for use as contaminant normalizing parameters. The data derived from the use of the above described techniques have been used throughout the Mussel Watch Project to aid in data interpretation.

Total Organic and Carbonate Carbon Content of Sediment

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ABSTRACT

This procedure describes the analytical method used to determine total organic and carbonate carbon in sediments collected as part of the NOAA National Status and Trends Mussel Watch Project effort along the Gulf of Mexico Coast.

1. INTRODUCTION

Total organic and carbonate carbon are parameters that are often useful in providing a better understanding of sediment contaminant data. The total carbon contained in estuarine sediment is divided into two fractions: the carbon that originates from plants and animals (organic) and carbon normally present as calcium carbonate (inorganic).

Carbon compounds in samples were decomposed by pyrolysis in the presence of oxygen and the CO_2 that formed was quantified by infrared detection. Total organic carbon (TOC) was determined by pyrolysis as above after sample acidification which converted carbonate carbon in samples to carbon dioxide. This was purged from the acidified sample prior to analysis. Carbonate carbon or total inorganic carbon (TIC) was determined as the difference between total carbon and total organic carbon.

2. APPARATUS AND MATERIALS

2.1. Equipment

Balance, analytical, capable of weighing to 1 mg, AC 1205. Sartorius, Bohemia, NY.
Crucibles, combustion, 528-018. LECO Corp., St. Joseph, MI.
Detector, infrared, Horiba PIR-2000, or other suitable detector. Horiba, Irvine, CA.
Flow controller, 42300513. Veriflo Corp., Richmond, CA.
Freeze drier, capable of freeze drying sediment at -40°C, Lyph-Lock 12. Labcon Co., Kansas City, MO.
Furnace, induction, 523-300. LECO Corporation, St. Joseph, MI.
Integrator, HP 3396A. Hewlett Packard, Avondale, PA.
Mortar and pestle, 500-mL, or other suitable container
Oven, drying, capable of maintaining 40° to 50°C, 1305M. VWR Scientific, West Chester, PA.
Pipettes, glass
Rotameter, 112-02. Cole-Palmer, Inc., Niles, IL.
Scoop, glass measuring, 503-032. LECO Corp., St. Joseph, MI.
Tubes, jet combustion, 550-122. LECO Corp., St. Joseph, MI.

2.2. Reagents

Accelerator, copper metal , 501-263. LECO Corp., St. Joseph, MI.
Accelerator, iron chip, 501-077. LECO Corp., St. Joseph, MI.
Catalyst pellets, platinized silica, 501-587. LECO Corp., St. Joseph, MI.
Hydrochloric acid (HCl) [7647-01-0], ACS reagent grade, A144-212. Fisher Scientific, Pittsburgh, PA.
Magnesium perchlorate (anhydrone) [$Mg(ClO_4)_2$] [10034-81-8], 501-171. LECO Corp., St. Joseph, MI.
Manganese dioxide (MnO_2) [1313-13-9], 501-060. LECO Corp., St. Joseph, MI.
Standards, pin and ring carbon, range 0.1 to 1.0% carbon, 501-502, 501-503, 501-504. LECO, Corp., St. Joseph, MI.
Water, HPLC grade, 6795-09. Malinckrodt, Paris, KY.

3. PROCEDURE

3.1. LECO system preparation

The LECO induction furnace was allowed to warm up for a period of at least 30 min. The oven was then closed and the oxygen cylinder valve was opened and the regulator set to 40 psi. The oxygen flow was allowed to stabilize for at least 15 sec before the flow was adjusted to approximately 800 mL/min using the flow controller. After 30 sec, the panel meter on the Horiba Infrared Analyzer was set to zero.

3.2. Total carbon determination

3.2.1. Sample preparation

Between 0.1 to 0.5 ± 0.001 g of freeze dried or oven dried, finely ground homogenized sediment was weighed on a calibrated balance into a tared, carbon-free combustion crucible. The amount of sample depended upon the expected carbon concentration. Ideally between 0.5 and 8.6 mg of carbon needed to be combusted to fall within the range of the standard curve.

One scoop (approximately 1.4 g) each of the copper and iron chip accelerators were added to each of the crucibles containing samples. All crucibles were kept covered with aluminum foil prior to analyses.

3.2.2. Sample analyses

The crucible was placed on the oven pedestal and sealed within the oven combustion tube. The oxygen flow was allowed to stabilize for about 15 sec, the flow rate checked on the rotameter and adjusted to 800 mL/min. The induction furnace was turned on and the HP integrator started. The carbon present in the sample began combustion after about 20 sec and the evolved CO_2 was analyzed by infrared detection. The oven was kept sealed until detector response returned to baseline. At this point, the HP integrator was stopped and the peak area for the sample was recorded. The oven was opened and the hot crucible was removed from the oven. This procedure was repeated for all samples in each run.

3.2.3. Standard analyses

Standard LECO pin and ring carbon standards were placed in an empty carbon free combustion crucibles and one scoop of the copper accelerator was added. LECO calibration standards consist

of 1 g steel rings or pins of precisely known carbon concentration. These calibration standards are traceable to NIST standard reference materials. Different LECO carbon standards were chosen to span from 0.1 to 1.0 percent range of carbon, and at least five different carbon standards were run with each sample set. Standards were analyzed using the same procedure as outlined in Section 3.2.2.

3.3. Total Organic Carbon determination

3.3.1. Sample preparation

Appropriate amounts of dried samples as per Section 3.2.1 were weighed into a tared crucible. The samples were acidified by adding 10% HCl in a dropwise fashion until all the bubbling stopped. The acidified samples were dried overnight at 50°C in the drying oven.

3.3.2. Sample analyses

The samples were analyzed as described in Section 3.2.2.

3.3.3. Standard analyses

The standards were analyzed as described in Section 3.2.3.

3.4. Total Carbonate Carbon content

Carbonate carbon was determined by difference between total carbon and total organic carbon in the samples.

4. STANDARDIZATION AND CALCULATIONS

Prior to analyzing samples, standards were analyzed to establish a standard curve on a daily basis. Standard curves varied slightly from day to day.

A set of five different LECO carbon standards containing a known range of carbon were analyzed to establish the curve. Several standard rings and/or pins may need to be run initially to bring the system to correct operating conditions. The calibration curve was prepared by plotting percent carbon versus standard peak areas.

The best fit equation for the calibration curve was determined by linear regression. If the correlation coefficient for the equation was less than 0.99, the standards data set was discarded and another set of five calibration points analyzed. The calibration curve was used to determine the carbon content of samples analyzed that day.

The sample peak areas obtained from the integrator were converted to percent carbon using the linear equation obtained from the calibration curve

$$A_S = m\,C_S + b$$

$$C_S = \frac{A_S - b}{m}$$

where A_S is the area of the sample peak, b is the intercept of the best fit line, m is the slope of the line and C_S is the concentration of carbon in the sample.

$$\text{Percent Carbonate Carbon} = \text{Percent Total Carbon} - \text{Percent TOC}$$

$$\text{Percent Calcium Carbonate} = \text{Percent Carbonate Carbon} \frac{\text{Molecular wt. } CaCO_3}{\text{Molecular wt. C}}$$

5. QUALITY CONTROL

Quality control samples were processed and analyzed in an identical manner to that used for the samples.

A method blank consisting of approximately 1.4 g each of copper and iron chip accelerators was run every 20 samples, or with every sample set, whichever was more frequent. Blank levels should be no more than three times method detection limit (MDL).

Duplicate samples were run every 20 samples, or with every sample set. Duplicates should agree within ± 20% for low level (<1.0% carbon) samples and ± 10% for normal/high level (≥1.0% carbon) sample. Duplicates were less precise for very inhomogeneous samples (i.e., peats, samples containing twigs, grasses, etc.).

LECO pin and ring carbon standards were used as reference materials and standards.

6. REPORTING AND PERFORMANCE CRITERIA

Reporting units were percent organic carbon on a dry weight basis, and percent carbonate carbon on a dry weight basis. Results were reported to two significant figures.

The minimum method performance standard for the method is detection of 0.02% carbon in a sample.

7. CONCLUSIONS

Total organic and carbonate carbon were determined in sediments collected along the Gulf of Mexico coast for use in the interpretation of organic contaminant levels of samples collected as part of the NOAA National Status and Trends Mussel Watch Project.

Tissue Lipid Determination Method

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ABSTRACT

The lipid determination procedure used for the NOAA National Status and Trends Mussel Watch Project Gulf Coast oyster tissue samples is described. Lipid content is operationally described as the weight of sample extracted using dichloromethane.

1. INTRODUCTION

Organic contaminants concentrate in body fat because of their similar chemical properties. Therefore, measurements of lipid content has been used as an aid for normalizing trace organic contaminants in tissue samples for different individuals. The lipid content is operationally defined by the method used to determine it. This section describes the method used by the Geochemical and Environmental Research Group to determine the lipid content, in percent, of oysters analyzed as part of the NOAA National Status and Trends Program Mussel Watch Project in the Gulf Coast.

2. APPARATUS AND REAGENTS

2.1. Equipment

Balance, analytical, capable of weighing to 0.0001 mg, Cahn 29. Cahn Instruments, Inc., Cerritos, CA.
Calibration weights. Cahn Instruments,Inc., Cerritos, CA.
Filter paper, Whatman GFC. Whatman Intl Ltd, Maidstone, England.
Flasks, flat bottomed, 125 mL.
Hot plate, capable of heating to 40°C.
Mixer, Vortex Genie, Model 6-560. Scientific Industries, Bohemia, NY.
Pipettor, 100 µL and 1 mL. American Dade, Miami, FL.
Rotary evaporator, Büchi Rotavapor Model R110. Brinkmann Instruments, Westbury, NY.
Vials, 7 dram, with Teflon-lined cap. Lab Products, Houston, TX.

2.2. Reagents

Methylene chloride (CCl_2H_2) [75-09-2], (pesticide quality or equivalent)
Sodium sulfate (Na_2SO_4) [7757-82-6], ACS Granular, anhydrous (purified by heating at 400°C for 4 hr)

3. PROCEDURE

After the oysters were extracted as described by Wade *et al.* (Volume. IV, this document), the total volume of extraction solvent was determined and recorded. A 20-mL aliquot was removed

and filtered through sodium sulfate which was pre-combusted 400°C for 4 hr, into a 125-mL flask. The aliquot was evaporated to remove the dichloromethane extraction solvent. The residue was dissolved and quantitatively transferred to a 7-dram vial, using dichloromethane. The dichloromethane in the 7-dram vial was evaporated with a stream of nitrogen. The volume of the aliquot was then adjusted to 1.00 mL. The vial was sealed and agitated using the Vortex Genie mixer until the residue was completely dissolved in the dichloromethane.

The analytical balance was calibrated before use with calibration weights. A 1 x 5 cm piece of filter paper was placed on the balance pan and tared. The filter paper was removed and placed on a hot plate that was heated to 40°C. One hundred microliters of the lipid aliquot was removed using a pipettor and placed on the hot filter paper. When the solvent has evaporated from the filter paper, the paper was allowed to cool and reweighed. This procedure was repeated for all samples in the sample set.

4. CALCULATION

The percent lipids is determined using the following formula:

$$\text{Percent lipids} = \left[\frac{[\text{LM (mg)}]\,[\text{TV (mL)}]\,[\text{FV (µL)}]}{[\text{DM (mg)}]\,[\text{AV (mL)}]\,[\text{VW (µL)}]} \right] 100\%$$

where LW is the lipid weight determined in mg, DW is the dry weight (mg) of sample (see Sweet *et al.*, this document), TV is the total volume (mL) of extract, AV is the volume (mL) of aliquot, FV is the final volume (µL) of aliquot, and VW is the volume (µL) weighed .

5. QUALITY CONTROL

Duplicate samples for percent lipid percent determinations are analyzed using approximately 10% of the samples. Duplicate analyses agreed with a 10% relative percent difference or better.

6. CONCLUSIONS

The lipid content of oysters is operatively defined. The values are normally between 2 to 20%. The lipid content can be useful in comparing contaminant concentrations of oysters with different lipid contents.

7. ACKNOWLEDGEMENTS

Thanks to the GERG laboratory personnel C. Frazier, Y. Yu, and G. Salata who do the tedious weighing of lipid extracts.

Lipid Weight Determination Procedures Followed by Battelle Ocean Sciences

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ABSTRACT

This document describes the procedures followed by Battelle Ocean Sciences to determine bivalve lipid weights for the years 1988 through 1993 of the National Oceanic and Atmospheric Administration (NOAA) National Status and Trends Mussel Watch Project.

1. INTRODUCTION

This document summarizes the procedures followed to determine and calculate lipid content in bivalve tissues for East and West Coast samples.

2. SAMPLE PROCESSING

This lipid weight determination procedure is an element of the Battelle tissue extraction procedure. The procedure presented below has been used since 1988 of the Mussel Watch Project.

2.1. Equipment

Aluminum foil
Balance, Mettler AC 100 or equivalent
Flask, Erlenmeyer, 500-mL with graduation marks
Pipette, 10-mL, Class A
Weighing pans, aluminum, baked at 120°C for 24 hr

2.2. Analytical procedures

Tissues were extracted according to protocols presented in Peven *et al*. (Volume IV, this document). The centrifuged extracts were combined in an 500-mL Erlenmeyer flask and the total volume recorded. A 10-mL aliquot was removed from the flask using a Class A glass pipette, the extract was evacuated into a pre-weighed aluminum weighing pan, and the pan covered with aluminum foil. After standing for approximately 24 hr at room temperature the pan was examined to ensure that the solvent (dichloromethane) has completely evaporated. The pan is then re-weighed, and the weight recorded.

2.3. Calculation

The lipid weight is calculated using the following formula:

$$\text{Total lipid weight (g)} = \frac{\text{Extracted sample volume (mL)}}{\text{Aliquot volume (mL)}} \text{ [Aliquot dry wt (g) - tare wt (g)]}$$

$$\text{Lipid weight (g/g)} = \frac{\text{Total lipid weight (g)}}{\text{Sample dry weight (g)}}$$

3. CONCLUSIONS

The lipid weight determination procedures described above were used by Battelle for the Mussel Watch Project. These same procedures are currently in use.

Gonadal Index and Histopathology for the East and West Coasts used in the National Status and Trends Mussel Watch Project

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ABSTRACT

This document describes procedures used by Battelle Ocean Sciences to prepare histological sections from which the gonadal index for each East and West Coast Mussel Watch Project site was determined, and from which histopathological observations were made. Procedures are documented for preparation of paraffin-embedded tissues sectioned at a 5 µm thickness and stained with hematoxylin and eosin.

1. INTRODUCTION

Gonad index determinations were included as part of the Mussel Watch Project to give an indication of the amount of gametic material in the bivalves' tissues at the time of chemical analysis. The intent of the determinations was to assure that sampling was conducted before spawning so that neither the lipophilic organic contaminants nor the trace element concentrations were influenced primarily by spawning. Sites were sampled from late fall through early spring, with the intention of collecting bivalves prior to their spawning and the subsequent loss of lipophilic contaminants that might have accumulated in the gametes, particularly the lipid-rich ova. A variety of techniques for determining gonadal index have been developed depending on the species observed. In mussels, for example, the gonadal follicles develop primarily within the mantle, whereas in oysters, as in most bivalves, they develop within the visceral mass. Virtually all techniques depend upon recognizing the changes in the germinal epithelium and germinal products that signal gonadal development. For the Mussel Watch Project, the technique developed by Seed (1975, 1976) for determining gonad index in mussels was adopted. The method can be used for all bivalve species sampled in the Project, thus making the derived numerical values consistent between the species. The method depends on a histological examination of the bivalves' tissues. Sections cut about midway through the specimens were stained and examined microscopically for stage of gonadal development. The histological examinations also provided an opportunity to observe a number of pathological conditions that might be present in the mollusks.

2. EQUIPMENT AND MATERIALS

2.1. Equipment

Blade Holder, microtome, Tissue Tek III Accu Edge, 4587. Scientific Products, McGraw Park, IL.

Blades, disposable, microtome, 819. American Optical is now Reichert-Jung). A. H. Thomas, Philadelphia, PA.

Cassettes, disposable. Surgipath, Grayslake, IL.

Dishes, staining, glass, A. H. Thomas, Philadelphia, PA.

Embedding center. Shandon/Lipshaw, Pittsburgh, PA

Embedding rings, Universal. Surgipath, Grayslake, IL.

Glass, cover No.1 thickness. Surgipath, Grayslake, IL.

Microtome, rotary, 820. A. H. Thomas, Philadelphia, PA.

Slide dryer. Shandon/Lipshaw, Pittsburgh, PA.

Slides, frosted glass, 1 x 3 in. Surgipath, Grayslake, IL.

Staining racks. A. H. Thomas, Philadelphia, PA.

Tissue processor, American Optical T/P 8000. (This instrument is no longer manufactured; American Optical is now Reichert-Jung). A. H. Thomas, Philadelphia, PA.

Water bath, Boekal, 14793. A. H. Thomas, Philadelphia, PA.

2.2. Chemicals

Acetic acid (CH_3COOH) [64-19-7], glacial, USP grade. Fisher Scientific, Boston, MA.

Aluminum ammonium sulfate [$AlNH_4(SO_4)_2 \cdot 12H_2O$] [7784-26-1], certified. Fisher Scientific, Boston, MA.

Ammonium hydroxide (NH_4OH) [1336-21-6], certified ACS. Fisher Scientific, Boston, MA.

Ethanol (CH_3CH_2OH) [64-17-5] USP grade. Pharmco Products, Norwalk, CT.

Formaldehyde (H_2CO) [50-00-0], 37% by weight, certified ACS. Fisher Scientific, Boston, MA.

Gelatin, USP grade. Fisher Scientific, Boston, MA.

Hydrochloric acid (HCl) [7647-01-1], certified ACS. Fisher Scientific, Boston, MA.

Mercuric oxide (HgO) [21908-53-2], certified ACS. Fisher Scientific, Boston, MA.

Methyl benzoate ($C_6H_5COOCH_3$) [93-58-3], certified. Kodak, Rochester, NY.

Polymount. Poly Scientific Research and Development Co., Bay Shore, NY.

Sodium acetate ($NaCH_3COO$) [127-09-3], certified ACS. Fisher Scientific, Boston, MA.

Tissue Prep 2 embedding media. Fisher Scientific, Boston, MA.

Xylenes [1330-20-7], histologic grade. Fisher Scientific, Boston, MA.

2.3. Staining solutions

2.3.1. Harris' hematoxylin

Hematoxylin	5.0 g
Absolute alcohol	50.0 mL
Aluminum ammonium sulfate	100.0 g
Distilled water	1000.0 mL
Mercuric oxide (red)	2.5 g

The hematoxylin was dissolved in the alcohol, and the aluminum ammonium sulfate in the water by heating as necessary. The two solutions were removed from the heat and mixed. The mixture was heated to a boil as rapidly as possible and removed from the heat long enough to allow the bubbling to stop. The mercuric oxide was added carefully. The solution was reheated until it became a deep purple. It was removed from the heat and the vessel immediately placed in very cold water and cooled as rapidly as possible. The stain was ready for use as soon as it cooled. The stain was filtered prior to use.

2.3.2. Eosin Y

Eosin Y ($C_{20}H_6O_5Br_4Na_2$)	35 mL
Sodium acetate	3.5 mL
Glacial acetic acid	1.7 mL
Distilled water	1000 mL
Thymol	Approximately 1 g

The 1.7 mL of acetic acid was dissolved in 295 mL of distilled or deionized water, and the 5.78 g of sodium acetate was dissolved in 750 mL of distilled water. The solutions were combined and 5 g of Eosin Y (dye content of 82%) was added as a preservative. The pH was approximately 4.98. The thymol, which was added as a preservative, was in large crystal form and is only slightly soluble in the eosin solution. Usually, one piece of thymol is sufficient to provide the necessary preservative protection.

3. METHODS

3.1. Gonadal index

3.1.1. Mussel preparation

The gonadal index determination for mussels is based on a histological evaluation of the maturation stage of mussel gonads, most of which are located in the mantle. Ordinary means of removal of mussels from their shells usually results in severe damage to the mantle tissue lying next to the shell. For that reason, it was considered necessary to preserve the mussels in their shells prior to shipment to the laboratory for histological evaluations. The procedure outlined below was designed to minimize damage to the mantle tissue and gonads.

Mussels usually grow in clusters. It was necessary that the mussels be separated from one another and treated individually. Each mussel was thoroughly rinsed of mud prior to its being opened. Mussels less than 7 cm across the long axis of the shell were not used for this procedure.

The tip of an oyster or clam knife was carefully inserted between the shells immediately posterior to the point where the byssus emerges, and rotated to pry the shells apart. A 90° rotation of the knife blade caused the blade to be wedged firmly in place so that a scalpel blade could be inserted and the posterior adductor muscle severed. This allowed the shells to open with little or no additional force being applied. The mussel was then placed in a wide-mouth jar filled with Dietrich's fixative. A wide-mouth one-liter jar labeled with an identification number was preferred. Ten mussels from each sample could be placed in the jar, assuring adequate fixation.

If the mantle tissue was damaged during the shell-opening procedure, the specimen was discarded because the wound could result in the loss of gametic material, and lead to an erroneous evaluation. Some damage to the gills and viscera was acceptable, but care was taken to minimize that damage, as the tissues were used for histopathological evaluation as well as gonadal index determination.

3.1.2. Oyster preparation

The gonadal index determination was based on a histological evaluation of the maturation stage of oyster gonads, which are located in the central portion of the visceral mass. It was important that each oyster to be evaluated be removed from the shell with no damage to the viscera. The procedure outlined below was designed to minimize visceral damage during removal of the oyster from the shell.

Oysters usually grow in clumps. It was necessary to separate the oysters from one another and to treat them individually. Each oyster was thoroughly rinsed with seawater prior to its being opened. Oysters less than 7 cm across the long axis of the shell were not used for this procedure.

The individual oyster was pressed on a firm flat surface with a flattened hand, i.e., the oyster was not grasped. An oyster knife, or other firm round-tipped blade, was inserted between the shells at the ligament and twisted firmly, thereby dislocating the hinge and slightly separating the shells. The shells were then held apart with firm pressure while the knife blade was carefully inserted between the mantle and upper shell in the area of the adductor muscle. The adductor muscle was carefully cut, the blade being pressed as closely as possible against the inner shell surface. Once the adductor muscle was cut, the upper shell was lifted off the oyster's body and discarded. The oyster was then removed from the lower shell by again carefully inserting the knife blade between the mantle and lower shell, cutting the adductor muscle, and gently prying the oyster from the shell.

The tissues were placed in Dietrich's fixative in a pre-filled half-liter wide-mouth plastic jar. Each jar was labeled with the sample identification number. Ten oysters from each station were placed in one jar.

3.1.3. Processing

Tissues fixed in the field (see Lauenstein and Cantillo, Volume I, this document) were returned to the laboratory in Duxbury, MA, for histological processing. All tissues intended for further processing must meet the following criteria:

- Fixation must be complete;
- Decalcification, if necessary, must be complete;
- Dimensions of the trimmed specimen must not exceed 5 mm in thickness; and
- Tissues must be suitable to fit into a processing cassette, the maximum planar dimensions of which are 31 x 26 mm.

While tissues may be trimmed immediately after removal from fixative, it is advisable to transfer them to 70-80% ethanol prior to trimming. Mussels (*Mytilus* spp.) and American oysters (*Crassostrea virginica*) were cross-sectioned by taking a 3-5 mm slice through the entire body mass at the center of the specimen. The Hawaiian oyster (*Ostrea sandvicensis*) is small enough to be used whole. The tissues were placed in cassettes that were numbered in pencil with the identifying number of the animal. The surface examined was placed face down in the cassette to enable the embedder to correctly orient the tissue in the block.

Tissues were processed for routine paraffin sectioning using an automatic tissue processor. The processor was designed to automatically carry tissue specimens, which have been previously trimmed and placed in numbered cassettes, through a series of solutions intended to dehydrate, clear, and infiltrate the tissues with an embedding matrix. The instrument was programmed by means of a timing disc that was notched to repeat the selected sequence at will.

Solutions for processing were stored in unbreakable beakers or electrically-heated paraffin baths. Each beaker position was referred to as a "station," and there were 12 stations available for use. The steps in processing are: 1) dehydration, 2) clearing, and 3) infiltration. The beakers were filled with the solutions and were arranged in the sequence found in Table II.1.

Table II.1. Fixation sequence.

Station 1	70-80% ethanol
Station 2	70-80% ethanol
Station 3	70-80% ethanol
Station 4	95% ethanol
Station 5	Absolute ethanol
Station 6	Absolute ethanol
Station 7	Methyl benzoate
Station 8	Clearant (Xylene is routinely used, but any of the commercially available proprietary clearing agents is acceptable.)
Station 9	Clearant
Station 10	Paraffin (Any of the commercially available embedding compounds is acceptable. The paraffin is kept in a molten state by means of an electric paraffin bath that is plugged into one of the outlets on the processor.)
Station 11	Paraffin
Station 12	Paraffin

A notched timing disc was used to define the length of time the tissues remain in each of the solutions. Each numbered segment on the disc represented one hour, which was divided into four 15-min intervals. The disc rotated in the same manner as a standard clock. Each time the operating pawl slipped into a notch on the disc, the rotatable top deck of the processor to which the tissue carrier was attached raised the carrier from the solution where it has been and rotated it to the next station. At the end of the cycle, the stop pawl slipped into a notch on the clear plastic disc covering the timing disc and prevented further rotation by stopping the clock mechanism. Thus, the risk of a tissue sample rotating beyond the final station and returning to the initial station was eliminated.

The routine timing schedule for processing tissues was as follows:

1.	70-80% ethanol	Indefinite. Tissues may be stored in 70-80% ethanol.
2.	95% ethanol	1-2 hr
3.	Absolute ethanol	Two changes, 1-3 hr total
4.	Methyl benzoate	1-2 hr
5.	Clearant	Two changes, 1-3 hr total
6.	Paraffin	Three changes, 2-4 hr total

As soon as the final paraffin bath was finished, the basket containing the cassettes was removed from the processor and the cassettes emptied into an electric bath containing molten paraffin maintained at a temperature of 58-65°C. The cassettes were then removed individually, and the paraffin-impregnated tissues were placed in a mold filled with embedding paraffin from an electric dispenser that maintains the paraffin at a temperature of 57-68°C. Using heated forceps, the tissue was oriented in the mold, a plastic ring was placed on the mold, and the ring filled to the top with paraffin from the dispenser. The completed block was set aside to cool at ambient temperature.

After cooling, the base mold was removed and the plastic ring inserted into a rotary microtome for sectioning at 5 µm. For sectioning, the tissue ribbons were floated on a warm (40 - 48°C) water bath filled with deionized or distilled water to which approximately 0.15 to 0.3 g

granular gelatin was added. One, two, or more sections, depending on the size of the block, were mounted on a 1 x 3 in-slide labeled in pencil with the specimen's identification number, usually the accession number. The slides were drained, placed in metal or glass staining racks, and dried in a 58 - 65°C paraffin oven or a forced hot air slide drier for at least 30 min. After drying, the slides were stored at room temperature until needed, or stained. Usually one or two slides were prepared from each block. The block was then filed for future reference.

Sections cut for routine gonadal index estimation or histopathological examination were stained with hematoxylin and eosin.

3.1.4. Staining procedure

The staining procedure sequence is outlined in Table II.2. Slides may be left longer than stated in any of the solutions except hematoxylin, eosin, or acid alcohol without changing the outcome of the stain.

The stained slides were removed from the final clearant bath, and a drop or two of mounting medium was placed on the slide. The stained tissue section was covered by a glass cover slip and dried flat for 24 hr. The slides were then examined under a compound microscope for assessment of gonad development stage and for any histopathological conditions.

Table II.2. Staining sequence.

1.	Xylene or other clearant	10 min
2.	Xylene or other clearant	10 min
3.	Absolute ethanol	2 min
4.	95% ethanol	2 min
5.	80% ethanol	2 min
6.	70% ethanol	2 min
7.	Distilled or deionized water	2 min
8.	Harris' hematoxylin	8-9 min
9.	Rinse in tap water	
10.	Acid alcohol (1% HCl in 70% ethanol)	2-4 dips
11.	Rinse *immediately* in running tap water	
12.	Saturated aqueous Li_2CO_3	1 min
13.	Buffered eosin	30-45 sec
14.	95% ethanol	5-8 dips
15.	95% ethanol	5-8 dips
16.	Absolute ethanol	5-8 dips
17.	Absolute ethanol	1-2 min
18.	Xylene or other clearant	5 min
19.	Xylene or other clearant	5 min
20.	Xylene or other clearant	5 min

Table II.3. Gonadal stages.

(a)	Resting or spent gonad:
	Stage 0. Inactive or neuter. This stage includes virgin animals as well as those which have completed spawning.
(b)	Developing gonad:
	Stage 1. Gametogenesis has begun although no ripe gametes are visible.
	Stage 2. Ripe gametes first appear. The gonad has developed to about one-third of its final size.
	Stage 3. The gonad has increased in mass to about half the fully ripe condition. Each follicle contains, in area, about equal proportions of ripe and developing gametes.
	Stage 4. Gametogenesis is still progressing, but the follicles contain mainly ripe gametes.
(c)	Ripe gonad:
	Stage 5. The gonad is considered fully ripe when early stages of gametogenesis are greatly reduced, and the follicles are distended with morphologically ripe gametes. Ova are compacted into polygonal configurations, and sperm have visible tails.
(d)	Spawning gonad:
	Stage 4. Active emission has begun, as evidenced by the general reduction in sperm density, or a rounding off of the ova as pressure within the follicles is reduced.
	Stage 3. The gonad is about half-empty.
	Stage 2. The area occupied by the gonad has been reduced, and the follicles are about one-third full of ripe gametes.
	Stage 1. Only residual gametes remain, some of which may be undergoing cytolysis.

3.1.5. Gonadal index determination

Four main stages in the reproductive cycle could be recognized in histological section: developing, ripe, spawning, and spent. Developing and spawning stages could then be further subdivided, resulting in a total of ten stages into which any individual can be assigned. Each stage was numbered and recognized as described in Table II.3.

For each bivalve population (i.e., at each site), a mean site Gonadal Index was determined from a sample of 10 or more individuals by multiplying the number of animals in each stage by the numerical ranking of the stage, and dividing the sum of those products by the total number of

individuals in the sample. For example, if, in a sample of 30 mussels or oysters, the specimens were rated as follows:

Condition	Stage Ranking	Number of Individuals
Spent	0	2
Developing	2	3
	3	5
	4	5
Ripe	5	9
Spawning	4	3
	3	2
	2	1

The mean gonadal index would be 3.5 is calculated as follows:

$$(2)(0) + (2)(3) + (3)(5) + (4)(5) + (5)(9) + (4)(3) + (3)(2) + (2)(1) = 106$$

$$\text{Gonadal index} = 106/30 \text{ specimens} = 3.5$$

The index can vary from zero, if the entire population is spent or resting, to five, if the population is fully ripe.

3.1.6. Quality control

The stage of gonadal development was determined by visual analysis. To determine the degree of subjectivity in assigning a particular stage ranking to a particular section, a specified amount of reanalysis of slides from each site was carried out. When 20 to 30 individuals were collected from each site, 10% of the slides from each site were randomly selected for reevaluation. If the quality control check resulted in a 6% difference in the overall sample readings, the entire population sample (i.e., all individuals collected for gonadal analysis at a given site) were reevaluated. When 15 individuals were collected, one slide from each site was randomly selected for reevaluation. If there was disagreement between an original reading and the reanalysis of the slide, an additional 5 slides from the population were randomly selected and reanalyzed.

3.2. Histopathology

3.2.1. Gross pathology

Gross observations were made when preparing the animals for fixation. Following are examples of conditions to look for closely when preparing the specimens for fixation:

- Malformations, especially in the gills and body mass;
- Parasites, both internal and external;
- Firmness of tissue; and
- Unusual coloration of internal organs, such as digestive diverticula and gonadal tissue.

3.2.2. Microscopic examinations

Tissue sections were first scanned broadly under a low-power objective (4X or 10X) for obvious areas of tissue discoloration or unusual architecture, or for the presence of larger parasites, such as trematodes. The sections were then scanned at higher magnification [e.g., 20X, 40X, 100X (oil immersion)] for tissue anomalies, such as neoplastic cells, or for the presence of microparasites such as *Minchinia nelsoni* or *Perkinsus marinus*.

4. CONCLUSIONS

The hematoxylin and eosin methods described in this document are standard histological techniques, and demonstrate clear, well-defined cell and tissue elements. Harris' hematoxylin is an excellent stain following fixation in Dietrich's fixative. The amount of eosin counterstaining is often at the preference of the histologist reading the slide. The 30- to 45-sec dip in eosin Y can be varied in either direction without affecting the amount of information to be gleaned from the slide.

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Gonadal Analysis - *Crassostrea virginica*

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ABSTRACT

This document describes the procedures followed for gonadal index determination of oyster specimens collected on the Gulf Coast of the US by Texas A&M University for NOAA's National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

Assessment of the physiological state of an oyster population requires an analysis of the state of gonadal development. Typically, oysters are undifferentiated in the winter. Gonads begin to develop in early spring and spawning occurs during late spring through early fall. Most Gulf Coast oysters spawn at least twice during this time period. Oyster gonadal tissue is distributed around the body mass and cannot be easily excised and weighed (Morales-Alamo and Mann, 1989). Therefore, a gonadal index based on histological examination is generally used. A dorsal-ventral slice of oyster tissue is taken just posterior to the palps and fixed in Davidson's fixative. Tissue samples are embedded in paraffin, sectioned, and stained using a hematoxylin-eosin protocol. Stained sections are examined under a compound microscope, and sex and the state of gonadal development determined. Fixation and staining follow methods described in Preece (1972). Characterization techniques were adapted from Ford and Figueras (1988).

2. EQUIPMENT, REAGENTS AND SOLUTIONS

2.1. Equipment

Microscope, compound, Zeiss, 12-070-20. Fisher Scientific, Pittsburgh, PA.
Microtome knife sharpener, automatic, Leica 903, 12-643-40. Fisher Scientific, Pittsburgh, PA.
Microtome, Leica Histocut 820 Rotary microtome, 12-603. Fisher Scientific, Pittsburgh, PA.
Paraffin pitcher, electric, Lepshaw 220, M7395. Baxter Scientific, McGaw Park, IL.
Rotary tissue processor, Tissue-Tek, M7286-1. Baxter Scientific, McGaw Park, IL.
Slide warmer for tissue embedding, M6420. Baxter Scientific, McGaw Park, IL.
Slide warmer, 12-594. Fisher Scientific, Pittsburgh, PA.
Vacuum oven, 13-264A. Baxter Scientific, McGaw Park, IL.

2.2. Reagents[◊]

Acid fuchsin ($C_{20}H_{17}N_3Na_2O_9S_3$) [3244-88-0], certified stain, A3908. Sigma Chemical Co., St. Louis, MO.
Albumin fixative (Mayer albumin-glyercine solution), M7651-16. Baxter Scientific, McGaw Park, IL.

[◊] Purity unimportant except where indicated.

Celloidin (as collodion solution) ($C_{12}H_{16}N_4O_{18}$) [9004-70-0], M7651-16. Baxter Scientific, McGaw Park, IL.

Eosin Y ($C_{20}H_6Br_4Na_2O_5$) [17372-87-1] certified stain. Sigma Chemical Co., St. Louis, MO.

Ethanol (C_2H_6O) [64-17-5], R8382. Sigma Chemical Co., St. Louis, MO.

Ethyl ether* ($C_4H_{10}O$) [60-29-7], 30,996-6. Sigma Chemical Co., St. Louis, MO.

Formaldehyde [CH_2O] 37% solution (50-00-0), F1635. Sigma Chemical Co., St. Louis, MO.

Glacial acetic acid [$C_2H_4O_2$] (64-19-7), A0808. Sigma Chemical Co., St. Louis, MO.

Glycerin ($C_3H_8O_3$) [56-81-5], G7893. Sigma Chemical Co., St. Louis, MO.

Hydrochloric acid (HCl) [7647-01-0], concentrated, 12N, H7020. Sigma Chemical Co., St. Louis, MO.

Lithium carbonate (Li_2C0_3) [554-13-2], 9020. Sigma Chemical Co., St. Louis, MO.

Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$) [1936-15-8], certified stain, O7252. Sigma Chemical Co., St. Louis, MO.

Paraffin (Ameraffin) [melting point between 55-57°C], M7347-1. Baxter Scientific, McGaw Park, IL.

Permount mounting media, SP15-500. Fisher Scientific, Pittsburgh, PA.

Picric acid ($C_6H_3N_3O_7$) [88-89-1], A253-500. Fisher Scientific, Pittsburgh, PA.

Sodium chloride (NaCl) [7647-14-5], S9625. Sigma Chemical Co., St. Louis, MO.

Xylenes (C_8H_{10}) (histological grade), X35-4. Sigma Chemical Co., St. Louis, MO.

2.3. Solutions

Harris' hematoxylin solution was purchased pre-mixed, and 200 mL of the stock solution was mixed with 14 mL of glacial acetic acid prior to use.

Davidson's fixative stock solution: 1 part glycerin, 2 parts 37-40% formaldehyde, 3 parts 95% ethanol, and 3 parts isotonic sodium chloride (usually 20-30 $^{o}/_{oo}$). The solution was stored at 4°C. Just before use, 1 part of glacial acetic acid was mixed with 9 parts of the stock solution.

Acid alcohol: 99 mL of 70% ethanol and 1 mL of 12 N HCl.

Collodion working solution: 0.5 mL of collodion stock solution in 50 mL of ether and 50 mL of ethanol.

Picro-eosin solution: 16 g of Eosin Y and 160 mL of a saturated aqueous solution of picric acid (~1.2 %) in 1440 mL of 80% ethanol.

Navy eosin solution: 10 g of Eosin Y, 2.5 g of Orange G, 1.26 g of acid fuchsin and 0.5 mL of acetic acid dissolved in 1 L of 70% ethanol.

Working solution - eosin stain: Mix 450 mL of the Picro-eosin solution and 20 mL of the Navy eosin solution.

Lithium carbonate: 1.25 g of lithium carbonate in 100 mL of water.

Ethanol solutions are prepared v/v with distilled water.

* Ethyl ether was kept refrigerated. One milliliter of water and an iron nail was added to absorb peroxides.

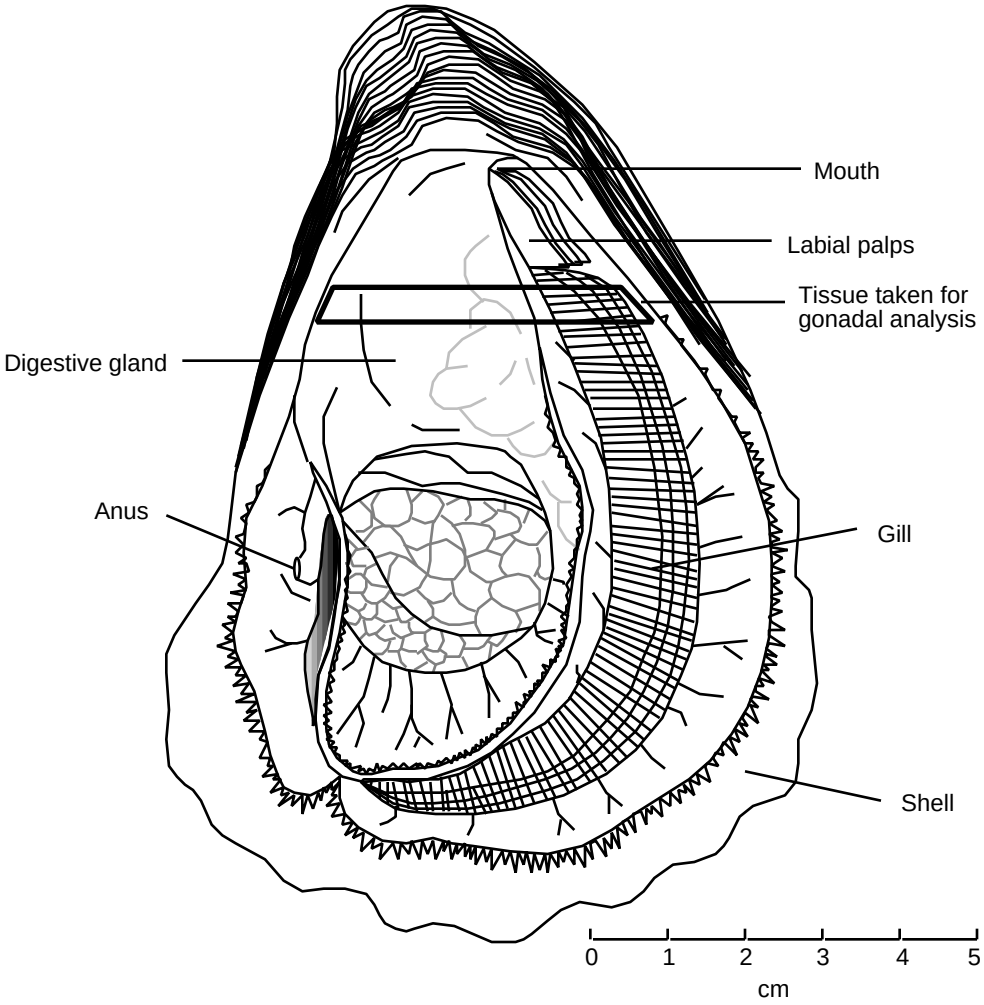

Figure II.3. Oyster tissue used for quantifying gonadal index (adapted from Galtsoff, 1964).

3. SAMPLE COLLECTION AND FIXATION

Ten of the twenty oysters to be pooled for the measurement of hydrocarbon body burden were first used for gonadal analysis. Each oyster was opened (as described in Wade *et al*., Volume IV, this document) and a 3 to 5-mm thick cross section of tissue removed from the oyster using a scalpel or scissors (Figure II.3). The section was obtained such that the dorsal-ventral aspect passed through the digestive gland and gill tissue just posterior to the palps. Each section was immediately placed in a scintillation vial, filled with refrigerated Davidson's fixative, and fixed for 48 hr. Bouin's fixative is an adequate alternative in most applications. The vials were shaken every 2 hr for the first 6 hr, and once every 6 hr thereafter. After the 48 hr, the fixative was decanted, 50% ethanol added and the tissues allowed to stand for 2 hr during which time the vials were occasionally shaken. After 2 hr, the solution was replaced with 70% ethanol, and the vials shaken every few hours. After 24 hr, each tissue slice was placed in an individual tissue cassette, which was then immersed in a larger vessel filled with 70% ethanol, where it can be kept indefinitely.

Table II.4. Tissue embedding sequence.

Dehydration		Clearing	
80% ethanol	60 min	xylene	60 min
95% ethanol	120 min	xylene	120 min
95% ethanol	120 min	xylene	120 min
100% ethanol	120 min		
100% ethanol	120 min	Infiltration	
100% ethanol	120 min	50:50 xylene:paraffin	120 min
100% ethanol	60 min	paraffin	120 min
		paraffin	Overnight in vacuum oven

The use of the same oysters for gonadal analysis and for the analysis of contaminant body burden potentially biases the latter analyses because digestive gland tissue and gonadal tissue, which contribute a disproportionate portion of the tissue taken for histological analysis, may contain higher than average body burden of certain contaminants. Sericano *et al.* (1993) showed that this source of error resulted in an underestimation of true body burden by no more than 10% under most conditions, if the slice of tissue removed was limited to a 3 to 5 mm section.

4. SLIDE PREPARATION

4.1. Tissue embedding

Individual tissue samples were prepared for embedding in paraffin using an established dehydration protocol (Table II.4). The solutions used for dehydration, clearing, and infiltration were changed frequently. The pure ethanol was stored in a glass bottle rather than in a plastic one to avoid introduction of water vapor into the ethanol.

The tissue embedding sequence used an automated tissue processor which automatically processes tissue through the alcohol-xylene series and into paraffin. Embedding can also be done manually by moving the tissues through the sequence. The paraffin was melted in an oven set at no more than 2°C above the melting temperature and kept at temperatures no higher than 2°C above melting during the infiltration process. Unused melted paraffin was discarded after 24 hr or used in the 50/50 xylene/paraffin mixture. Newly melted paraffin was always used in the final infiltration and embedding steps.

After the tissues were infiltrated with paraffin (Table II.4), they were transferred to new paraffin and placed in a vacuum oven overnight. The vacuum oven and warming plate were set to maintain the paraffin at the same carefully-controlled temperature. It is useful to place the tissues in beakers in aluminum blocks specially designed to hold them above melting point temperature of the paraffin once the tissue is removed from the oven. The aluminum block containing tissues in beakers was removed from the vacuum oven and placed on a warming plate. The tissues were transferred to aluminum molds filled with newly melted paraffin, oriented cross-sectional face down for sectioning, and a plastic mold backing placed on top. The mold was moved to a cold plate and the plastic backing filled with paraffin. As the

tissue/paraffin cooled and hardened, the paraffin shrank. Care was taken to use sufficient paraffin to cover the tissue after it cooled and hardened. Occasionally, more paraffin had to be added. After the paraffin cooled, the mold was placed in a refrigerator for a few hours or cooled overnight, whereupon the tissue-paraffin block was removed from the mold and sectioned.

4.2. Tissue sectioning

The paraffin blocks were sliced at 6 µm using an AO microtome. Microscope slides were thinly coated with albumen fixative, placed on a slide warmer, a small amount of water was placed on the slide, and the slide warmed. A string of contiguous sections was layered onto the water layer, so that the entire string of sections floated without contact with the slide. The sections were allowed to expand. Once the sections expanded to full size, the water was carefully decanted onto a Kimwipe tissue and the slide returned to the slide warmer for further drying. Once the sections were firmly attached to the slide, the slide was taken off the slide warmer, placed in a slide box, and allowed to dry overnight. After drying overnight, the slides were ready to stain.

4.3. Tissue staining

A standard hematoxylin and eosin staining procedure adapted from Preece (1972) was used. Times required for each step are flexible in both the staining procedures discussed here and in the previous embedding protocol. Different tissue types may require different times. All tissue baths, especially xylene and ethanol ones, should be changed frequently. Slides should not be allowed to dry during transfers. Problems and solutions to standard embedding, sectioning, and staining procedures are discussed in Preece (1972) and most other manuals of histological technique.

Sections were deparaffinized and hydrated using a xylene-ethanol series (Table II.5). Gently moving the slides up and down in the staining dish increased the rate of diffusional exchange and shortened the time required for hydration and dehydration through the alcohol series. Following deparaffinization, slides were dipped once quickly in a Collodion solution and plunged immediately into 80% ethanol. The Collodion coat serves to keep the section on the slide during staining but lengthens the staining time. Sections were hydrated and then stained in Harris' hematoxylin solution. We found it more convenient to purchase this stain pre-mixed. However, the formula is found in Preece (1972). Staining times varied from batch to batch and tissue to tissue. The procedure involved the initial over-staining of the tissues and their subsequent destaining in acid alcohol. The tissues can be stained and destained several times until the desired color is obtained.

Following decolorization, the sections were blued in lithium carbonate solution and counterstained with Picro-Navy eosin, dehydrated in an ethanol-xylene series, and mounted in a standard mounting medium. The final series of 100% ethanol baths also removed the Collodion coat.

5. ANALYSIS

Each slide was examined microscopically to determine sex and stage of gonadal development. A histopathological examination can also be made at this time if desired. Careful examination of early developmental stages may be needed to positively distinguish males or females in early

Table II.5. Tissue staining sequence.

Deparaffinization		Rinsing	
xylene	5 min	running tap water	1min
xylene	5 min		
ethanol	5 min	Blueing	
ethanol	5 min	Lithium carbonate solution	0.5-1 min
Colloidionization		Washing	
Collodion solution	quick dip	water	2 min
80% ethanol	2 min		
		Counterstaining	
Hydration		Picro-eosin/Navy eosin	1 min
50% ethanol	2 min		
30% ethanol	2 min	Dehydration	
water	5 min	95% ethanol	several dips
		95% ethanol	several dips
Staining		100% ethanol	1 min
Harris' solution	2-4 min	100% ethanol	1 min
		100% ethanol	1 min
Rinsing			
water	5 min	Clearing	
		xylene	5 min
Decolorization		xylene	5 min
acid alcohol	2-3 dips		
		Mounting	
		Permount	24 hr to dry

stages of development from individuals as yet undifferentiated. Occasional hermaphrodites will also be found. The stage in the gametogenic cycle is assigned based on the maturity of the follicles and gametes and a numerical value assigned as described in Table II.6.

6. CONCLUSIONS

The procedure described provides a qualitative ranking of gonadal stage but no quantification of the amount of gametic tissue present. Its strengths are that it provides a concomitant assessment of sexual stage in the gametogenic cycle, and, if desired, allows for histopathological analysis, with a single sample preparation protocol. The procedure cannot be performed on pooled samples, thus necessitating individual analysis of a fraction of the samples pooled for other purposes or analysis of separate individuals. Thus a direct correspondence between, for example, hydrocarbon body burden and stage in the gametogenic cycle may be difficult. If direct comparison is required, the procedure requires the removal of some tissue from the animal that cannot be part of a subsequent analysis of contaminant body burden. This subsampling results in a certain degree of bias in the measurement of contaminant body burden, normally around 10% as described for NS&T samples by Sericano *et al.* (1993),

Table II.6. Development stages.

Developmental Stage	Value	Comments
Sexually undifferentiated	1	Little or no gonadal tissue visible
Early development	2	Follicles beginning to expand
Mid-development	3	Follicles expanded and beginning to coalesce; no mature gametes present
Late development	4	Follicles greatly expanded, and coalesced, but considerable connective tissue remaining; some mature gametes present
Fully developed	5	Most gametes mature; little connective tissue remaining
Spawning	6	Gametes visible in gonoducts
Spawned	7	Reduced number of gametes; some mature gametes still remaining; evidence of renewed reproductive activity
Spawned	8	Few or no gametes visible; gonadal tissue atrophying

because the digestive gland and gonadal tissues preferentially removed for histology are enriched in some contaminants relative to the remaining tissues and deficient in others (Ellis *et al.*, in press). This bias, therefore, will also be size-, contaminant-, and time of year-dependent.

If a quantitative gonadal/somatic index is desired, the technique of Choi *et al.* (this volume) should be used. The latter, however, is not compatible with a concomitant histopathological analysis in that the standard histological preparation for assessing gametogenic stage is not used in the quantitative analysis and tissue subsampling for histology cannot be done on the same individuals to be analyzed quantitatively for gonadal-somatic index (Ellis *et al.,* in press; Choi *et al.*, in press).

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Collection of Fish Tissues for the National Benthic Surveillance Project: Necropsy Procedure, Tissue Processing, and Diagnostic Procedure for Histopathology

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ABSTRACT

This document describes the standard necropsy procedure for the collection of fish tissues for chemical and histopathological analyses for the NOAA National Status and Trends Program National Benthic Surveillance Project. Tissue processing and diagnostic procedures for histopathological analyses are also described.

1. INTRODUCTION

Standard techniques have been used to collect fish tissue samples for chemical and histopathological analyses since the inception of the NOAA National Status and Trends Program's National Benthic Surveillance Project (NBSP) to ensure collection of high quality samples resulting in accurate chemical analyses and histopathological diagnoses. Procedures used by the NOAA/National Marine Fisheries Service/Northwest Fisheries Science Center for performing fish necropsies, tissue collection and handling for all routine NBSP analyses, processing tissues for histopathological examinations, and diagnosing histopathological conditions are described in this document.

2. PREPARATION OF FISH FOR SAMPLE COLLECTION

Fish that were within the appropriate size range for each species were placed into a live holding container with fresh seawater. The minimum fish length was determined by the size of the animal when a given species reached sexual maturity. To minimize artifacts of holding, fish were maintained either in flowing or frequently replenished seawater. Dead animals or animals severely damaged during the capture process were not necropsied. Each animal selected for necropsy was weighed, measured, and assigned a unique specimen number.

3. RECORDING OF FIELD DATA

Information on animals sampled for histopathological examination or chemical analyses was maintained in a field notebook and on individual field cards. For each animal collected for pathological examination, the following information was noted and recorded in the project field notebook:

- unique specimen number assigned to the animal
- length (total fork length, mm)
- weight (g)
- taxonomic identification
- site number and name at which the animal was captured
- date.

Each animal collected had an individual field card that was filled out at the time of necropsy and contained the following information:

- unique specimen number
- taxonomic identification
- gender
- location, type, and approximate number of visible parasites
- location and description of grossly visible anomalies and lesions
- samples collected for analyses other than histopathology (e.g. chemical analysis, stomach content analysis)
- identity of person performing necropsy.

4. NECROPSY PROCEDURE

4.1. Collection of structures for fish age determination

Fish were sacrificed by severing the spinal cord just posterior to the brain; and otoliths, scales or spines were collected for age determination at this time. Otoliths from flatfish were stored dry in a test tube labeled with the specimen number, and otoliths from round fish were stored in 70% ethanol. Scales or spines may have been collected from species that had otoliths that were difficult to assess for age, or where these structures provided a more accurate assessment of fish age. Collection of other fish tissues began immediately after collection of structures for aging.

4.2. Necropsy tools

Three separate sets of necropsy tools were used:

1) One set of large scissors and forceps was used for external work on the fish, including collecting fins and skin sections (if fin erosion or other grossly visible lesions were present) and for opening the body cavity.

2) For those fish where liver was being collected for metals analysis, polyamide forceps and a titanium knife were used.

3) A third set of necropsy tools was used only to collect internal tissues for histology and chemical/biochemical analyses.

To prevent transfer of contaminant residues, all necropsy tools and the Teflon cutting surfaces were cleaned as follows:

Between individual fish of one species at the same site, the tools and the Teflon cutting surfaces were wiped and rinsed with distilled water.

Between sites, and between different species within sites, the tools were washed and rinsed using a thorough soap and water wash, followed by a distilled water rinse; an isopropanol rinse; another distilled water rinse; and air drying.

Necropsy was performed, whenever possible, at a clean air work station under a laminar flow hood.

4.3. Observation and collection of external lesions

Immediately following sacrifice and collection of structures for aging, any grossly visible external lesions on each animal was described and recorded on the field card. Using the set of external tools, tissue specimens from grossly visible external lesions on the fin and skin were excised to a 3-mm thickness, and placed into tissue cassettes and preserved in Dietrich's fixative (Gray, 1954), no warmer than 22°C (normal room temperature). The body cavity was then opened using the same external tools. Any grossly visible anomalies and lesions in the internal organs were described on the field card.

4.4. Collection of bile

Using the separate set of isopropanol-rinsed dissection instruments designated for internal tissues, the entire liver was excised, with gall bladder attached, and placed on an isopropanol-rinsed Teflon board. The gall bladder was then carefully separated from the liver: gripped by the bile duct to prevent bile from flowing out of the bladder, and care was exercised not to touch the liver with the scissors. Care was taken not to spill any bile onto the liver, otherwise the tissue might have deteriorated due to direct contact with the bile. If bile did inadvertently contact the liver, the affected portion of the liver was not used for histopathology. The bladder was held over the mouth of the amber vial designated for bile collection. A clean #11 scalpel blade was used to puncture the bladder and the bile was allowed to drip down the tip of the blade into the vial.

4.5. Collection of liver for histology

Since tissue degradation of cellular structures begins immediately after death of the fish, all histology samples were collected as soon as possible after sacrifice. If liver tissue was needed for metals analyses, it was collected before the histopathology sample was removed. This prevented the metal "internal" set of dissecting tools from touching the liver before the metal sample was collected (see Section 4.7 for protocol on collecting liver for metal analyses).

A pair of sharp scissors from the set of internal tools was used to cut a routine section for histology (no thicker than 3 mm) from the central longitudinal axis of the liver (Figure II.4). The cut side of the liver tissue was placed face down into a cassette, with care taken to avoid mechanical damage such as tearing, poking, pulling, or stretching of tissues. For optimal fixation, tissues in the cassette should not contact one another or the sides of the cassette. As soon as a cassette was filled, it is immediately placed into Dietrich's fixative.

If grossly visible lesions were noted in the liver, sections for histology were also taken for as many of the differing lesion types as possible. Whenever grossly visible nodules were noted in the liver, sections no thicker than 3 mm are also collected from the heart, upper intestine and spleen for histology. These tissue samples were used to verify presence/absence of metastatic foci from primary neoplasms in the liver.

4.6. Collection of kidney and ovary tissues for histology

After the histology liver samples had been collected, additional tissues such as kidney and gonad could be collected for histology. The internal tools were used to collect sections less than 3-mm thick of these organs. The kidney histology sample was collected as a sagittal or transverse section from the posterior kidney depending on the species, and the gonad sample as a transverse section from the middle of the ovary or testis. Additional cassettes were used if the cassette containing the liver was full. The kidney and gonad sections were placed flat in the cassette, the cassette was securely snapped shut, and then placed in fixative.

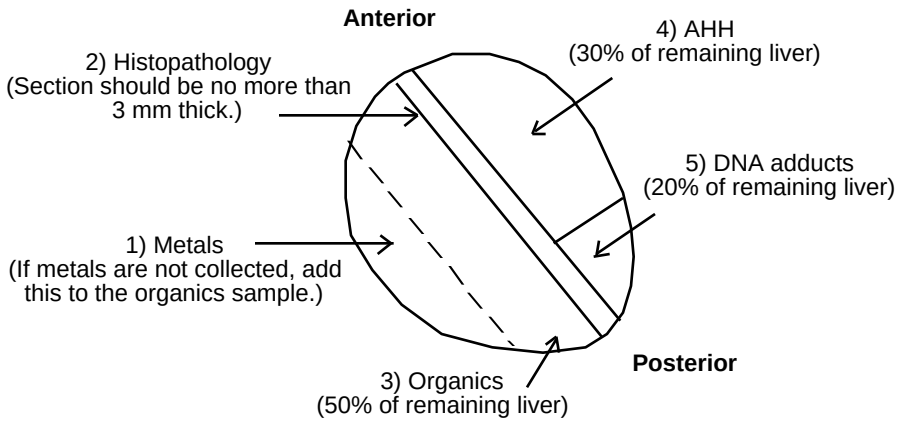

Figure II.4. Generic fish liver showing where various samples were collected, and the order in which they were collected. Percentages show approximate amount of liver needed after collection of the histopathology and metal samples.

4.7. Collection of liver tissue for metals

If liver tissue needed to be collected for metal analyses, this was quickly performed just after bile collection such that the liver was not contaminated with metal dissecting tools. The liver was carefully manipulated with polyamide forceps previously rinsed with isopropanol, followed by distilled, deionized water. The titanium knife was rinsed by the same procedure and was used to cut a portion from one side of the liver for metals analysis, and polyamide forceps were used to place the tissue into the plastic acid-rinsed vial. Samples were frozen at -20°C. Liver samples for metal analyses were generally from the same area of the liver for each fish, as shown in Figure II.4.

4.8. Collection of liver tissues for organic chemical analyses

After samples for histology trace metal analysis were collected, the remaining liver was divided into proportions for the following analyses: 50% of the liver was collected for organic chemical analyses, 30% was collected for aryl hydrocarbon hydroxylase (AHH), and 20% was collected for xenobiotic-DNA adducts. Liver samples for organic chemistry analyses were also routinely collected from the same area of the liver for each fish, as shown in Figure II.4. The minimum amount of tissue needed for each analysis was: for organics, 1 g; for AHH, 500 mg; and for DNA adducts, 200 mg. If an individual liver was too small to provide this minimum amount of liver for each sample, then these samples may have been composited back in the lab. If the size of individual samples were so small that they would have been freezer burned, compositing needed to take place in the field.

AHH and DNA adduct samples were placed into cryovials labeled with the unique specimen number and immediately frozen in liquid nitrogen. These samples were later transferred to -80°C freezers for storage. Tissues collected for organic chemical analyses were placed into similarly labeled dichloromethane-rinsed scintillation vials with Teflon-lined lids. These samples could have been kept on ice at the necropsy station for up to 15 min until they could be placed into a -20°C freezer for storage on the research vessel. These samples were stored in a -80°C freezer after they had been transferred to the lab.

4.9. Collection of stomach contents

Contents of the stomach (the portion between the esophagus and the pyloric ceca) were removed by grasping one end of the stomach with internal dissecting tools and gently squeezing the contents into the appropriate vial. Stomach contents for organic analyses were placed into dichloromethane-rinsed, 20-ml vials with Teflon-lined lids, and held on ice for a few minutes until they could be placed into a -20°C freezer, followed by transfer to a -80°C freezer back at the laboratory. Stomach contents collected for taxonomy were composited with 5 individuals of the same species in one 4 oz glass jar, and preserved with neutral buffered Formalin.

5. HANDLING HISTOPATHOLOGY TISSUES FOLLOWING NECROPSY

To achieve optimal fixation, the volume of fixative to tissue should be 1:20, therefore no more than 30 cassettes were placed into a 2-L container of Dietrich's fixative. Tissues were allowed to remain in this fixative for at least 48 hr and no more than five days. Fixation at normal room temperature was optimal. However, if fixation at 4-5°C was necessary, then a minimum of 72 hr was required for complete fixation. Periodic agitation of containers was important during the first 48 hr to assist uniform penetration of the fixative. Once fixation was complete, cassettes containing tissues were transferred to 70% ethanol for storage until being processed in the laboratory.

6. PROCESSING AND ANALYZING HISTOPATHOLOGY TISSUES

Tissue specimens were routinely dehydrated in ascending concentrations of ethanol, cleared in xylene, infiltrated and embedded in paraffin, sectioned at 4-5 mm thickness, and stained with Gill's hematoxylin and alcoholic eosin-Y. For further characterization of specific lesions, additional sections were stained using various special staining methods such as Gmori's Iron Hematoxylin for hemosiderin, periodic acid-Schiff for glycoproteins and mucopolysaccharides, and Masson's trichrome for collagen (Thompson, 1966; Armed Forces Institute of Pathology, 1968; Preece, 1972; and Sheehan and Hrapchak, 1980). Histologic slides were coded with a randomly generated 'blind' number so that the histopathologists did not know the collection site for that specimen. Slides were then examined by light microscopy and any histopathological conditions observed in tissues were recorded and entered into a computer database. Lesion classifications followed previously described, standardized diagnostic criteria in rodents and other mammals (Frith and Ward, 1980; Jones and Butler, 1975; Ward and Vlahakis, 1978; Stewart *et al.*, 1980; and Robbins *et al.*, 1984), and fish (Hendricks *et al.*, 1984; Murchelano and Wolke, 1991; Myers *et al.*, 1987; and Hinton and Lauren, 1990a and 1990b). Consistency of diagnostic criteria among the several examining histopathologists was ensured by extensive training and periodic meetings with the chief histopathologist to review and discuss unusual and/or problematic cases.

Histopathologic diagnoses were encoded using a lesion classification code modeled after the Systematized Nomenclature of Pathology (SNOP) code, and identified as File Type 13 by the NOAA/National Ocean Data Center. Site, year, and species-specific lesion prevalences were generated from this database and then linked in parallel to the chemical contaminant data determined for each year a particular site was sampled.

7. CONCLUSIONS

A number of necropsy and sample processing techniques were employed to prevent degradation or contamination of samples. Necropsies were conducted immediately after sacrificing each fish to prevent degradation of tissue due to cell death. Separate sets of necropsy tools were used for external and internal procedures to prevent transfer of chemicals from the skin of the specimen to the internal tissue collected for analyses. Tissues for chemical analyses were quickly frozen at appropriate temperatures, or immediately placed in fixative for histopathology to prevent tissue degradation that might have interfered with accurate analyses. Proper necropsy techniques were necessary to ensure the collection of high quality samples.

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Procedure for Estimating *Clostridium perfringens* in Sediments

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ABSTRACT

This document describes the method used to assess the presence of *Clostridium perfringens* spores, an indicator of fecal contamination, in sediments collected during 1990 and 1991 for the National Oceanic and Atmospheric Administration's National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

The use of *Clostridium perfringens* as an indicator of fecal contamination has been described by Duncanson *et al.* (1986). Determination of *C. perfringens* on sediments from selected sites was conducted during 1990 and 1991 of the NOAA Status and Trends Program Mussel Watch Project. The analyses were performed by MTH Environmental Associates, Marstons Mills, MA, under subcontract to Battelle Ocean Sciences.

2. EQUIPMENT AND MATERIALS

2.1. Equipment

Anaerobic jar system, nonvented, 11-814-21. Fisher, Pittsburgh, PA.
Anaerobic gas generator, B71040. Fisher, Pittsburgh, PA.
Autoclave, 14-460-10. Fisher, Pittsburgh, PA.
Balance, analytical, 01-913-2A. Fisher, Pittsburgh, PA.
Filters, membrane, 0.45 µm, sterile, 09-730-104. Fisher, Pittsburgh, PA.
Filtration manifold, 6-place, with trap
Filtration units for 47-mm membrane filters, 09-753-1A. Fisher, Pittsburgh, PA.
Forceps, for membrane filter, 09-753-50. Fisher, Pittsburgh, PA.
Incubator, 244-925. Curtin Matheson, Wilmington, MA.
Petri dishes, sterile, 08-757-105. Fisher, Pittsburgh, PA.
Pipets, serological, sterile, disposable: 1-mL, 13-678-25B; 2-mL, 13-676-27C; 10-mL, 13-678-25E; and 25-mL, 13-678-14B. Fisher, Pittsburgh, PA.
Sample collection containers, sterile, 14-375-147. Fisher, Pittsburgh, PA.
Spatula, sterile, 14-372-1A. Fisher, Pittsburgh, PA.
Timer, 14-649-14. Fisher, Pittsburgh, PA.
Tubes, centrifuge with caps, 50 mL, 28.5-mm o.d. x 104-mm length, 05-529C. Fisher, Pittsburgh, PA.
Tubes, culture, and caps, 75-mm o.d. x 150-mm length, 14-957M. Fisher, Pittsburgh, PA.
Vacuum pump, xx5500000. Millipore Corp., Bedford, MA.
Vortex mixer, 12-812. Fisher, Pittsburgh, PA.

2.2. Chemicals

Ammonium hydroxide (NH_4OH) [1336-21-6]. Sigma Chemical Co., St. Louis, MO.
Deionized water, sterile
Sodium hexametaphosphate, sterile ($Na(PO_3)_6$) [10124-56-8]
Phosphate buffered saline (PBS) solution, sterile
The phosphate buffered saline solution was prepared by dilution from a stock solution. The stock solution contained 28.81 g anhydrous sodium phosphate monobasic (NaH_2PO_4) [7558-80-7] and 125 g anhydrous sodium phosphate dibasic (Na_2HPO_4) [7582-85-6] in 1000 mL deionized water. The working solution was prepared by mixing 20 mL of the stock solution and 8.5 g NaCl in 1000 mL of deionized water (Miescier and Cabelli, 1982).

3. PROCEDURE

The analytical procedures for *Clostridium perfringens* were based on the procedures described by Bisson and Cabelli (1979) and Emerson and Cabelli (1982), as modified by Saad and Cabelli (submitted for publication). Aseptic technique was strictly followed.

3.1. Sample collection and preservation

Samples were stored as recommended by Duncanson *et al.* (1986). The sediment samples for *C. perfringens* analysis were frozen immediately after collection, and were stored frozen until analyzed. If they were not analyzed immediately after defrosting, they were stored at 1-4°C. Once thawed, samples cannot be refrozen, but can be held ar 1-4°C.

3.2. Sample extraction

Sample extraction was performed according to Emerson and Cabelli (1982), as modified by Saad and Cabelli (in preparation). The modification substitutes a metaphosphate solution for sonication as the removal agent.

After defrosting, the sediment sample was homogenized. An aliquot of known weight was transferred to a sterile centrifuge tube, 10 mL of sterile metaphosphate solution was added, and the mixture was placed in a vortex mixer and mixed at high speed for 15 sec. Twenty five milliliters of sterile deionized water was subsequently added and the mixture was again mixed in the vortex mixer for 15 sec. The sample was allowed to settle for 10 min, and 30 mL of the supernatant removed to a sterile 25-mm test tube. The supernatant was stored on ice and analyzed within 30 min.

3.3. Sample preparation and counting procedures

The sample extract was analyzed by membrane filtration (MF) by using serial half-log dilutions of the extract according to the method described by Bisson and Cabelli (1979). The extract was filtered by using sterile membrane filters and a rinse solution of sterile phosphate buffered saline (PBS). The filters were incubated anaerobic. The filter was incubated at 44.5°C for 18 to 24 hr. After incubation, the filters were exposed to ammonium hydroxide, and all visible *C. perfringens* colonies were counted.

3.4. Calculations

Plate counts, counts of spore colonies on each plate, were converted from the number of *C. perfringens* colonies per mL to the number of spores per gram wet weight using the formula:

$$M_w = \frac{\left(\frac{N\ V_a}{V_f}\right)}{M_a}$$

where M_w is the number of spores per gram wet weight, N is the total number of spores counted, V_a is the total volume added (mL), V_f is the total volume filtered (mL), and M_a is the wet weight of sediment analyzed (g).

4. QUALITY CONTROL

The analysis for *C. perfringens* in sediments included monitoring for interference and contamination, and analytical accuracy and precision. Replicate samples from each sampling site were analyzed, but no analytical acceptance/rejection criteria were established.

4.1. Interference and contamination

Two procedural blanks were processed with each set of sample analyses; one set of samples consisted of four to six samples (12 individual assays). One blank consisted of all solutions used during sample preparation and processing in the absence of any sediment. The second blank was a 100-mL aliquot of the sterile PBS solution. This blank was used to monitor contamination of the filtration apparatus. To be acceptable, blank cultures had to have shown no growth of *C. perfringens* or any non-target organisms. If evidence of contamination appeared, that set of samples was reanalyzed.

4.2. Accuracy and precision

All samples were analyzed in duplicate, and a coefficient of variation (%CV) was determined as follows.

$$\%CV = \frac{SD}{\bar{x}}\ 100\%$$

where %CV is the coefficient of variation (%) between duplicate samples, $\bar{x}$ is the mean value of the five sets of duplicate measurements, and SD is the standard deviation of the five sets of duplicate measurements.

The acceptable limit of the %CV between the duplicate analyses was <30%. If the %CV exceeded 30%, a minimum of three assays per sample were performed. A minimum of 10% of all plates were recounted, one recount was conducted by the same analyst and a second count was done by a second analyst.

Following extraction, 2 to 10 filtrations per dilution were performed. The number of filtrations was determined based on the turbidity of the sample and the expected *C. perfringens* density.

The internal standard consisted of a sediment sample containing a known concentration of *C. perfringens.* One internal standard was processed with every 12th sample. The results had to

be within 10% of the known concentration. If this limit was exceeded, the 12 samples analyzed since the last acceptable internal standard analysis were reanalyzed.

5. CONCLUSIONS

The techniques described above effectively provide reliable estimates of the number of *C. perfringens* spores in Mussel Watch sediment samples. The presence of *C. perfringens* is an indicator of fecal contamination.

6. REFERENCES

Bisson, J. W., and V. J. Cabelli (1979) Membrane filter enumeration method for *Clostridium perfringens.* Appl. Environ. Microbiol., 37:55-66.

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Method Estimation of *Clostridium perfringens* in Sediments

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ABSTRACT

This procedure describes the method used for estimating *Clostridium perfringens* in sediments collected as part of the NOAA National Status and Trends Mussel Watch Project effort along the Gulf of Mexico Coast.

1. INTRODUCTION

This document describes the procedures for estimation of *Clostridium perfringens* in sediments used by Texas A & M University for the National Oceanic and Atmospheric Administrations' (NOAA) National Status and Trends Program. Estimates of the number of *C. perfringens* spores are used as indicators of sewage contamination of aquatic environments. *C. perfringens* spores have been used extensively as a monitor for sewage particulate deposition and movement in aquatic systems (Cabelli, 1976). The *C. perfringens* spores were extracted by the method of Emerson and Cabelli (1982) and analyzed by the mCP membrane filter method of Bisson and Cabelli (1979).

2. APPARATUS AND MATERIALS

2.1. Equipment and supplies

Anaerobic jar (GasPak anaerobic unit). Baltimore Biological Laboratory.
Membrane filter, HCWG. Millipore Corp., San Francisco, CA.
Petri dishes, 50 x 12 mm
Sonifier, 350 with ~1/8 in micro tip. Branson Ultrasonics Corp., Danbury, CT.
Test tube, 50-mL. Baxter Diagnostic, Inc., McGaw Park, IL.
Vortex Genie mixer 6-560. Scientific Industries, Bohemia, NY.

2.2. Reagents

Agar [9002-18-0]. Sigma Chemical Co., St. Louis, MO.
Ammonium hydroxide (NH_4OH) [1336-21-6]. Sigma Chemical Co., St. Louis, MO.
Bromocresol purple [115-40-2]. Aldrich Chemical Co., Milwaukee, WI.
D-Cycloserine ($C_3H_6N_2O_2$) [68-41-7]. Sigma Chemical Co., St. Louis, MO.
Ferric chloride ($FeCl_3 \cdot 6H_2O$) [10025-77-1]
Indoxyl ß-D-glucoside ($C_{14}H_{17}NO_6$) [487-60-5]. Sigma Chemical Co., St. Louis, MO.
L-cysteine hydrochloride. Sigma Chemical Co., St. Louis, MO.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) [7487-88-9]. Sigma Chemical Co., St. Louis, MO.
Polymyxin-B sulfate [1405-20-5]. Sigma Chemical Co., St. Louis, MO.
Sucrose ($C_{12}H_{22}O_{11}$) [57-50-1]. Sigma Chemical Co., St. Louis, MO.

Tryptose. Sigma Chemical Co., St. Louis, MO.
Water, sterile distilled
Yeast extract [8013-01-2]. Sigma Chemical Co., St. Louis, MO.

3. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

3.1. Sample Collection

Sediments were collected in precleaned and/or precombusted (400°C) glass jars.

3.2. Sample Preservation and Storage

Sediment samples were shipped frozen to the laboratory and stored at -20°C until analysis. After subsampling, excess sample was archived at -20°C in the dark.

4. PROCEDURES

4.1. Sample preparation

Prior to extraction, samples were thawed, mixed, and a weighed portion (5 to 10 g) was added to a sterilized 50-mL test tube containing 5 mL of sterile distilled water. Sediment samples were extracted using the method of Emerson and Cabelli (1982) prior to analysis by the *C. perfringens* mCP assay.

The distilled-water suspension of the sediment sample was pulse sonicated for 10 sec using twenty 0.5-sec pulses at 1,400 W/in^2. Another 35 mL of sterile distilled water was added to the tube and the contents of the tube were blended in a vortex mixer for 10 sec, then set aside for 10 min. Portions of the supernatant were then removed for membrane filtration.

4.2. mCP Membrane filter method

The membrane filter procedure provided a method for the rapid quantification of *C. perfringens* in aquatic environments. Background growth was inhibited by the use of D-cycloserine, polymyxic-B sulfate, and incubation at 45°C. The medium was prepared by adding the following ingredients to 90 mL distilled water:

3 g tryptose
2 g yeast extract
0.5 g sucrose
0.1 g L-cysteine hydrochloride
0.01 g $MgSO_4 \cdot 7H_2O$
0.004 g bromocresol purple
1.5 g agar

The ingredients were dissolved in the water, and the pH of the solution was adjusted to 7.6. After autoclaving the media at 121°C for 15 min, the medium was allowed to cool to 50°C and the following ingredients were added:

40 mg D-cycloserine
2.5 mg polymyxin-B sulfate
60 mg indoxyl ß-D-glucoside dissolved in 8.0 mL of sterile distilled water

2.0 mL filter-sterilized 0.5% solution of $FeCl_3 \cdot 6H_2O$

Once the medium had cooled to 50°C, it was dispensed in 5-mL quantities into sterile petri dishes (50 x 12 mm). The poured plates were stored in an anaerobic jar until use.

An aliquot of the sediment extraction (section 4.1) was then filtered. Extracts were filtered 2 to 5 times, with the number of filtrations based on the turbidity of the sample. Serial half-log dilutions of the sample extract were also analyzed by the mCP procedure. The sample or sample dilution was passed through a membrane filter, and the filter placed in a media-prepared petri dish (mCP plates). The plates were incubated anaerobically at 45°C for 18-24 hours.

The plates were exposed to ammonia vapors from ammonium hydroxide for 20-30 sec, which caused the colonies to turn a red to pink, not purple color. The colonies were then counted. A typical recovery for this procedure is about 90%.

5. CALCULATIONS

Plate counts, counts of spore colonies on each plate, were converted from the number of *C. perfringens* colonies per mL to the number of spores per gram wet weight using the formula:

$$M_w = \frac{\left[\frac{N \quad V_a}{V_f}\right]}{M_a}$$

where M_w is the number of spores per gram wet weight, N is the total number of spores counted, V_a is the total volume added (mL), V_f is the total volume filtered, and M_a is the wet weight of sediment analyzed (g).

6. QUALITY CONTROL

The analysis for *C. perfringens* in sediments included monitoring for interference and contamination, and analytical accuracy and precision. Replicate samples from each sampling site were analyzed, but no analytical acceptance/rejection criteria were established.

6.1. Interference and contamination

Procedural blanks were processed with each set of sample analyses. The blank consisted of the sterile, deionized water used during sample preparation and processing. Acceptable blank cultures showed no growth of *C. perfringens* or any non-target organisms. If evidence of contamination appeared, that set of samples was reanalyzed.

6.2. Accuracy and precision

All samples were analyzed in duplicate, and a coefficient of variation (%CV) was determined as follows:

$$\%CV = \frac{SD}{\bar{x}} 100\%$$

where %CV is the coefficient of variation (%) between duplicate samples, $\bar{x}$ is the mean value of the duplicate measurements, and SD is the standard deviation of the duplicate measurements.

The acceptable limit of the %CV between the duplicate analyses was <30%. If the %CV exceeded 30%, a minimum of four assays per sample were performed. A minimum of 10% of all plates were recounted; one count was done by the same analyst, and a second count was done by a second analyst.

The internal standard consisted of a sediment sample containing a known concentration of *C. perfringens*. One internal standard was processed with every 12th sample. The results had to be within 10% of the known concentration. If this limit was exceeded, the 12 samples analyzed since the last acceptable internal standard analysis were reanalyzed.

7. CONCLUSION

The presence of *C. perfringens* spores was determined in sediments for the NOAA NS&T Mussel Watch Project effort along the Gulf Coast as an indicator of fecal contamination.

8. REFERENCES

Bisson, J. W., and V. J. Cabelli. (1979) Membrane filter enumeration method for *Clostridium perfringens.* Appl. Environ. Microbiol., 37:55-66.

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Perkinsus marinus Assay

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ABSTRACT

This document describes the procedures followed for the assay of *Perkinsus marinus* prevalence and infection intensity in oyster specimens collected on the Gulf Coast of the US by Texas A&M University for the National Oceanic and Atmospheric Administration's National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

The determination of the health of oyster populations requires an analysis of *Perkinsus marinus* prevalence and infection intensity. *P. marinus* is the most serious disease-producing pathogen of Gulf coast oysters. This standard operating procedure is the accepted best method for quantitating the presence of this parasite when an approximate or exact quantification of disease intensity is required. A tissue homogenate or a section of mantle tissue is incubated in thioglycollate medium for 14 days according to the method of Ray (1966). A semiquantitative (Craig *et al.*, 1989) or quantitative (Choi *et al.*, 1989) assessment of hypnospore number is then made microscopically.

2. EQUIPMENT AND SUPPLIES

2.1. Reagents

Chloramphenicol (chloromycetin), ($C_{11}H_{12}C_{12}N_2O_5$) [56-75-7], 1 g lyophilized powder vials; only available from veterinary supply houses.

Fluid thioglycollate medium, powder, T9032. Sigma Chemical Company, St. Louis, MO.

Iodine, (I_2) [7553-56-2], I3380, purity 99%. Sigma Chemical Company, St. Louis, MO.

Nystatin ($C_{47}H_{75}NO_{17}$) (mycostatin) [1400-61-9], 500,000 unit lyophilized powder, N3503. Sigma Chemical Company, St. Louis, MO.

Potassium iodide (KI) [7681-11-0], P8256. Sigma Chemical Company, St. Louis, MO.

Potassium phosphate, monobasic, (KH_2PO_4) [7778-77-0], purity 99+%, P5379. Sigma Chemical Company, St. Louis, MO.

Sodium chloride (NaCl) [7647-14-5], Purity > 99.5%, S9625. Sigma Chemical Company, St. Louis, MO.

Sodium hydroxide (NaOH) [1310-73-2], S5881. Sigma Chemical Company, St. Louis, MO.

2.1.1. Thioglycollate medium preparation

A mixture of 20 g NaCl, 29.3 g dehydrated fluid thioglycollate medium, and 1 L distilled water was heated while stirring until the medium dissolved and the solution become a transparent golden-yellow color. After cooling, the solution was dispensed, 10 mL at a time, into 18-mL culture tubes which were subsequently autoclaved and sealed.

Thioglycollate maintains anaerobic conditions in the culture tube as well as providing needed nutrients and an appropriate osmotic environment. Therefore, tubes were sealed tightly and opened only briefly for injection of antibiotic and addition of tissue as subsequently described. After autoclaving, prepared tubes were stored in the dark until use and then returned immediately to the dark for tissue incubation. Unused, autoclaved tubes of medium can be stored for many months in the dark without deterioration. Occasionally, tubes became cloudy or the medium congealed. In both cases, these individual tubes were discarded. However, the remaining tubes in a batch remained usable.

2.1.2. Antibiotic solution

Five milliliters of sterile water were added to a 1-g vial of chloromycetin (chloramphenicol) sterile powder using a sterile needle and syringe. Ten milliliters of sterile water were added to a 500,000-unit vial of mycostatin (nystatin) powder with a sterile needle and syringe. Both vials were shaken to dissolve the solids. Using a sterile needle and syringe, 2.5 mL of chloromycetin solution were added to the mycostatin vial, the mixture shaken, and refrigerated until use. The solution can be stored safely for several weeks if refrigerated.

2.1.3. Lugol's iodine solution

Four grams of potassium iodide and 2 g of iodine crystals were dissolved in 100 mL of distilled water, allowed to stand 24 hr, and filtered. The solution was stored in a dark bottle. The solution remained stable for many weeks.

2.1.4. PBS(II)

PBS(II): 0.15M NaCl, 0.01M KH_2PO_4, pH=7.4

2.2. Equipment

Autoclave
Automatic pipette or repipettes, 50 µL and 200 ml capacity, 13-707-30. Fisher Scientific, Pittsburgh, PA.
Brinkmann polytron tissue homogenizer, Brinkman Instrument Co., Westbury, NY.
Heater/stirrer, Corning, 11-495-52C. Fisher Scientific, Pittsburgh, PA.
Microscope, Wild dissecting. Fisher Equivalent Stereomaster Zoom, 12-598-14. Fisher Scientific, Pittsburgh, PA.
Repipettes, 10-50 ml adjustable volume, 13-688-70. Fisher Scientific, Pittsburgh, PA.
Slide warmer, catalogue number 12-594. Fisher Scientific, Pittsburgh, PA.
Vortex mixer, Vortex-Genie 2 mixer, 12-812. Fisher Scientific, Pittsburgh, PA.

3. TISSUE COLLECTION

Twenty large oysters were collected from each field location. Analysis of twenty oysters was sufficient to determine prevalence and infection intensity. Comparisons of replicate samples of twenty specimens have consistently shown significant differences no more frequently than expected by chance (Craig *et al.*, 1989; Wilson *et al.*, 1990). Oysters were immediately put on ice but not allowed to be submerged in fresh water by allowing meltwater to drain continuously from the containers, and returned to the laboratory where they were assayed within 24 hr of collection.

Each oyster was opened with an oyster knife following the protocol used for hydrocarbon tissue collection (Wade *et al.*, Volume IV, this document). Maximum anterior-posterior length, as defined by Morales-Alamo and Mann (1989), was recorded by visual inspection. Using sterile dissecting scissors and forceps, a small piece of approximately 1 cm^2 of mantle tissue was excised from the lower valve just posterior to the palps. The tissue was placed in a culture tube containing the thioglycollate medium to which 50 µL of chloramphenicol-nystatin antibiotic solution was added.[Δ]

The tube was immediately recapped and shaken by inversion several times to assure that the tissue was submerged in the medium. When processing many samples, it was convenient to add the antibiotic solution to the vials prior to, but not earlier than, 24 hr before opening the oysters. The culture tubes were placed in the dark at room temperature and incubated for 14 days. At the end of 14 days, the tissue was analyzed or, if inconvenient, the tube was placed in a refrigerator in the dark. Tissues ready to be analyzed can be kept for at least 3 months without deterioration if the culture tubes are kept dark and refrigerated.

4. TISSUE ANALYSIS

4.1. Semiquantitative method

After the incubation period, the oyster tissue was removed from the culture tube using a sterile needle and placed on a microscope slide. The tissue sample was teased apart using sterile needles to assure even staining with Lugol's iodine solution. The tissue was flooded with Lugol's solution using a pasteur pipette, covered with a cover slip and examined microscopically. *P. marinus* hypnospores appear as black spheres of 10 to 200 µm diameter when viewed through a dissecting microscope. Usually, hypnospores exceed 40 µm in size, however, occasionally hypnospores develop without enlargement. An infection intensity was assigned to each tissue sample based on the number or coverage of enlarged *P. marinus* hypnospores observed in the tissue using the scale in Table II.7. Note that the scale ranks tissue by the number or coverage of hypnospores after enlargement. Accordingly, those tissues where enlargement occasionally fails to occur must be ranked on Mackin's scale as if the cells were enlarged.

The technique depends on the assumption discussed by Ray (1954) that hypnospores develop from single *P. marinus* cells without replication and that all *P. marinus* cells develop into hypnospores. Gauthier and Fisher (1990) discuss an adaptation of this method using oyster hemolymph which can be used to non-destructively follow the progression of *P. marinus* infection.

To maintain quality control, blind assays were conducted among the readers to correct for any technician bias that may be present with any semiquantitative technique. We encourage other users to standardize their analyses with laboratories already using the technique so that data are comparable.

[Δ] Ray (1966) discusses the use of various antibiotics. Although certain laboratories use alternative antibiotics, both Ray (1966) and Quick and Mackin (1971) concluded that the recommended combination of nystatin and chloramphenicol was superior.

Table II.7. Semiquantitative scale of infection intensity for *Perkinsus marinus* [adapted from Mackin (1962) by Craig *et al.*, (1989)].

Letter designation	Infection intensity	Numerical value	Description
N	Negative	0.0	No hypnospores present
VL	Very light	0.33	1-10 hypnospores
L-		0.67	11-74 hypnospores
L	Light	1.00	75-125 hypnospores
L+		1.33	> 125 hypnospores but much less than 25% of tissue is hypnospores
LM-		1.67	< 25% of tissue is hypnospores
LM	Light/moderate	2.00	25% of tissue is hypnospores
LM+		2.33	> 25% but much less than 50% of tissue is hypnospores
M-		2.67	> 25% but < 50% of tissue is hypnospores
M	Moderate	3.00	50% of tissue is hypnospores
M+		3.33	> 50% but much less than 75% of tissue is hypnospores
MH-		3.67	> 50% but < 75% of tissue is hypnospores
MH	Moderately heavy	4.00	75% of tissue is hypnospores
MH+		4.33	> 75% but much less than 100% of tissue is hypnospores
H-		4.67	> 75% of tissue is hypnospores but some oyster tissue is still visible
H	Heavy	5.00	Nearly 100% of tissue is hypnospores

4.2. Quantitative method.

The assessment of infection intensity using a piece of mantle tissue is rapid, inexpensive, and can be done with little tissue loss on animals destined for body burden analysis, permitting a direct comparison. However, use of a single tissue section introduces three potentially significant inaccuracies.

The single-tissue section method does not quantitatively assess cell number although an approximate conversion to cell number can be used (Section 5).

Tissue-to-tissue heterogeneity in infection intensity was typically as large as two levels on Mackin's scale (Table II.7) (e.g., from L- to L+).

False negatives were frequently encountered at low infection levels. A lethal infection from a single infective cell required on the order of 22 to 28 doublings of the *P. marinus* population. The first 10 to 12 were likely to be read as negative because cell density was low enough that

a piece of mantle tissue examined may easily not contain any cells. This error reduced the value of prevalence as a primary determinant of health because the method used was particularly poor at identifying truly uninfected oysters.

A quantitative method measuring the total body burden of parasites resolves these three inaccuracies but requires substantially more technician time and, obviously, the use of entire animals.

After shucking, the oyster meat was homogenized using a Brinkmann Polytron tissue homogenizer at level 3 (moderately-low speed) for 2 min. The homogenized oyster tissue was incubated in thioglycollate medium as described previously. After 14 days, the volume of the mixture in each flask was measured using a graduated cylinder. The mixture was poured back into the original flask and stirred vigorously. A 30-mL subsample was immediately removed and placed into a 50-mL tube. The subsample was centrifuged at 6000 rpm for 10 min and the supernatant discarded. Approximately 30 mL of 2 M NaOH were added to each pellet and the mixture incubated at 50°C in a water bath for 1 hr. After incubation, the sample was centrifuged at 6000 rpm for 15 min and the supernatant was removed. The pellet was resuspended in 30 mL of phosphate buffered saline (PBS II) and centrifuged using the same conditions. The pellet was washed two additional times. The pellet volume was measured. This required the addition of some PBS II in some instances. The suspension was stirred using a Vortex mixer. Aliquots of 100-µL were taken and 100 µL of Lugol's solution was added to each. The number of hypnospores was counted in at least 10 100-µL aliquots from each sample using a hemacytometer and the mean number of hypnospores reported.

5. CALCULATIONS

Data were reported as prevalence (percent infected) and mean or median infection intensity calculated using the semiquantitative scale in Table II.7. The calculation of median infection was frequently desirable because the semiquantitative scale used was truncated at both extremes and infection intensity in a sampled population was often not normally distributed. In particular, a few relatively heavily infected individuals were frequently encountered in populations characterized by low overall infection intensities, and uninfected individuals could be collected in most populations at certain times of the year because transmission rates are sufficiently slow. If the quantitative scale was desired, data were reported as number of *P. marinus* cells per gram wet weight of oyster tissue and then converted into the semiquantitative equivalent using the formula:

$$\text{number of hypnospores} = 1409.9\ (10^{0.64296x}),$$

where x is the semiquantitative designation (Choi *et al.*, 1989).

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Development of an Immunological Probe for Quantification of Oyster Gonad

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ABSTRACT

This document presents a quantitative method to measure the amount of gamete tissue in the oyster, *Crassostrea virginica,* based on the use of polyclonal antibodies developed from oyster eggs and sperm. Eggs and sperm are purified using a Percoll gradient and injected into rabbits. The specificity of antibodies harvested from rabbit serum was assayed by an Ouchterlony double immunodiffusion test and cross-reacting antibodies absorbed with acetone-dried somatic tissue powder. Single ring immunodiffusion (SRID) was used in the quantitation of gamete tissue. Rabbit anti-oyster egg IgG used in SRID detected 50 µg/mL to 10 mg/mL oyster egg protein present in oyster homogenate obtained from the National Oceanic and Atmospheric Administration's National Status and Trends Program Mussel Watch Project samples used for hydrocarbon analysis. This technique provided a significant improvement in the assessment of reproductive condition over the standard histological examination of gonadal stage.

1. INTRODUCTION

Immunological techniques are excellent tools for investigating diverse research interests in biological and environmental sciences. Immunological techniques are based on an antigen-antibody reaction. Antigen is a foreign material, usually one or more proteins isolated from a subject species and introduced into a host animal. As a result of a periodic exposure to the antigen, antibodies are formed in the host animal. This antibody will then react with the antigen in the subject species using various immunological methods (Garvey *et al.*, 1977; Yentsch *et al.*, 1988; Ward, 1990). The attractiveness of immunological methods arises from the high specificity and sensitivity for specific antibody molecules. Immuno-precipitation and immuno-electrophoresis have been successfully employed in the study of phylogenetic relationships among species and races of oysters (Numachi, 1962; Li *et al.,* 1967; and Brock, 1990), as well as in the studies of gut-content analysis and marine food-webs (Feller *et al.,* 1979 and 1985; Feller 1984a. and 1984b; Feller, 1986; Feller and Gallagher, 1982; and Hentschel and Feller, 1990). Immuno-fluorescence assay has become a popular method in studying marine microbial diversity and productivity (Ward, 1982; Ward and Carlucci, 1985; Campbell *et al.,* 1983; Currin *et al.,* 1990; Shapiro *et al.,* 1989; Dahale and Laake, 1982; and Smith, 1982).

In bivalves, with few exceptions, gonadal tissue cannot be cleanly excised and weighed. Accordingly, a quantitative gonadal index has not been available. The most widely and extensively used method for determining reproductive condition is histological examination of gonadal tissue to determine gonadal stage. In brief, a section of oyster tissue is fixed, embedded in paraffin, sliced, stained, and examined under a microscope. From the histological examination, the stage in the gametogenic cycle (early development, late development, fully developed, etc.), is scored as a numerical code (Loosanoff, 1965; Gauthier and Soniat, 1988). A semiquantitative improvement to this quantitative score is obtained by measuring the percent area of gonad that occupies the prepared slide, or the thickness of the gonad (Hays and Menzel, 1981; Morales-Alamo and Mann, 1989). These techniques provide semi-quantitative

information on oyster reproduction at best. They do not provide a quantitative measurement of gametic tissue.

One way to obtain a quantitative measure of gametic tissue is to induce spawning. Animals are induced to spawn using chemicals or thermal shock, and the number of gametes released are counted or the weight of gametes is measured (Davis and Chanley, 1956; Sprung 1983). However, this method can only be used at the latest stage of the gametogenic cycle and it may underestimate absolute reproductive output because spawning is not always complete (Lucas, 1982).

We have developed immunological probes for oyster *(Crassostrea virginica)* female and male gametic tissue and used them to quantitatively measure the amount of gametic tissue present. This quantitative technique can be used throughout the gametogenic cycle and, as importantly, can utilize a pooled tissue homogenate as obtained for hydrocarbon extraction using the NS&T protocol. As a result, a direct comparison of gamete content and contaminant body burden is available. This chapter describes the procedures involved in probe development and the quantitative measurement of reproductive tissue.

2. EQUIPMENT AND REAGENTS

2.1. Instrumentation

Aspirator

Balance, Satorius portable PT120. Sartorius Corp., Bohemia, NY.

Centrifuge, Dynac Centrifuge. 0065; Clay Adams, Parsippany, NJ; or, IEC B-22 Centrifuge, 3493; Needham Heights, MA.

ELISA plate, 12 x 8 well, 2580596. Corning Inc., Corning, NY.

Gel puncher; Gel Puncher 4.0 mm diameter. Bio-Rad lab., Richmond, CA.

Glass plate, 10 cm x 10 cm

Hemacytometer, Reichert-Jung, Bright-Line Hemacytometer. Cambridge Instruments, Inc., Buffalo, NY.

Humidity chamber: A rectangular plastic container (30 cm x 20 cm x 5 cm) with a lid and sheets of paper towels soaked with water and placed on the bottom of the container.

Leveling table, Bio-Rad 20 x 30 cm. Bio-Rad Lab., Richmond, CA.

Microscopes, Zeiss or equivalent: 25x magnification required for dissecting scopes; and 100x required for compound microscopes.

Tissue grinder, Pyrex Brand Ten Broeck Tissue Grinder, 77277. Corning Inc., Corning, NY.

Ultrasonifier, Branson Sonifier II Cell Disrupter (Sonifier 250), 101-063-196. Branson Ultrasonic Co., Danbury, CT.

2.2. Chemicals

Standard reagent grade is acceptable, except where otherwise indicated.

Acetic acid, glacial ($C_2H_4O_2$) [64-19-7], A6283. Sigma Co., St. Louis, MO.

Acetone (CH_3COCH_3) [67-64-1], A5765. Sigma Co., St. Louis, MO.

Agarose [9012-36-6] 162-0100. Bio-Rad Lab, Richmond, CA.

Barbital (Barbiton) ($C_8H_{12}N_2O_3$) [57-44-3], B0375. Sigma Co., St. Louis, MO.

BCA Protein Assay, 23225. Pierce Co., Rockford, IL.

Bovine serum albumin, A2153. Sigma Co., St. Louis, MO.

Citric acid ($C_6H_8O_7$) [77-92-9], C0759. Sigma Co., St. Louis, MO.

Coomassie Brilliant Blue ($C_{26}H_{16}N_3Na_3O_{10}S_3$) [6104-58-1], 161-0400. Bio-Rad, Richmond, CA.

Dextrose (a-D glucose) ($C_6H_{12}O_6$) [492-62-6], G7528. Sigma Co., St. Louis, MO.

Ethanol (C_2H_5OH) [64-17-5]

Freund's adjuvant complete (FAC) [9007-81-2], F4258. Sigma Co., St. Louis, MO.

Freund's adjuvant incomplete (FAI), F5506. Sigma Co., St. Louis, MO.

Percoll (colloidal PVP coated silica for cell separation) [65455-52-9]. Sigma Co., St. Louis, MO.

Potassium dihydrogen phosphate (KH_2PO_4) [7758-11-4], P3785. Sigma Co., St. Louis, MO.

Sodium azide (NaN_3) [26628-22-8], S2002. Sigma Co., St. Louis, MO.

Sodium barbital ($NaC_8H_{12}N_2O_3$) [144-02-5], B0500. Sigma Co., St. Louis, MO.

Sodium chloride (NaCl) [7647-14-5] S7635. Sigma Co., St. Louis, MO.

Sodium citrate ($C_6H_6Na_2O_7$) [6132-04-3], S4644. Sigma Co., St. Louis, MO.

Sodium dihydrogen phosphate (NaH_2PO_4) [7558-79-4], S9763. Sigma Co., St. Louis, MO.

Tannic acid ($C_{76}H_{52}O_{46}$) [1401-55-4], T0125. Sigma Co., St. Louis, MO.

2.3. Reagents

2.3.1. Reagents to adjust buffer pH

HCl: 1 to 12 N for buffer pH adjustment
NaOH: 1 to 7 N for buffer pH adjustment

2.3.2 Other reagents

Phosphate-buffered saline (PBS) solution (used in the preparation of tanned blood cells)[◊]

0.15M NaCl
0.003M KCl
0.01M NaH_2P0_4
0.01M KH_2P0_4, pH should be 7.4.

10X PBS

1.5 M NaCl, 0.03 M KCl, 0.1 M sodium phosphate, 0.1 M potassium phosphate, pH 7.4.

100% Percoll [Δ]

Mix 9 parts of stock Percoll and 1 part 10X PBS.

70% Percoll

Mix 3 parts 10X PBS and 7 parts 100% Percoll.

60% Percoll

Mix 4 part 10X PBS and 6 parts 100% Percoll.

[◊] All buffers mixed to achieve final molarities.

[Δ] Even though stock Percoll has been diluted 9 parts Percoll to 1 part 10X PBS this solution will be referred to as "100% Percoll" to agree with immunological nomenclature.

Protein Assay Standard

2 mg/mL, 1.5 mg/mL, 1 mg/mL, 0.7 mg/mL, 0.5 mg/mL, 0.3 mg/mL., and 0.1 mg/mL bovine serum albumin. Obtained by serial dilution using distilled water.

McIlvain Buffer

0.05 M citric acid, 0.10 M sodium phosphate, pH is adjusted to 4.9.

PBS(I)

0.75 M NaCl, 0.075 M Potassium dibasic phosphate, pH is adjusted to 7.4.

PBS(II)

0.15 M NaCl, 0.01 M potassium dibasic phosphate, pH is adjusted to 7.4.

PBS(III)

0.1 M NaCl, 0.01 M potassium dibasic phosphate, pH is adjusted to 7.4.

Alsever's solution

20.5 g dextrose
8 g of sodium citrate (dihydrate)
0.55 g of citric acid (monohydrate)
4.20 g of NaCl

The listed chemicals were dissolved in 1 L of distilled water. After dissolving the chemicals, the solution was autoclaved for 15 min to eliminate any microorganisms and the pH adjusted to 6.1. Alsever's solution is an anticoagulant blood preservative that permits the storage of whole blood at refrigerator temperature for about 10 weeks.

Tannic acid

0.05% (w/v) tannic acid in PBS (II) solution. Tannic acid permits antigen molecules to adhere to the surface of red blood cells.

1.5% Agarose gel

Dissolve 1.5 g of agarose in 100 mL distilled water heated to boiling. During cooling, 0.5 g sodium azide was added to the solution as a preservative. Twenty-mL aliquots of dissolved agarose were placed in glass tubes, cooled until solidified and then stored at 4°C. The agarose gel can be liquified by placing the solidified gel tube in boiling water.

Staining solution (0.5% Coomassie brilliant blue)

5 g Coomassie brilliant blue dissolved in 450 mL distilled water, 450 mL ethanol, and 100 mL glacial acetic acid.

Destaining solution

450 mL distilled water, 450 mL ethanol, and 100 mL acetic acid

Barbital buffer

0.017 M barbital and 0.083 M sodium barbital. The solution was prepared by dissolving barbital in boiling water. The solution must be completely dissolved to avoid recrystallization of the buffer. Sodium barbital was added while the solution cooled. After cooling, the solution pH was adjusted to 8.6.

1.5% agarose in barbital buffer

1.5 g of agarose was dissolved in 100 mL boiling barbital buffer while stirring and 0.5 % (w/v) sodium azide was added as a preservative.

1.5% agarose in PBS

1.5 g of agarose was dissolved in 100 mL boiling PBS while stirring and 0.5% (w/v) sodium azide was added as a preservative.

Sodium azide solution

0.5% (w/v) sodium azide.

2.4. Animals

New Zealand white rabbits
Sheep (for collecting red blood cells for hemagglutination)
Oyster *(Crassostrea virginica)* (for gonadal tissue and somatic tissue such as gill, mantle, and adductor muscle)

3. PROCEDURE

3.1. Collection of gametic tissue

3.1.1. Gametic tissue probe development

It is essential to collect a large quantity of oyster eggs or sperm for the development of an immunological probe. Since reproductive effort of oysters is often higher during the spring spawning pulse than the fall spawning pulse, collection of reproductively-active oysters should be done during spring to early summer.

3.1.2. Collection of gametic tissue for NS&T Program

Samples for analysis were obtained as a 1-mL aliquot of the homogenate used for hydrocarbon analysis (Wade *et al.*, Volume IV, this work) and frozen at -40°C until analysis. Analysis was performed within 6 months. Holding tissues for more than a year may result in loss of antigenicity.

3.1.3. Purification of oyster eggs

Ripe oysters were shucked, and the meats rinsed with PBS solution to remove associated sediment. The presence of eggs was then confirmed by examining a smear slide using a compound microscope.

Gonadal tissue containing ripe eggs was excised using scissors and forceps. Gonadal tissue around the adductor muscle (i.e. posterior) usually provides the most eggs because more eggs are present in this region than more anteriorly, when oysters are reproductively mature.

Gonadal tissues were placed in a petri dish and some PBS solution was added. Gonadal tissue was gently squeezed using a rubber-headed syringe piston (5 to 10-mL syringe) to extract the eggs from the connective tissue. The extracted eggs were transferred to another petri dish using a pasteur pipette. Some PBS solution was added to the remaining gonadal tissue and the tissue squeezed again to extract any residual eggs. The extracted eggs were pooled into one container.

The crude egg extract was filtered through a 100-µm mesh screen to remove tissue debris.

The oyster egg filtrates were diluted using 1 part PBS to 1 part filtrate, and 10 mL of the diluted oyster egg filtrate was placed in a 15-mL polystyrene centrifuge tube with a conical bottom. The solution was centrifuged at 700 xg* for 10 min. After centrifugation, any tissue debris or egg fragments which are sedimented over intact eggs were removed using a pasteur pipette connected to an aspirator. Ten mL of PBS solution was added to the tube and the intact eggs were resuspended. The tube was centrifuged at the same speed and for the same time, and the tissue debris removed. This procedure was repeated 3 to 4 times. After final rinsing, the egg pellet was resuspended by using 2 parts PBS solution per 1 part oyster egg pellet.

Thirty-five mL 60% Percoll solution was mixed with 5 mL of the oyster egg suspension in a 50-mL conical-bottomed polystyrene centrifuge tube, and centrifuged at 900 xg for 15 min. After spinning, isolated eggs form an aggregate at the top of the tube and impurities such as sediment particles and tissue debris are pelleted at the bottom.

Isolated oyster eggs were harvested using a pasteur pipette and transferred into a centrifuge tube.

One part of the isolated egg pellet was resuspended with 5 parts of PBS solution, placed in a centrifuge tube, and centrifuged at 900 xg for 10 min to remove silicon particles in the Percoll solution. This rinsing step was repeated 3 times.

Some purified eggs to be used for counting and measuring dry weight and protein content of individual eggs were stored at 4°C. The remaining eggs were frozen at -40°C until used.

3.1.4. Purification of oyster sperm

Ripe male oysters were shucked, and the meats rinsed with PBS solution to remove associated sediment.

Gonadal tissue containing ripe sperm was excised using scissors and forceps. Gonadal tissues around the adductor muscle (i.e. posterior) normally contain more sperm than other parts of the body mass and should be used selectively.

Gonadal tissues were placed in a petri dish and enough PBS solution added to provide an extraction medium. Gonadal tissues were gently squeezed using a rubber-headed syringe piston (5 to 10-mL syringe) to extrude sperm from the connective tissue. The extracted

* xg - each unit equals the acceleration of 980 cm/sec2

sperm were pipetted to another petri dish. Some PBS was added to the remaining gonadal tissue and the tissue squeezed again to extract any residual sperm. The extracted sperm were pooled into one container.

The crude sperm extract was filtered through a 30-µm mesh screen to remove tissue debris. An equal volume[◊] of PBS solution was added to the sperm filtrate, and 10 mL crude sperm filtrate was placed on a 15-mL polystyrene centrifuge tube with a conical bottom. The solution was centrifuged at 700 xg for 15 min. After centrifugation, tissue debris sedimented over the sperm was removed with a pasteur pipette connected to a vacuum pump and the sperm resuspended in 10 mL of PBS solution. The tube was then centrifuged at the same speed and for the same time, and the tissue debris found over the sperm removed. This procedure was repeated 3 to 4 times. After final rinsing, the sperm preparation was resuspended into an equal volume of PBS.

Thirty-five milliliters 70% Percoll was added to 5 mL sperm suspension in a 50-mL polystyrene centrifuge tube. The tube was then centrifuged at 900 xg for 20 min. Oyster sperm aggregate and form a layer in the Percoll column, while sediment particles and other impurities are sedimented at the bottom of the tube.

Isolated oyster sperm was pipetted into a centrifuge tube. Silicon particles contained in the Percoll fluid were removed by adding 5 parts of PBS solution to 1 part of the isolated sperm tissue and centrifuging at 700 xg for 10 min.

Purified oyster sperm was stored at -40°C until used.

3.2. Determination of protein content in oyster eggs and sperm

3.2.1. Determination of egg dry weight

The number of eggs present in the egg suspension was determined using a hemacytometer. The egg suspension was then centrifuged at 700 xg for 10 min. After centrifugation, the supernatant was discarded and the egg pellet frozen at -40°C.

The frozen egg pellet was lyophilized.

Lyophilized egg dry weight was determined using a balance. The individual egg dry weight was calculated by dividing the dry weight of the egg pellet by the number of eggs present in the egg pellet. An individual oyster egg weighs approximately 13 ng dry weight.

3.2.2. Determination of egg protein content

A known quantity (e.g., 500 mg purified oyster eggs) was added to a 50 mL polystyrene centrifuge tube and mixed with a known quantity of PBS (e.g,. 20 mL PBS) solution.

Oyster eggs in PBS were sonicated using an ultrasonifier using a Branson sonifier 250 equipped with microtip set at level 6 for 2 min. To prevent excessive warming of oyster egg homogenate during sonication, the tube was placed in an ice-filled beaker. After sonication, egg homogenate was centrifuged at 6000 xg for 20 min to precipitate the insoluble material.

[◊] The procedure is based on unknown or flexible volumes of sperm filtrate. Subsequent volumes are then provided in equal portion to the starting sperm filtrate volume.

The quantity of soluble protein contained in the egg homogenate was determined using the BCA Protein Assay. Total egg protein generally accounts for 37% to 40% of egg dry weight.

3.3. Determination of protein content in oyster sperm

The sperm preparation was lyophilized and its dry weight determined using a balance.

A known quantity of oyster sperm was resuspended in a known volume of PBS solution (e.g., 200 mg purified oyster sperm in 20 mL PBS).

The sperm suspension was then homogenized using an ultrasonifier as previously described. After sonication, the sperm homogenate was centrifuged at 6000 xg for 20 min to remove the insoluble material.

The quantity of soluble protein in the homogenate was measured using the BCA Protein Assay.

3.4. Immunization of rabbits with purified oyster eggs and sperm

New Zealand white rabbits were selected as a host animal to raise antibodies against oyster egg or sperm protein. The protocol used in the production of antiserum is summarized in Table II.8. Freund's adjuvant complete, which contains a water-in-oil emulsion and a mycobacteria cell suspension as an antibody production stimulant, was used for the initial injection. Subsequent injections used Freund's adjuvant incomplete which lacks mycobacteria. For injection, the antigen (i.e., oyster egg or sperm protein) was well-mixed with Freund's adjuvant and injected into the rabbit subcutaneously at the hind legs. The total volume of injected material was adjusted to 1 mL. This 1 mL was injected in 200 µL aliquots at several different sites around the hind legs.

Table II.8. Summary of immunization protocol.

1st Week	Initial injection	1 mg Antigen in 500 µL + 500 µL FAC
2nd Week	Booster	500 mg Antigen in 500 µL + 500 µL FAI
3rd Week	None	None
4th Week	Booster and test bleeding	500 mg Antigen in 500 µL + 500 µL FAI
5th Week	Booster	500 mg Antigen in 500 µL + 500 µL FAI
6th Week	Booster	500 mg Antigen in 500 µL + 500 µL FAI
7th Week	None	None
8th Week	Test bleeding	None

FAC: Freund's adjuvant complete. FAI: Freund's adjuvant incomplete.

At the 4th and 8th week after injection, 10 mL of test blood was withdrawn from the rabbit and the antiserum isolated from the red cells. To do this, the blood was collected in a clotting vial which was then stored at 4°C overnight or at room temperature for 10 hr. After clotting, the blood was centrifuged at 700 xg for 15 min, the antiserum pipetted into a centrifuge tube, and stored at 4°C.

The immune response of the rabbit was identified using passive hemagglutination and ring immunodiffusion techniques for examining the strength of the antibody according to Garvey *et al.* (1977). The technique is described below.

3.4.1. Ring immunodiffusion test

This is the simplest method to detect the antibody-antigen reaction.

A 300-µL aliquot of the antiserum to be tested was placed in a test tube (5 cm long, 5 mm diameter).

A 300-µL aliquot of antigen (e.g., 3 mg/mL oyster egg protein or sperm protein) was carefully layered on top of the antiserum. The tube was incubated at room temperature for at least 2 hr.

A positive reaction resulted in precipitation at the antiserum-antigen inter-layer. The degree of precipitation varies depending on the strength of the antiserum.

3.4.2. Passive hemagglutination test

3.4.2.1. Preparation of formalinized sheep erythrocytes

Freshly-drawn sheep blood was mixed in an equal volume of Alsever's solution to prevent coagulation.

The sheep blood was centrifuged at 700 xg for 10 min. The erythrocytes were rinsed 4 times with 7 to 10 volumes of PBS(I).

After final washing, the erythrocytes were brought up to a 10% concentration in PBS(I).

An equal volume of 3.7% formaldehyde was added to the erythrocyte preparation.

The suspension was allowed to stand, with occasional stirring, at room temperature for 4 to 6 hr. After the initial incubation, the suspension was continuously stirred at either 30°C or 37°C for 14 to 18 hr.

The formalinized sheep erythrocytes were washed 4 times, each time with 10 volumes of PBS(I), and then stored as a 10% concentration in PBS(I) at 4°C.

3.4.2.2. Preparation of antigen-coated cells

The formalinized sheep red blood cells (RBC) were centrifuged and washed once with PBS(II).

A 0.5-mL aliquot of packed RBC were suspended in 25 mL 0.05% tannic acid and incubated for 45 min at 37°C, with intermittent mixing.

The RBC in tannic acid were washed 3 times with 20 volumes of PBS(II), centrifuging at 700 xg for 10 min after each wash. The final wash was done using McIlvain Buffer.

The RBC were resuspended in 12 mL of McIlvain Buffer. The tanned RBC remain usable for periods up to 18 hr if kept at 4°C.

Antigen (i.e., oyster egg protein or sperm protein) was added to the tanned RBC in the proportion 3.5 mg antigen: 0.5 mL tanned RBC. The mixture of antigen and RBC were incubated at 37°C for 1 hr.

After incubation, the antigen-coated RBC were rinsed 3 times, each time with 3 volumes of PBS(II).

The antigen-coated RBC were resuspended in PBS(II) with 0.07% bovine serum albumin at 1% concentration to avoid agglutination of the RBC.

3.4.2.3. Test procedure

Freshly-harvested antiserum to be tested was generally diluted to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256; 1:512, 1:1024, and 1:2048 in PBS(II).

A 50-µL aliquot of the serially-diluted antiserum was added to each well of a 12 x 8 well microplate.

A 50-µL aliquot of the antigen-coated RBC was added onto each well containing serially-diluted antiserum and the plate shaken using a microplate shaker to mix the RBC and the antiserum.

The plate was incubated for 2 to 4 hr at room temperature.

After incubation, the pattern formed by the RBC at the bottom of each well was examined according to the following criteria.

Positive: compact granular agglutination of diffuse film of agglutinated cells covering the bottom of the well; edges of film either folded or somewhat ragged.

Neutral: narrow ring of cells surrounding a diffuse film of agglutinated cells.

Negative: heavy ring of cells or a discrete smooth button of cells in the center of well.

The titre of the antiserum, which is the greatest dilution of a given antiserum that still elicits a positive reaction to antigens, increases as the immunization procedure progresses. However, the titre attains a stable level and will not increase with further booster injections at 2 to 3 months following the initial injection (Figure II.5). Once a host animal shows a maximum immune response, the animal is either exsangunated or maintained at the same high level of antiserum by booster once each 4 to 6-month period.

3.4.3. Evaluation of specificity of antiserum

Polyclonal antibodies often exhibit cross-reactions with non-target tissues. Rabbit antiserum raised from oyster egg or sperm protein normally exhibits a slight cross-reactivity with somatic tissue, especially mantle. The following procedure describes Ouctherlony's double diffusion method (Ouctherlony and Nilsson, 1973), and the removal of non-specific antibody present in the antiserum using absorption with acetone-dried tissue powder.

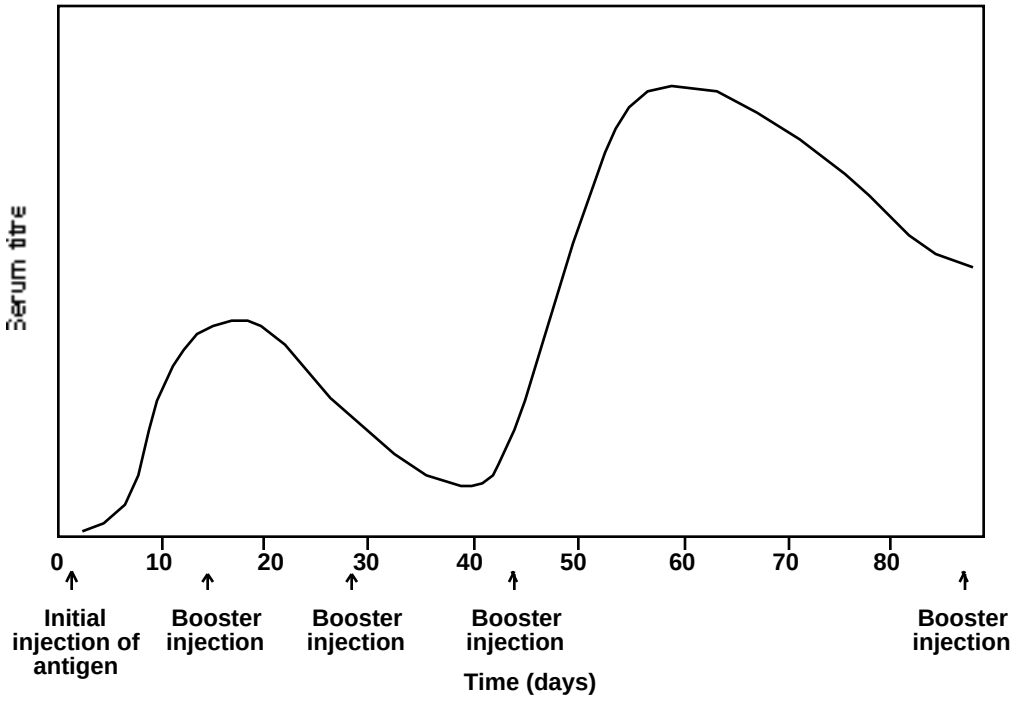

Figure II.5. Response of antibody as immunization progresses.

3.4.4. Ouchterlony double immunodiffusion test for antiserum specificity

The double immunodiffusion test is used for visualizing the antibody-antigen reaction. The antibody-antigen reaction in double immunodiffusion results in precipitation lines of the antibody-antigen complex in the gel matrix. The complex can be identified later by staining the gel. The following procedure describes how the cross-reaction of rabbit anti-oyster egg or sperm serum to oyster somatic tissue was identified.

Gill, adductor muscle, and mantle tissues were excised from reproductively-inactive oysters and ground using a glass syringe-piston tissue grinder. Two parts PBS solution were added to 1 part somatic tissue in the grinder during homogenization.

After homogenization, the tissue homogenate was pipetted into a polystyrene centrifuge tube. The tissue homogenate was further homogenized using an ultrasonifier. The tube was placed in an ice-filled beaker during ultrasonication to prevent excessive heating, which may result in protein denaturation.

The ultrasonicated oyster tissue homogenate was centrifuged at 7000 xg for 10 min to remove tissue debris. The supernatant was decanted and stored at -40°C until used.

A 1.5% agarose gel in PBS was liquefied again by placing the solidified gel tube in boiling water. The tube containing liquefied gel was temporarily stored in a water bath at 50°C.

A 10 x 10-cm glass plate was placed on an adjustable horizontal table and the surface of the glass plate leveled. The prepared agarose gel was then poured onto the middle of the plate. After the gel solidified, the plate was placed at 4°C for 15 min to harden the gel matrix.

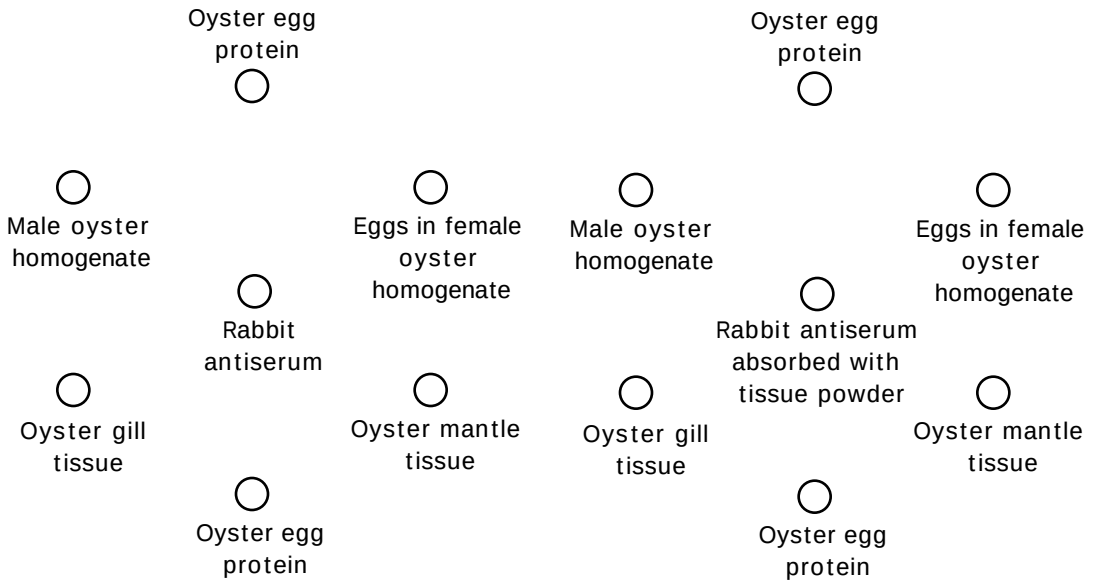

Figure II.6. An array of antiserum and antigens used in the test of cross-reactivity of rabbit anti-oyster egg serum using Ouctherlony's double diffusion method.

Using a gel puncher (4-mm diameter) connected to an aspirator, a hexagonal array of wells was punched in the plate (Figure II.6).

A 20-µL aliquot of the antiserum to be tested was placed in the well in the middle of the hexagonal pattern, and 20-µL samples and control antigens were added to the wells around the center well.

The gel plate was placed in a humid chamber and incubated at 4°C for 3 to 6 days. The humid chamber could be made by placing a layer of paper towels on the bottom of a rectangular plastic container, adding some distilled water to the paper and sealing the container with the cover.

After 2 days of incubation, a precipitation line will begin to develop between a well containing antigen and a well containing antiserum.

After a certain period of incubation (at least 2 days), wells on the plate were rinsed with distilled water to remove any impermeable matter left in the wells. The incubation period must be determined empirically for every antibody type.

A 10.2 x 10.2-cm filter paper was cut and soaked with distilled water. The gel plate was covered with the wet filter paper, avoiding air bubble formation.
The gel plate covered with wet filter paper was placed on a 0.5-cm thick layer of paper towels and covered with a stack of paper towels 2.5 cm thick. The plate was then pressed by placing several volumes of books on the top of the paper towels for 1 hr.

After pressing the gel, the plate was dried at 45°C until the filter paper could be pulled off.

The plate was stained with 0.5% Coomassie Brilliant Blue staining solution for 2 to 4 hr, and destained with destaining solution for 1 to 2 hr.

The pattern and the number of precipitation lines was examined to determine whether any cross-reactivity existed between the antiserum and non-gonadal tissue.

3.4.5. Preparation of acetone-dried somatic tissue powder

To remove cross-reacting antibodies, the serum was absorbed with acetone-dried somatic tissue powder.

Mantle, gill, and adductor muscle tissues were excised from reproductively-inactive oysters collected during December or February. Care was taken to be sure the oysters are free of gonadal tissue. The harvested somatic tissue was rinsed with PBS several times to remove associated sediment.

The tissues were ground using a blender (food processor) and placed in a beaker.

An equal volume of acetone was added to the tissue homogenate, and the diluted slurry stirred for 5 hr.

The acetone-tissue mixture was then centrifuged at 700 xg for 15 min and the supernatant containing acetone and water discarded.

The tissues were resuspended in 2 volumes of acetone, stirred for 3 hr, centrifuged at 700 xg for 15 min, and the supernatant discarded. This step was repeated 3 times.

Acetone-treated oyster tissues were rinsed twice with distilled water by resuspending the tissues in an equal volume of distilled water and centrifuged at 700 xg for 15 min.

The distilled-water rinsed oyster tissues were frozen at -40°C and lyophilized.

3.4.6. Removing non-specific antibody using acetone-dried tissue powder.

A 75-mg aliquot of tissue powder prepared from the above procedure was added to 1 mL of antiserum, placed in a centrifuge tube, and incubated at room temperature for 2 hr.

After incubation, the tube was centrifuged at 7000 xg for 20 min, and the antiserum transferred to another centrifuge tube.

Another 75-mg aliquot of tissue powder was added to the tube and incubated for 2 hr.

The tube was centrifuged at 7000 xg for 20 min, and the antiserum collected and stored at 4°C until used. The serum can be stored for up to 2 weeks at 4°C.

3.5. Quantification of oyster eggs or sperm using single ring immunodiffusion

The radial immunodiffusion method of Mancini *et al.* (1965) was used for the quantitation of oyster egg protein. This section describes steps involved in Mancini's single radial immunodiffusion technique (SRID).

Oysters to be analyzed were shucked and their wet weight recorded. Dry weight was later obtained from the empirical equation:

Dry wt. = Wet wt. (0.196).

Oysters were first homogenized using a glass syringe-piston tissue grinder and further homogenized using an ultrasonicator. The oyster homogenate was placed in an ice-filled beaker during sonication.

The homogenate was centrifuged at 900 xg for 15 min after sonication and the volume of supernatant recorded.

Twenty-mL of agarose gel in barbital buffer was liquefied by placing the gel in boiling water. The tube containing gel was then placed in a water bath adjusted to 50°C. Above 56°C, antiserum can be denatured and may lose its specificity.

One to 2 mL antiserum, either rabbit anti-oyster egg or sperm serum, cross-absorbed with oyster somatic tissue powder, was added to the gel tube and agitated until the serum was completely mixed with the gel.

A 10 x 10-cm glass plate was placed on a horizontal table and the plate leveled. The gel containing antiserum was poured on the plate and allowed to solidify for 10 min. The plate coated with gel was then placed at 4°C for 15 min to further solidify the gel.

Four-mm diameter wells were made in the gel plate using a gel puncher connected to an aspirator. Each well was 1.8 mm in depth and 4 mm in diameter, and held up to 25 µL antigen.

Twenty µL of antigen was added on each well. The plate was placed in a humid chamber, and incubated at 4°C for 3 to 6 days. Each plate contained standards, a negative control, and the samples to be analyzed. For analyzing female oysters, standards were prepared using oyster egg protein in a range of 10 to 100 µg/mL by making serial two-fold dilutions down to 1/256 of the original 10 mg/mL oyster egg protein solution. Protein extract of gill or mantle tissue was used as a negative control (Figure II.7).

After incubation, the plate was washed with distilled water to remove any residual antigen in each well and a 10.2 x 10.2-cm wet filter paper was placed on the plate.

The plate was then placed on a 0.5-cm thick layer of paper towels and covered with a layer of paper towels 2.5 cm thick. The plate was pressed with several volumes of books for 1 hr.

The plate was dried at 45°C until the filter paper could be pulled off.

The plate was stained with 0.5% Coomassie brilliant blue staining solution for 2 to 3 hr and destained with destaining solution for 1 to 2 hr.

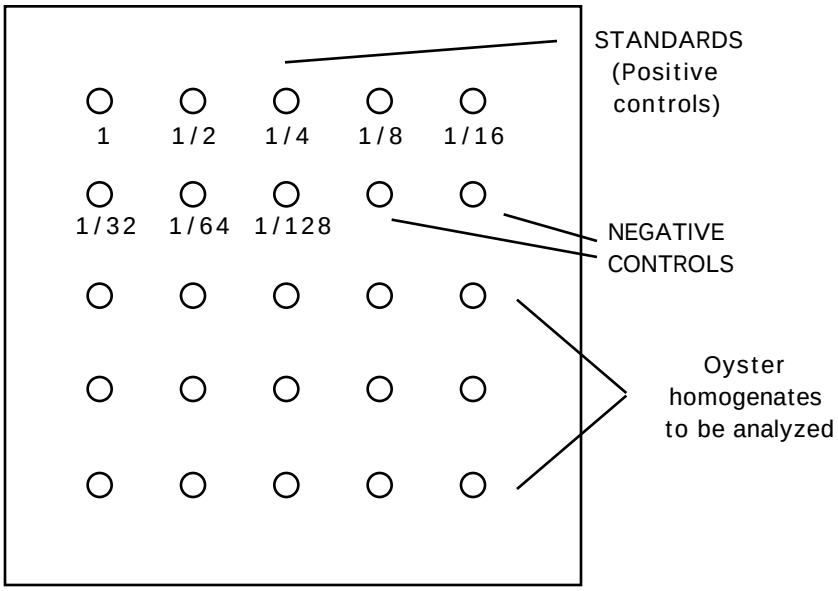

Figure II.7. An example of a gel plate used in the analysis of oyster egg protein using the single ring immunodiffusion assay.

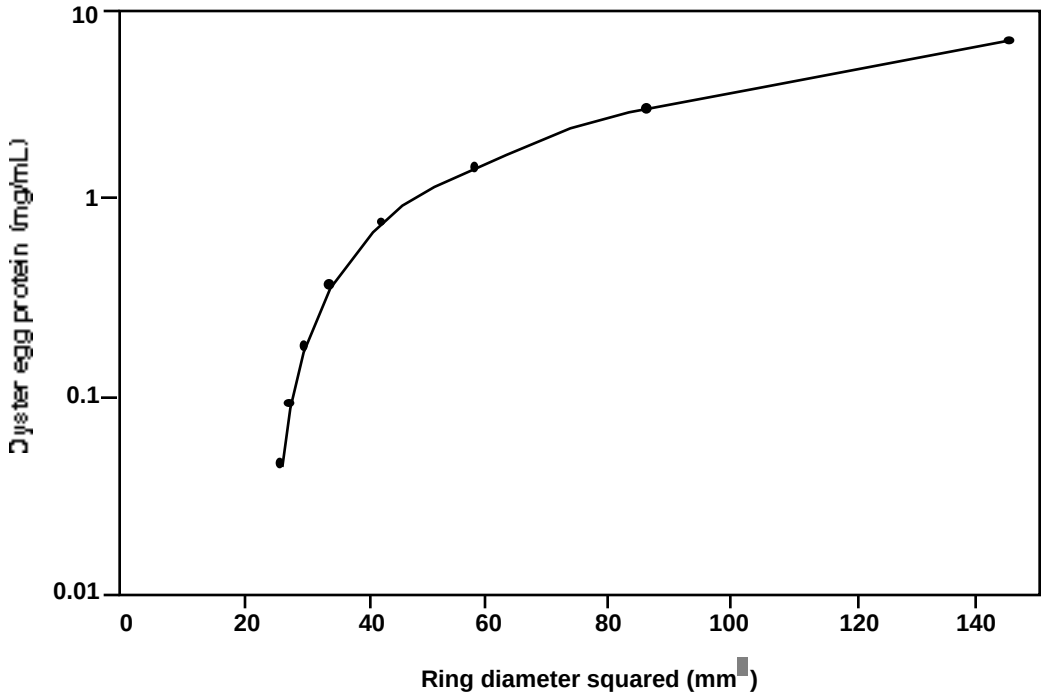

Figure II.8. A standard curve used in the quantitation of oyster egg protein in the single ring immunodiffusion assay.

The diameter of the precipitation ring was measured to 0.1 mm using a vernier caliper. A standard curve was constructed as the ring diameter squared (mm^2) versus the standard concentration (mg/mL). The concentration of gonadal protein in an oyster homogenate was interpolated from the standard curve and expressed as mg gonadal protein per mL oyster homogenate. Figure II.8 shows an example of a standard curve constructed using SRID data.

The total amount of gonadal protein was estimated by multiplying the SRID value (mg gonadal protein per mL oyster homogenate) by total homogenate volume (mL). The total dry weight of oyster eggs was estimated by dividing the total amount of egg protein measured from SRID by percent weight of protein in oyster egg (0.37 to 0.4). The total dry weight of oyster sperm was estimated by dividing the total amount of sperm protein measured from SRID by the percent weight of protein in oyster sperm (0.14 to 0.16).

A gonadal-somatic index (GSI) was established as a ratio of the total dry weight of gametic tissue to total oyster biomass.

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