# National Centers for Coastal Ocean Science

# Coastal Ecosystem Assessment Program:

# A Manual of Methods

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**Executive Summary**

Environmental managers strive to preserve natural resources for future generations but have limited decision-making tools to define ecosystem health. Many programs offer relevant broad-scale, environmental policy information on regional ecosystem health. These programs provide evidence of environmental condition and change, but lack connections between local impacts and direct effects on living resources. To address this need, the National Oceanic and Atmospheric Administration/National Ocean Service (NOAA/NOS) Cooperative Oxford Laboratory (COL), in cooperation with federal, state, and academic partners, implemented an integrated biotic ecosystem assessment on a sub-watershed 14-digit Hydrologic Unit Code (HUD) scale in Chesapeake Bay. The goals of this effort were to 1) establish a suite of bioindicators that are sensitive to ecosystem change, 2) establish the effects of varying land-use patterns on water quality and the subsequent health of living resources, 3) communicate these findings to local decision-makers, and 4) evaluate the success of management decisions in these systems. To establish indicators, three sub-watersheds were chosen based on statistical analysis of land-use patterns to represent a gradient from developed to agricultural. The Magothy (developed), Corsica (agricultural), and Rhode (reference) Rivers were identified. A random stratified design was developed based on depth (2m contour) and river mile. Sampling approaches were coordinated within this structure to allow for robust system comparisons. The sampling approach was hierarchal, with metrics chosen to represent a range from community to cellular level responses across multiple organisms. This approach allowed for the identification of sub-lethal stressors, and assessment of their impact on the organism and subsequently the population. Fish, crabs, clams, oysters, benthic organisms, and bacteria were targeted, as each occupies a separate ecological niche and may respond dissimilarly to environmental stressors. Particular attention was focused on the use of pathobiology as a tool for assessing environmental condition. By integrating the biotic component with water quality, sediment indices, and land-use information, this holistic evaluation of ecosystem health will provide management entities with information needed to inform local decision-making processes and establish benchmarks for future restoration efforts.

**Forward**

The purpose of this manual is to provide a framework for the assessment of ecosystem health that may be adapted, or used in whole or part for application to other regions. Methods are provided for all aspects from sampling design to laboratory analysis in great detail. The manual is divided into two sections, stressors and bioindicators, which are broken down into target components, with each component further broken down into sub-sections, as follows:

Introduction: familiarizes the reader with the component or species, its background or ecological significance in the localized ecosystem, and why it was chosen as an indicator.

Sampling Design: describes how sampling was conducted for each component including numbers of samples collected, seasons sampled, and site selection.

Metrics: provides a brief overview of each protocol used when collecting samples, with references and links to more specific methodology.

Methods: describes specific protocols for collecting, processing, and ingredients and formulas which are specific to species/sample and equipment. Minor modifications may be necessary when adapting methods to specific needs. An appendix with field data sheets and photographs that illustrate crab shell lesions is also included.

## Introduction

Land use affects many characteristics of adjacent waters; for example, the practice of irrigation for agriculture or municipal water withdrawals influences hydrology and in-stream flow and thus alters habitat availability (Van Sickle et al. 2004). Urbanization generally leads to increased concentrations of chemical contaminants (Comeleo et al. 1996), alterations in the direction and magnitude of storm water flow (Klein 1979), and subsequent reduced water quality (USEPA 1995). Effects of land-use choices on the ecology of adjacent ecosystems is complicated, due in part to the diffuse nature of non-point sources, buffering capacity of the land, and the natural variability of ecosystems in general (Paul et al. 2002). Effective ecosystem management depends on understanding these impacts. The National Oceanic and Atmospheric Administration (NOAA) 2005-2010 strategic plan's (http://www.midatlanticoceanresearchplan.org/strategic-plan-2005-2010-national-oceanic-and-atmospheric-administration) key objective is to "protect, restore, and manage the use of coastal and ocean resources through an ecosystem approach to management (EAM)". This approach attempts to view an ecosystem in a holistic framework and incorporates all aspects of biological, climatological, and social data relevant to defining and quantifying ecosystem stressors and their impacts to allow for making informed management decisions. However, there are critical information gaps in understanding linkages between anthropogenic influences and ecosystem outcomes. To address this need, COL and federal, state, and academic partners initiated an Ecosystem Assessment Program with the intent of providing information pertinent to management on local scales by connecting the influence of land-use sources to impacts on living resources.

## Concept

The principle of ecosystem indicators has received considerable attention in recent years as a means of characterizing ecosystem health or change by measuring a few key biotic and abiotic factors. Several programs have been developed to provide these characterizations at regional or national scales. The U.S. Environmental Protection Agency's Environmental Monitoring and Assessment Program, EMAP, was initiated in the 1980s to develop the science needed to conduct regional and national level assessments (USEPA 2002). NOAA's National Status and Trends Program has similarly monitored the health of our nation's resources since 1984. This program employs both ecological indicators (relevance at the population, community, or

ecosystem level) and biomarkers (individual, cellular, or sub-cellular level of change, principally used in reference to contaminant exposure). In Chesapeake Bay, the Atlantic Slope Consortium (www.asc.psu.ed) completed an ecosystem assessment relying principally on ecological indicators with application on small sub-watersheds (Brooks et al. 2006). By reducing the scale of assessment to the sub-watershed level, both local and regional impacts are examined.

These programs have served to identify indicators appropriate for examining ecosystem level change over various scales that are useful to regional managers in describing the ecological health of the system. However, these programs are generally not designed to provide detailed information at the local, land-use decision-making scale. In addition, ecosystem indicators provide evidence of holistic change (i.e., fish community structure), but are less robust in their ability to associate change with specific causes, or predict the biotic impact of local scale land use change.

Adams et al. (2000) and Adams (2005) provide a conceptual framework for addressing these shortcomings through the use of biotic indicators. To effectively assess the variability in estuarine/coastal systems requires the use of multiple indicators at varying levels of organization and comparison to reference sites. The use of reference sites offers a baseline of current climatic and social conditions and obtainable goals for restoration, in contrast to reliance on historical conditions. The hierarchical approach to sampling organisms from the sub-cellular level to populations provides resolution not possible with more typical community-based approaches or those relying principally on abiotic factors.

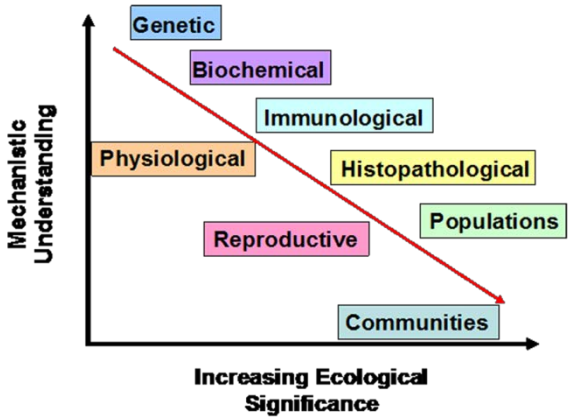


Figure 1. Functional framework for investigating ecosystem response through bioindicators.

Stress is a departure from homeostasis in an organism and is the first stage of impairment. Identification of physiological stress may provide an early warning, and indicators at this level can be fairly specific for individual stressors (e.g., acetylcholinesterase inhibition and pesticides). However, the ecological relevance of stress in an organism cannot be determined solely based on

physiological change. Connecting changes at the sub-cellular level through pathological change in the organism to subsequent population level impacts allow for the realization of assessing the impact of specific anthropogenic influence on living resources and, thus ecosystem health (Figure 1).

Expanding on the conceptual framework of Adams et al. (2000) and Adams (2005), the choice of sentinel or indicator organisms is critical to the interpretation of results. Indicator organisms should possess the qualities of being ubiquitous in the systems of study and sensitive to the metrics examined. However, differing stressors impart differential response among species so that a single species model may not respond adequately to each stressor signal presented. Thus, the use of multiple organisms occupying different ecological niches, and applying hierarchal sampling to these species or groups could provide a broader picture while increasing the range of signal detection.

**Project Goals**

The specific bjectives of the project were to:

1. Evaluate linkages among land use, habitat quality, and biotic health through the development and implementation of a multivariate ecosystem health indicator package;
2. Demonstrate the utility of the approach for ecosystem management through application to select Chesapeake Bay watersheds;
3. Inform local decision-making processes as to the environmental impacts of changing land-use patterns; and
4. Transfer technology for application throughout Chesapeake Bay and other coastal systems.

Completion of these objectives support the overall goal of this assessment, which was to provide local managers and decision-makers with the information necessary to make informed decisions on the environmental influence of altering land-use patterns.

**Site Selection**

Site selection was based on the following criteria for the initial development of the indicator package:

1. Divergent land-use characteristics and reference site;
2. Small watersheds without extensive upstream hydrology (20-30,000 acres);
3. Similar salinity range;
4. Accessible by small boat and without extensive travel from lab;
5. Availability of historic and/or other monitoring data in system.

For site selection, sub-watershed boundaries (14 digit HUC) were overlaid on the 2001 National Land Cover Dataset (Homer et al. 2007) to obtain comparative land-use information. Land-use categories were combined into urban, forest, agricultural, wetlands, or barren for subsequent analysis. A total of 12 sub-watersheds in Maryland's Chesapeake Bay were included in the initial analysis. Seven were subsequently excluded based on salinity, since we restricted the range to 6-12ppt to allow for consistency in habitat Principle Component Analysis (PCA) (S-Plus v. 8.0) was used to identify divergence in land-use patterns (Figure 2). Results demonstrated that the Magothy and Severn rivers tended toward urban patterns and are most different from the Corsica (agricultural) along principle component 1 (PC1 x axis). The Rhode River and Monie Bay are somewhat intermediary in terms of PC1, and tended toward forested patterns. Of these five systems, the Severn River and Monie Bay are excluded due to

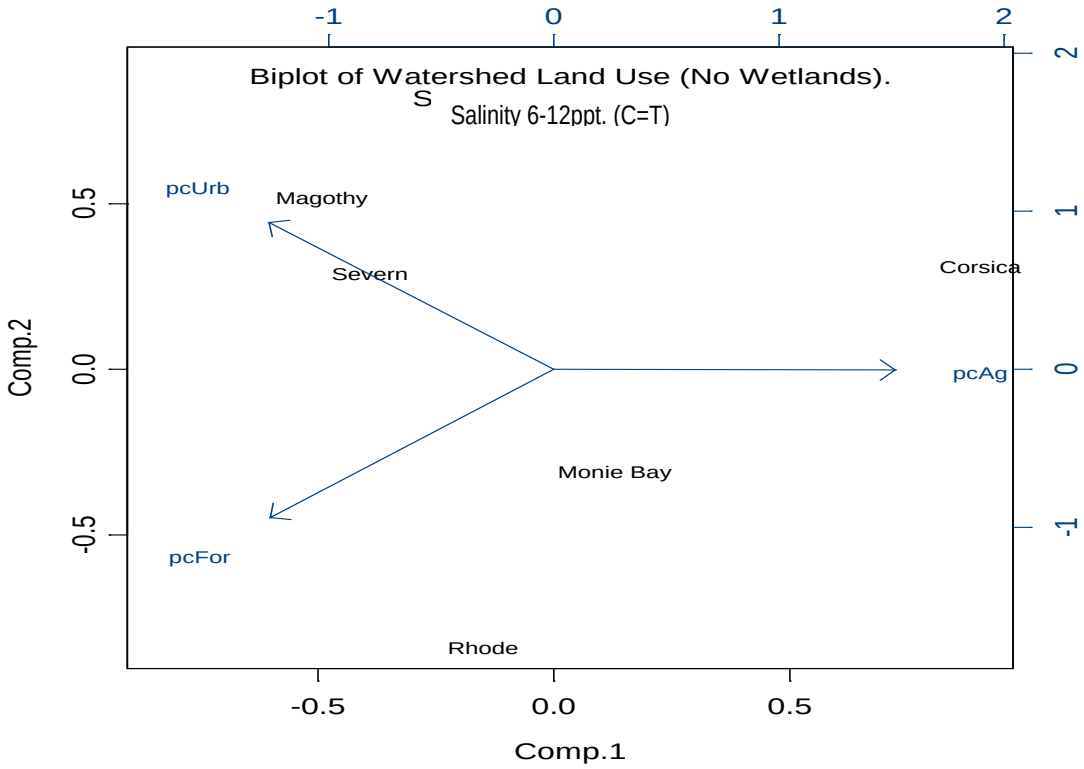


Figure 2. Biplot of land use for five sub-watersheds meeting selection criteria.

size and depth differences. The Corsica and Magothy are chosen as agricultural and developing basins respectively, with the Rhode River selected as a forested, balanced, reference system.

**Sampling Design**

A random, stratified design was employed to fully characterize each sub-watershed. Stratification was accomplished by using the 6ft contour to separate deepwater from nearshore, shallow water habitats and river mile as a surrogate for salinity. Samples are also taken from upstream tributaries and the mouths of each system to provide source information and the influence of tidal flux. This approach allows for random characterization of the system as a whole, as well as within system comparison. Sample sites are fixed for the year and are visited precisely for water quality and benthic assessment. The stratification scheme divides each system into nine blocks or sections (Figure 3 ), and water quality and benthic characteristics for each block are represented by the sampling site. Collection of organisms was conducted within each river mile segment, with the appropriate segment determined by the physiology or behavior of the organism (i.e., mummichogs tend to inhabit nearshore zones) for full analysis. Sampling was conducted in all three systems during 2 week windows for direct comparison. Each location was visited a minimum of three times per year. The intensive sampling periods are based on mean water temperature to reflect spring conditions, maximum summer temperatures, and subsequent impacts in the fall.

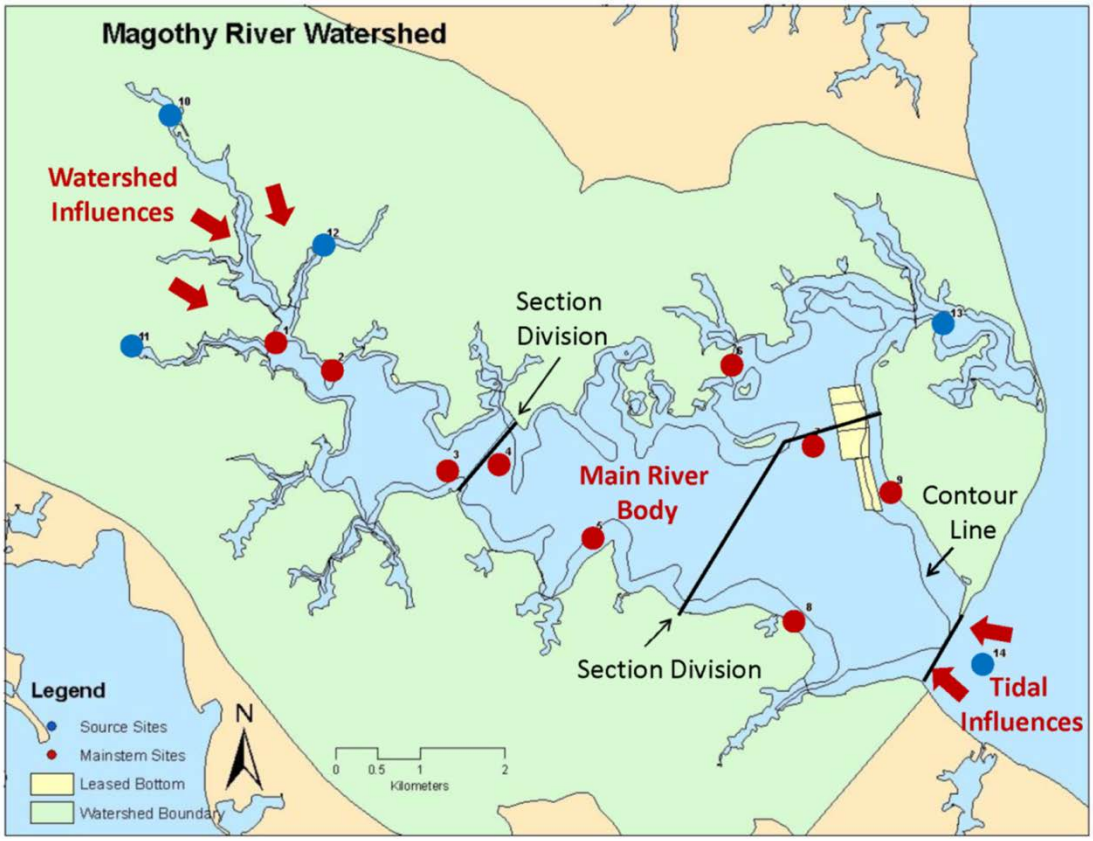


Figure 3. Graphic representation of sampling design framework applied to Magothy River. Tidal influences at the mouth of the river and watershed influences at the head of the river are shown with red arrows. The 6ft contour line is shown on each side of the river. Water sampling stations are represented by red or blue dots.

# Stressors

## Water Quality

**Introduction**

The suitability of our waterways to support aquatic life and provide recreational services is fundamental in evaluating ecosystem health. Although many estuarine organisms are adapted to the continual flux in water conditions, extreme or persistent conditions can cause serious detriment to the health of organisms. Many coastal areas suffer from anthropogenic inputs of nutrients, contaminants, and sediments, which can cause low dissolved oxygen, habitat loss, bioaccumulation of toxins, and loss of benthic organisms.

**Sampling Design**

Water samples along with associated physical water quality data are collected at each of the 12-15 random stations per system. Physical data was collected at 0.5m depth increments. Two liters of water are taken at the surface (0.5m). Filtration for chlorophyll and DNA extraction are conducted on site, while the remaining water was kept on wet ice and delivered within hours to the laboratory for subsequent processing. Samples for full nutrient analysis, microbial community structure, specific pathogen detection, chlorophyll A concentrations, and fecal coliform bacteria are collected only during spring, summer, and fall.

**Metrics**

*Physiochemical Characteristics* - Water quality monitoring, the physiochemical characteristics of the entire water column, forms the backbone of the assessment as it links land use and organism to community response. A full suite of physical and chemical parameters are collected. **Method Water Quality 1.**

*Chlorophyll A* - Chlorophyll A is found in all eukaryotic photoautotrophs. Its presence in surface waters is an indicator of the relative level of phytoplankton densities, which are related to a number of factors such as nutrient concentrations, water temperature, hydrodynamics, and zooplankton grazing opportunities. A relatively low concentration of chlorophyll A tends to indicate low productivity, while a very high amount usually indicates eutrophication and overproduction, often leading to reduced water clarity and the formation of hypoxic bottom waters. **Method Water Quality 2.**

*Nutrients* - All organisms require nutrients. In the aquatic environment, nutrient levels often vary from runoff and precipitation and are indicators of water quality. The main compounds of interest contain nitrogen or phosphorous. Estuaries typically have high concentrations of nutrients compared to oceanic waters. On a broad scale, the lack of nutrients results in low densities of organisms and smaller organisms, while excessive nutrients result in population blooms often followed by hypoxia. **Method Water Quality 3.**

*Microbial Community Composition* - Microbial community structure in estuaries changes seasonally and in response to natural gradients (Crump et al. 2004). This effort will establish whether anthropogenic gradients result in community shifts, and identify specific groups of bacteria associated with environmental degradation. **Method Water Quality 4.**

*Individual Pathogens* – Preliminary data from an assessment conducted in the coastal bays of the Delmarva Peninsula (USA) indicate that some species of bacteria that are pathogens of humans and/or fish may vary in density or physiology in response to eutrophication (Jacobs et al. 2009). Small scale, intensive sampling will allow further evaluation of these findings. Pathogen analysis use polymerase chain reaction (PCR) assays to quantify the abundance of *Vibrio vulnificus*, total *Mycobacterium* spp., total *Vibrio parahaemolyticus* and virulent strains of *V. parahaemolyticus*. **Method Water Quality 5.**

*Fecal Coliform Bacteria* – Measurement of fecal indicator bacteria is the standard for assessing the overall risk to human health from the consumption of shellfish or recreational water use. The U.S. federal government establishes water quality criteria based on densities of indicator bacteria (USEPA 1986), and individual states use these to regulate shellfish beds and bathing beach closures. Densities of fecal indicator bacteria are be determined and compared against these standards and between watersheds. **Method Water Quality 6.**

**Method Water Quality 1: Datasonde**

*Metadata*

1. At each station, data related to the climate and hydrodynamic conditions are recorded (Appendix I: Water Quality - Field Sheet).
2. Weather conditions include qualitative assessments of precipitation for day of sampling and the preceding day, wind velocity and direction, and cloud cover.

*Physio-chemical Characteristics*

1. Datasondes measure water temperature, depth, conductivity, dissolved oxygen, pH, and turbidity.
2. Specific conductance and salinity are estimated based on conductivity and temperature.

3. Depth is calculated based on barometric and ambient pressure.
4. Datasondes are maintained and calibrated according to manufacturer's recommendations. Conductivity, pH, and turbidity probes are calibrated at least once per month. Depth and dissolved oxygen probes are calibrated at least 1/wk.
5. At each station, physicochemical metrics are measured just below the surface, at 0.5m increments in depth, and just above the bottom.
6. All data are saved electronically to the datasonde.
7. At a minimum, surface and bottom data are recorded on data sheets (Appendix I: Field Sheeet - Water Quality), along with the metadata described above.

**Method Water Quality 2: Chlorophyll Analysis**

1. Chlorophyll A concentrations are determined by the Analytical Services Laboratory at University of Maryland's Horn Point Laboratory.
2. Surface water samples are collected in acid-washed (soaked in 10% hydrochloric acid for 4-8h, then rinsed twice with deionized water) 1L polyethylene jars.
3. Water for nutrient analysis is placed immediately on ice.
4. Water for chlorophyll A analysis is filtered through a 25mm round glass fiber (GF/F) filter with 0.7µm pore size using a pre-rinsed 50mL syringe until the filter is discolored (typically between 50 and 100mL); volume filtered is recorded on field data sheet.
5. These filters are then wrapped in aluminum foil and placed immediately on dry ice.
6. Samples are delivered to the service laboratory the same day as collection, and processing of nutrient samples begins that day.
7. Chemical analysis for chlorophyll A concentrations largely follows the methods described in Van Heukelem and Thomas (2001).
8. Upon receipt by the service lab, samples are logged and stored in a -80°C freezer with accompanying paper work.
9. Chlorophylls are extracted from the GF/F filters using acetone and sonication.
10. Extracts are then analyzed using high performance liquid chromatography, with duplicates and blanks.

**Method Water Quality 3: Nutrient Analysis**

1. Nutrient concentrations are determined by the Analytical Services Laboratory at University of Maryland's Horn Point Laboratory.
2. Surface water samples are collected in acid-washed (soaked in 10% hydrochloric acid for 4-8hr, then rinsed well with deionized water) 1L polyethylene jars.
3. Water for nutrient analysis is placed immediately on ice.
4. Samples are delivered to the service laboratory the same day as collection, and processing of nutrient samples begins that day.
5. Ammonium, nitrate, nitrite, silicate, phosphate, total dissolved nitrogen and total dissolved phosphorous are determined using a four channel continuous flow automated analyzer.
6. Ammonium is determined by the Berthelot reaction in which a blue colored chromophore similar to indophenol forms when a solution of ammonium salt is added to sodium phenoxide, followed by the addition of sodium hypochlorite.
7. The addition of sodium citrate prevents precipitation of hydroxides of calcium and magnesium.
8. Nitrate and nitrite are determined from filtered samples passed through a granulated copper-cadmium column to reduce nitrate to nitrite.
9. The nitrite concentration (originally present, plus the reduced nitrate) is then determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl) ethylenediamine dihydrochloride to form a colored azo dye.
10. Nitrate concentration is obtained by subtracting the corresponding nitrite value from the nitrite and nitrate concentrations.
11. The determination of silicate is based on the reduction of silicomolybdate in acid solution to "molybdate blue" by ascorbic acid. Oxalic acid is introduced to eliminate interference from phosphate.
12. Phosphate is determined by how ammonium molybdate and antimony potassium tartrate react in an acid medium to form an antimony-phosphomolybdate complex, which, in the presence of ascorbic acid, is reduced to form an intense blue complex. The intensity of the blue complex is proportional to the amount of reactive phosphate in the water sample.

13. Total Dissolved Nitrogen and Phosphorous are measured by digestion obtained through persulfate oxidation of the sample. The reaction began under alkaline conditions oxidizing nitrogen to nitrate, as $SO_4^{-2}$ is produced the solution became acidic and phosphorus is then converted to phosphate.
14. These conditions are obtained by a borate-sodium hydroxide system.
15. The previously described nitrate and phosphate tests are used to obtain total concentrations.
16. Particulate nitrogen and carbon are measured by combustion in pure oxygen ($O_2$) under static conditions.
17. The combustion products are then completely oxidized.
18. Oxides of nitrogen are then reduced under high temperature.
19. The sample is then sent through a series of thermal blocks and absorption traps, and the differential levels of compounds before and after the traps are used to determine the amount of particulate carbon and nitrogen in the original sample.
20. Dissolved organic carbon is determined by high-temperature oxidation following the methods of Sugimura and Suzuki (1988).
21. It involved filtering the water sample through a membrane filter (0.4µm) followed by acidification and degassing with an oxygen stream.
22. An aliquot is then injected into the oxidation column and the dissolved carbon is oxidized to carbon dioxide.
23. Carbon dioxide levels are measured using an infrared gas analyzer, which provided a quantitative determination of the amount of dissolved carbon in the original sample.

**Method Water Quality 4: Microbial Community Structure**

*Freshwater Microbial Diversity Sample Collection*

1. Freshwater samples are collected from streams adjoining river sampling stations.
2. Depending on the river, number of samples ranges from 1-4 per sampling effort.
3. Polypropylene acid-washed bottles (1L), attached to an extendable pole, are submerged and filled by the water source, being careful not to disturb rocks and sediment, but also not taking water directly from the surface.

4. Bottles are rinsed twice with water from the water source before final collection, pouring water off to the side to not disturb sediment under the sampling site.
5. Water samples are filtered through 0.22µm Sterivex (Millipore, Billerica, MA) filter cartridges (SVGP01050) with a 60mL syringe to obtain 200mL samples.
6. To prevent contamination, 60mL portions of sample are poured into the back of the syringe after removing the plunger.
7. After filtering each portion, the filter is detached from the syringe, the plunger is removed, the filter is reattached, sample is poured into the back of the syringe, and the plunger is replaced and used to force sample through the filter.
8. After 200mL are filtered, air is pushed through the filter to remove water droplets.
9. The filter is then wrapped in parafilm and sealed in a sealable bag.
10. Sealed filters are stored on wet ice until archived at -20°C.

*ARISA Community Fingerprinting*

1. Bacterial communities are fingerprinted for all DNA samples collected from freshwater and estuaries using automated ribosomal intergenic spacer analysis (ARISA).
2. DNA samples are extracted from Sterivex (Millipore) filters using PowerSoil DNA extraction kits (PowerSoil, MoBio Laboratories, Inc., Carlsbad, CA), with modifications by Jacobs et al. (2009).
3. Following extraction, PCR amplification is conducted using a master mix composed of 1X buffer, 0.2mm of dNTPs, 0.25µm of each primer (16s-1392f, 23s-125r), 40ng/µL of BSA, 0.78125µL of Taq polymerase and 2.5mm of MgCl2, plus 1µL template per 25µL reaction.
4. PCR conditions for ARISA are as follows: 94°C for 2min, followed by 30 cycles of amplification at 94°C for 15s, 55°C for 15s, and 72°C for 45s and a final extension of 72°C for 2min (Fisher and Triplett 1999).
5. PCR products are then run on 2% acrylamide gel.
6. Upon confirming PCR amplification, 2.0µL of PCR products are combined with 0.5µL of fluorescent DNA size standard (base pair sizes including 100, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 990, 1000, 1050, 1100, 1150, 1200) and 9.5µL formamide.

7. Samples are sent to University of Wisconsin, Madison, for automated electrophoresis processing. Sample electropherogram analysisis useconducted using Applied Biosystems Peak Scanner (Life Technologies, Grand Island, NY) software.

*Processing Electropherogram Data Using Peak Scanner* (Life Technologies, Grand Island, NY) *and a Macro*

1. Start new project. Click "add samples to project" under "setup" tab
2. Import samples by clicking "add files" (files are be in .fsa format)
3. Choose sample type "sample" from drop down bar
4. Choose the project-specific size standard from drop down bar
    a. In order to make a size standard entry, click "new size standard" under "resources" tab on left
    b. Name the size standard and choose dye color "red" (or other color as appropriate for the size standard used)
    c. Enter the sizes of the size standard under "size standard definition" and add to the current size standard definition
    d. Save to get a new, project-specific size standard
5. Choose the project-specific analysis method from the drop down bar.
    a. In order to develop an analysis method, click "new analysis method" under "resources" tab on left
    b. Name the analysis method and add description as needed
    c. Peak detection -> peak smoothing ->default is none
    d. Peak detection -> range -> default analysis is full range and all sizes; in this study, we used partial range 1000 to 12000
    e. Peak detection -> peak characteristics -> 2, 3, 15, 0, 0 for all columns
    f. Peak detection -> size calling method -> local southern
    g. Peak detection -> baselining -> baseline window size -> 51
    h. Peak detection -> minimum peak heights -> peak threshold -> 50, 50, 50, 500, 50 (determine which analysis method accurately finds the size standards (red or other color as appropriate) – for this example, 500 is the minimum threshold for red.)

Note: The off scale and quality passes and fails are determined by the ability of the program to find the peaks that it associated with the size standard; therefore, be certain to set the limits at a low enough height that size standards can be detected accurately.

6. Go through each sample to check where the size standards are called, to edit which peaks are selected as your size standards:
    a. To see peak label, click on “edit size standards” under “quality control” tab on left side
    b. To see what a peak is designated, drag the zoom icon on the “plot view” up and over on the plot; peak’s height, area, size, etc. will be displayed.
    c. To replace the peak label for size standards, highlight the peak and choose “replace label” or “add label” on the plot view menu. It is possible to change the label to any other size standard value included in the size standard method.
    d. If a peak is not called and it is actually one of the size standard peaks:
        1. Go to “edit peaks” under the “review data” tab
        2. Zoom in on peak
        3. Right click on “peak” and choose “range select”
        4. Drag over peak to highlight it
        5. Right click on “peak” and choose “add peaks”
        6. Go to “edit size standards” under “quality control”
        7. Zoom back in on peak
        8. Right click on box under peak and choose “add” and the size standard that appropriate for that peak
    e. Once size standard peaks have been recalled to accurately depict where peaks should be in the sample, press the analyze key (select ONLY single sample, highlight it and choose “analyze selection” under analysis key) again under the “setup” tab. This reanalysis should improve the status of the off scale and quality boxes. SQI will be checked because peak values have been manually adjusted.
7. To export values, under “sizing table view” tab, sort all samples by “dye color” and select all blue values. Highlight all blue samples, copy and paste to Microsoft Excel (Microsoft Corporation, Redmond, WA) spreadsheet.

8. To analyze samples using macro program in Microsoft Excel (Microsoft Corporation, Redmond, WA), put sample file name data in column A, size data in column C, and area in BP data in Column E. Open macro (simply open file), highlight all columns and select "tools" tab, select "macros", and open macro will be shown. Select "run" and macro will provide an array spreadsheet of all data. To transpose array, copy array, paste special and check "transpose."

9. Macro tip: for especially large spreadsheets, macro must be rewritten to accommodate the large amount of data. All "integer" language must be replaced as "long". Simply run a "search and replace all" integer to "long" in rewriting the macro.

*Bacterial Community Beta Diversity Calculations*

1. ARISA fingerprint data are organized in a spreadsheet, and band density data (i.e., the area under the peak) are binned to account for small variations in the fragment sizing among samples using the methods of Hewson and Fuhrman (2006).
2. These binned data are assumed to represent the relative abundance of operational taxonomic units (OTUs) of bacteria in the samples.
3. The resulting array used to calculate pairwise similarity between samples using the Bray-Curtis equation: $S_{jk} = 100\{1- \sum (|y_{ij}-y_{ik}| / y_{ij} + y_{ik})$ where $S_{jk}$ is the percent similarity between the *j*th and *k*th sample, $y_{ij}$ is the binned band density of the *i*th band in the *j*th sample, and $y_{ik}$ the binned band density of the *i*th band in the *k*th sample.
4. Similarity matrices are visualized using multiple dimensional scaling (MDS) diagrams. Similarity matrices, MDS diagrams, and analysis of similarity (ANOSIM) statistics are carried out using PRIMER v6 filter (PRIMER-E Ltd., Plymouth, UK) for Windows.

**Method Water Quality 5: Specific Pathogen Detection**

*Sample Collection*

1. At each water quality station, a 500mL water sample is collected in a sterile container at 0.5m depth.
2. Samples are gently shaken, inverted to mix, and filtered immediately in the field.
3. A total of 200mL of sample is passed through a 0.22µm Sterivex (Millipore, Billerica, MA) filter attached to a sterile 60mL syringe.

4. Water is completely removed from each filter, with filter housings wrapped tightly in parafilm and stored in a sealable plastic bag on dry ice.
5. In heavily turbid areas, water is filtered until no more can pass and volume noted to the nearest 1mL.
6. Filter housings are subsequently stored at -80°C until extraction.

*DNA Purification*

1. Sterile pliers are used to forcibly crack the plastic outer covering of the Sterivex filters.
2. Filters are then removed from the frame by cutting five lengthwise strips (approximately 6.0mm) using sterile razor blades.
3. Strips are then folded lengthwise and all five placed into a MoBio PowerSoil kit bead tube (PowerSoil kit (MoBio Laboratories, Inc., Carlsbad, CA).
4. The manufacturer's protocols are modified to obtain constant volumes, increase cell lysis, and maximize removal from the filters.
5. Sixty microliters of manufacturer's solution C1 is added to each bead tube; tubes are then incubated at 70°C and shaken at 500rpm in an Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany) for 10min, removed, inverted, vortexed at maximum speed on a bench top vortexer, and then re-incubated under the same conditions for an additional 10min.
6. Following incubation, 700µL of phenyl-chloroform-isoamyl alcohol is added to each tube to dissolve the filters and all tubes are vortexed at max speed for 10min. Tubes are then centrifuged at 10,000 X *g* for 30s and 800µL of supernatant removed for further DNA extraction per manufacturer's instructions.

*qPCR* - All primers and probes listed in this section are ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). On occasion, products from all assays are run on a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Sybr®Safe, Life Technologies) at 84V for 1.75hr to ensure proper sized products are amplified by comparison to a known molecular weight marker.

*qPCR Mycobacterium spp*.

1. A genus level assay for *Mycobacterium* spp. targeting the internal transcribed spacer (ITS) region and partial 23S gene developed by Bruijnesteijn van Coppenraet et al. (2004) is evaluated for use on environmental samples and optimized for efficiency and sensitivity (Table 1) (Jacobs et al. 2009).
2. Every sample is evaluated for the presence of inhibitors using a unique manufactured control (Nordstrom et al. 2007).
3. qPCR for *Mycobacterium* spp. is performed by using 2.5µL of 10X PCR buffer (Invitrogen Corporation, Carlsbad, CA), 1.50µL of 50.00mM $MgCl_2$, 0.50µL of 10.00mM dNTP solution (Roche Diagnostics, Inc., Indianapolis, IN) (mixed equal concentration of each), 0.50µL of 10.00µM of each primer, 0.50µL of 10.00µM FAM-labeled probe, 0.25µL of 5U/µL Platinum hot start Taq (Invitrogen Corporation, Carlsbad, CA) and 5µL of sample template per reaction. DNase/RNase-free water is added in quantity sufficient for 25µL of total reaction volume.
4. The two-stage qPCR cycling parameters are
    i. initial denaturation of template at 95ºC for 180s.
    ii. 40 cycles of denaturation at 95ºC for 30s and combined annealing and extension at 62ºC for 40s.

*qPCR Vibrio vulnificus* - Primers vvh_F and vvh_R are used in conjunction with the probe vvh874 for the detection of *V. vulnificus* (Table 1) (Panicker and Bej 2005). The same internal control as used for *Mycobacterium* spp. is incorporated simultaneously into the assay to test for the presence and influence of inhibitors (Table 1) (Nordstrom et al. 2007).

1. qPCR is performed by using 2.50µL of 10X PCR buffer (Invitrogen Corporation, Carlsbad, CA).
2. 1.25µL of 50.00mM $MgCl_2$ (Invitrogen Corporation, Carlsbad, CA).
3. 0.50µL of 10.00mM dNTP solution (Roche Diagnostics, Inc., Indianapolis, IN) (mixed equal concentration of each).
4. 1.00µL of 10.00µM vvh primer (each).
5. 0.60µL of 10.00µM vvh874 probe.
6. 0.19µL of 10.00µM internal control primers (each).
7. 0.38µL of 10.00µM of ICCy5 probe.

8. 2.00µL of IC DNA template ($10^5$ng/µL).
9. 0.40µL of 5U/µL Platinum hot start Taq (Invitrogen Corporation, Carlsbad, CA).
10. 3µL of sample template.
11. Quantity sufficient of PCR grade water for 25µL of total reaction volume.
12. Two-stage qPCR cycling conditions are optimized with
    a. Initial denaturation of template at 95ºC for 60s.
    b. 50 cycles of denaturation at 95ºC for 5s and combined annealing and extension at 59ºC for 45s.

*qPCR Total Vibrio parahaemolyticus* - A species specific primer/probe combination is employed for detection of total *V. parahaemolyticus* (Nordstrom et al. 2007). This assay incorporated a unique internal control for the detection of any inhibitors within each sample (Nordstrom et al. 2007). Primers tl_F and tl_R are used with the probe VptlTexRed for the detection of total *V. parahaemolyticus* (Table 1).

1. qPCR for *V. parahaemolyticus* is performed by adding 2.5µL of 10X PCR buffer (Invitrogen Corporation, Carlsbad, CA).
2. 1.5µL of 50.00mM $MgCl_2$ (Invitrogen Corporation, Carlsbad, CA).
3. 0.50µL of dNTP solution (Roche Diagnostics, Inc., Indianapolis, IN) (mixed equal concentration of each).
4. 0.50µL of each 10.0µL of tl primer.
5. 0.375µL of 10.0µM VptlTexRed probe.
6. 0.188µL of each 10.0µM IC primer.
7. 0.375µL of ICCy5 probe.
8. 2.00µL of IC DNA ($10^5$ng/µL).
9. 0.45µL of 5U/µL Platinum hot start Taq (Invitrogen Corporation, Carlsbad, CA).
10. 3µL of sample template
11. Quantity sufficient of PCR grade water for 25µL total reaction volume.
12. Two-stage qPCR cycling parameters are optimized with
    a. Initial denaturation of template at 95ºC for 60s.
    b. 45 cycles of denaturation at 95ºC for 5s.
    c. Combined annealing and extension at 66ºC for 45s.

*qPCR trh/tdh positive Vibrio parahaemolyticus* - A combination of two sets of primer and probe are used for the detection of the genes *tdh* and *trh* in *V. parahaemolyticus* (Table 1) (Nordstrom et al. 2007). The same internal control mentioned above is also used in this assay (Nordstrom et al. 2007).

1. qPCR is performed by using 2.5µL of 10 X PCR Buffer (Invitrogen Corporation, Carlsbad, CA).
2. 2.0µL of 50.0mM $MgCl_2$ (Invitrogen Corporation, Carlsbad, CA).
3. 1.0µL of 10.0mM dNTP solution (mixed equal concentration of each) (Roche Diagnostics, Inc., Indianapolis, IN).
4. 0.50µL of 10.00µM *tdh* and *trh* primers (each).
5. 0.19µL of 10.00µM *tdh*_269-20and *trh*_133-23 probes (each).
6. 0.19µL of 10.00µM IC primers (each).
7. 0.38µL of 10µM ICCy5 probe.
8. 2.0µL of IC DNA ($10^5$ng/µL).
9. 0.45µL of 5U/µL Platinum hot start Taq (Invitrogen Corporation, Carlsbad, CA).
10. 3µL of sample template
11. Quantity sufficient of PCR grade water for 25µL of total reaction volume.
12. Two-stage qPCR cycling parameters are optimized.
    a. Initial denaturation of template at 95ºC for 60s.
    b. 50 cycles of denaturation at 95ºC for 5s.
    c. Combined annealing and extension at 59ºC for 45s.

**Table 1. Oligonucleotide sequences used in Method Water Quality 5**

| Pathogen target | | Oligonucleotide sequence (5' -3') |
|---|---|---|
| *Mycobacterium spp.* | Myco_F | GGGGTGTGGTGTTTGAG |
| | Myco_R | CTCCCACGTCCTTCATC |
| | MycoFAM | FAM/TGGATAGTGGTTGCGAGCATC/BHQ |
| Total *Vibrio vulnificus* | vvh_F | TTCCAACTTCAAACCGAACTATGA |
| | vvh_R | ATTCCAGTCGATGCGAATACGTTG |
| | vvh874 | FAM/AACTATCGTGCACGCTTTGGTACCGT/BHQ |
| Total *V. parahaemolyticus* | tl_F | ACTCAACACAAGAAGAGATCGACAA |
| | tl_R | GATGAGCGGTTGATGTCCAAA |
| | VptlTexRed | TxRed/CGCTCGCGTTCACGAAACCGT/BHQ |
| *V. parahaemolyticus trh* | trh_F | TTGCTTTCAGTTTGCTATTGGCT |
| | trh_R | TGTTTACCGTCATATAGGCGCTT |
| | trhTET | TET/AGAAATACAACAATCAAAACTGA/BHQ |
| *V. parahaemolyticus tdh* | tdh_F | TCCCTTTTCCTGCCCCC |
| | tdh_R | CGCTGCCATTGTATAGTCTTTATC |
| | tdhFAM | FAM/TGACATCCTACATGACTGTG/BHQ |
| Internal control | ic_F | GACATCGATATGGGTGCCG |
| | ic_R | CGAGACGATGCAGCCATTC |
| | ICCy5 | Cy5/TCTCATGCGTCTCCCTGGTGAATGTG/BHQ |

**Method Water Quality 6: Fecal Coliform Bacteria**

1. Surface water samples are collected in sterile 500mL polypropylene jars.
2. Place immediately on ice and in the dark.
3. Upon return to the lab, aliquots of samples are filtered through 0.45µm membrane filters in accordance with standard methods (APHA 1998).
4. Filters are placed on *m-Enterococcus* agar in 15mm petri dishes.
5. The dishes are placed in sealable bags and sealed.
6. Bags are incubated in a water bath at 35°C for 48±8hr.
7. Positive colonies on each filter are counted and an estimate of colony forming units (CFUs) per 100mL of water is calculated based on standard methods (APHA 1998).

# Benthic Contaminants

**Introduction**

Sediment, water, and organisms associated with benthic habitats form a very important ecological component of aquatic systems. Benthic structures support microbial communities with critical roles, including biogeochemical processing of nutrients and production of organic matter. Communities of macroscopic benthic organisms include animals involved in the processing of detritus, stabilizing sediments, and filtering water. More significantly, the benthos, those organisms which live in the benthic habitat, are an important component of the trophic system for most aquatic systems.

**Sampling Design**

Sediment samples are collected and analyzed according to established NOAA/NCCOS protocols (Fulton et al. 2007). At each water quality station, one replicate sediment sample for benthos per site was collected using a 0.044$m^2$ surface area Young grab. If the bottom type prevents sampling with a Young grab (hard bottom or leased/prohibited areas), a new position was chosen by randomly moving away from the original location until sampling was possible. Cross contamination was minimized by rinsing sample equipment between stations and/or using pre-cleaned supplies for each station. Sampling occurs in all systems over a 2d period in late August of each year so the data are compatible with Chesapeake Bay Long-Term Benthic Monitoring Program results.

**Metrics**

*Benthic Contaminants* - Anthropogenic contaminants are common in aquatic environments and their impacts to aquatic communities have been well documented. Many contaminants entering water bodies are hydrophobic and therefore accumulate in benthic sediments. Field collection and sample storage are provided in **Method Benthic Habitat 1,** and the methods for chemical contaminant analysis are provided in **Method Benthic Habitat 2 .**

*Benthos* - Benthic indices of biotic integrity are a common community level metric of ecosystem health and calculated annually for the entire Chesapeake Bay along with several other measures of benthic health (www.baybenthos.versar.com/docs/ChesBayBIBI.PDF) provided in **Method Benthic Habitat 3.**

*Sediment Toxicity* - Chemical contaminants within benthic sediments may be toxic to aquatic life. Measurements of chemical contaminant concentrations do not provide sufficient evidence to determine relative toxicity levels, as some chemicals may not be bioavailable or mixtures of chemicals may be more or less toxic than the sum of their individual toxicities. Details are provided in **Method Benthic Habitat 4**

**Method Benthic Habitat 1: Field Collection and Sample Storage**

1. Label EPA-certified jars with project name, date, time, location, and type of analysis. Need one glass jar for metals, one glass jar for Microtox (SDIX, Carlsbad, CA) and one plastic jar for organics.
2. Collect sediment using bottom sediment grab sampler; make sure grab closes completely.
3. Allow water to drain, then remove the superficial 2-3cm of sediment with an acetone-washed stainless steel or sterile disposable plastic spoon or scoop and transfer sediment to an acetone-washed stainless steel bowl. Homogenize sediments in bowl with spoon.
4. For benthic analysis, place remaining sediment into labeled plastic sealable bags, hold on ice, and store samples at -20°C until shipped to diagnostic laboratory of choice.
5. For contaminant analysis, transfer sediments into jars leaving 1-2cm space at top and close lid. Use care not to touch outside of jar with spoon.
6. Wrap electrical tape around lid to completely seal; place jars into plastic bags and store on ice.
7. At each new site, rinse sediment grab, bowl, and spoon with water from that site and follow with acetone washes to begin procedure again.

Microtox (SDIX, Carlsbad, CA) samples can be stored at 4°C; organics and metals samples can be stored -20°C until seasonal sampling is complete then shipped overnight to diagnostics laboratory of choice

**Method Benthic Habitat 2: Chemical Contaminant Analysis**

1. Sediments are thawed and then extracted with methylene chloride, concentrated by nitrogen blow-down, and cleaned by gel permeation chromatography.
2. Concentrations of PAHs, PCBs, organochlorine pesticides, and polybrominated diphenyl ethers are measured using gas chromatography/ion trap mass spectrophotometry, gas chromatography/mass spectrophotometry with negative chemical ionization, and gas chromatography/mass spectrophotometry with electron ionization sources.
3. Trace metals are measured using a combination of inductively coupled plasma spectrophotometry and graphite furnace atomic absorption.
4. Mercury is analyzed by direct mercury analysis using atomic absorption.

5. Quality control blanks, spikes, duplicates, and standard reference materials are analyzed for each group of samples.

**Method Benthic Habitat 3: Benthic Index of Biotic Integrity**

1. Benthic fauna are identified to the lowest practical taxonomic level and counted.
2. For most organisms, identification is to genus or species level.
3. Pelagic, epifaunal, and nonfaunal organisms are excluded from the analysis.
4. Ash-free dry weight is calculated for each species by drying the organisms to a constant weight at 60°C and ashing in a muffle furnace at 500°C for 4hr and reweighing (ash weight).
5. The condition of the benthos is determined using the Chesapeake Bay Benthic Index of Biotic Integrity (B-IBI) (Llansó et al. 2003).
6. The index includes the parameters, abundance, species diversity, biomass, total number of pollution-tolerant species, and number of pollution-sensitive species.
7. For measurements of numbers of species and diversity, general taxonomic designations at the generic, familial, and higher taxonomic levels are dropped if there is one valid lower level designation for that group.
8. Criteria for assessing non-degraded, marginal, and degraded benthos are based on sediment composition (the percent of silt and clay) and salinity.

**Method Benthic Habitat 4: Sediment Toxicity**

1. Sediment samples for toxicity testing are collected in pre-cleaned 4oz jars and placed in 4°C storage until time of analysis.
2. Microtox (SDIX, Carlsbad, CA) assays are conducted according to the standardized solid-phase protocols with the Microtox Model 500 (SDIX, Carlsbad, CA) analyzer and all materials and reagents are purchased from SDIX (SDIX, Carlsbad, CA).
3. Sediment from each station is homogenized and a 7.05±0.05g sediment sample is used to make a series of sediment dilutions with 3.5% NaCl diluent.
4. Test samples are placed in a 15°C water bath for 10min incubation.
5. Luminescent bacteria (*Vibrio fisheri*) are then added to the test concentrations for 20min incubation.

6. At the end of the incubation period, a column filter separates the liquid phase from the sediment phase, and bacterial post-exposure light output is measured using Microtox Omni Software (SDIX, Carlsbad, CA).
7. An EC50 (the sediment concentration that reduces light output by 50% relative to the controls) value is calculated for each sample.
8. Triplicate samples are analyzed simultaneously.
9. Sediment samples are classified as either toxic or nontoxic using criteria developed by Ringwood et al. (1997), taking into consideration the sediment composition.
10. Sediment from any station with a combined silt and clay content greater than or equal to 20% and an EC50 less than 0.2%, or sediment from a station with a silt-clay content less than 20% and an EC50 less than 0.5% is considered toxic.
11. These criteria are developed by comparing Microtox® (SDIX, Carlsbad, CA) results against sediments classified as degraded and under-graded based on contaminant content (Ringwood et al. 1997).

# Bioindicators

## Fish Indicators

**Introduction**

Fish communities have long been used as indicators of ecosystem health. Distribution, diversity, and abundance of populations are the cornerstones of ecological principles. Comparison of diversity indices, presence/absence of rare species, and trophic balance are all meaningful in identifying ecosystem change. However, many of these indices do not lend themselves to understanding the mechanistic cause of change or identifying individual stressors within a population. More recently, biomarkers and bioindicators have gained favor as a means of evaluating biological response of organisms. Biomarkers tend to be associated with contaminant

exposure and generally represent lower levels of biological organization, while bioindicators encompass all levels of response and are thus representative of ecosystem status (Adams 2005).

The choice of an indicator species is critical and is based on the diversity of fish that would be expected in chosen river systems. The organism must be sensitive to the stressors encountered as well as have broad spatial and temporal distribution to allow for system comparison. In the Chesapeake Bay, perhaps the most ubiquitous and abundant fish is the white perch (*Morone americana*). White perch are a semi-anadromous species which migrate to tidal fresh waters to spawn, and spend the remainder of their lives in brackish waters. While overwintering in deeper, downstream sections of the Bay's tributaries, white perch exhibit a high degree of system fidelity throughout their lives. Ecologically, these fish are zooplanktivorous as larvae then transition to benthic predators as juveniles and adults. In addition, river specific differences in growth and survival have been well documented relating to the availability of resources (Seltzer-Hamilton 1992). Finally, the species is highly susceptible to a variety of pathogens and sensitive to oxygen concentrations <5mg/L. Because of the ubiquitous nature of the white perch, system fidelity, mid-level trophic positioning, and responsiveness to environmental stressors, this species has been chosen as the predominant species for fish bioindicators.

White perch inhabit both deepwater channels and nearshore areas during the year, and exhibit random movements, making the signal obtained somewhat of a composite for the system of study. In contrast, the mummichog (*Fundulus heteroclitus*) inhabits nearshore vegetated areas and has less than a 50m home range. A wealth of data has been obtained from this species because of its ubiquitous distribution, relative abundance, ease of capture, and hearty nature. As a benthic detritivore, the mummichog has been chosen to complete the spatial habitat coverage of each system and allow for identification of direct land margin interface stressors.

**Sampling Design**

For the 3yr developmental stage of this program, white perch, mummichogs, and fish community composition data are collected from each of the three river mile segments during the spring, summer, and fall. White perch and mummichogs are collected randomly from each segment, (**Method Fish 1**) from each system within each of the three river mile segments, and across all

seasons. Where possible, two sites are used within each river mile to obtain the fish. Fish community composition was conducted from May through October on a two week rotation in each system.

**Metrics**

**White Perch**

*Gross Pathology/Field and Observational Data* – External lesion prevalence, gross abnormalities, and internal and external parasite burden are common endpoints in both IBIs and biomarker studies, as elevated prevalence of any is broadly indicative of environmental stress. Additionally, the proportion of the viscera covered in lipid stores, the ratio of weight to length, and the relative weight of the liver all provide measures of the nutritional status of the organism. These observations are conducted in the field, and necropsy techniques and qualitative methods for ranking are included as **Method Fish 1**.

*Histopathology* – Routine histopathology of spleen, liver and kidney provides information on parasite burden and disease state, which cannot be fully determined by gross examination during necropsy, bacteriology (infection) or other observations. Intersex, the co-development, determined by histological examination of the gonads, is an indicator of exposure to endocrine disrupting compounds. Methods for fish tissue histopathology preparation and staining are included as **Method Fish 2**, and specific techniques for enumeration and severity ranking of inflammation and other changes in tissue structure are provided as **Method Fish 3**. The size and concentration of macrophage aggregates have been demonstrated to be useful indicators of low dissolved oxygen and chemical contaminant stress (Fournie et al. 2001, Meyers and Fournie 2002). Methods for measuring macrophage aggregate density are provided as **Method Fish 4**.

*Hemotology and Plasma Protein* – Packed cell volume and hemoglobin are useful measures of environmental stress, particularly exposure to low oxygen conditions (Houston 1990). Plasma protein can also indicate general nutritional status. All blood work was provided in **Method Fish 5**.

*Bacteriology* – Culture and identification of bacteria within fish may be related to environmental stressors through enhancing susceptibility to, or concentration of, pathogens in the environment. The same specific pathogens targeted in water sampling are selectively cultured from specific organs of fish in combination with general bacteriology. Methods for aseptic sampling of fish are included with field **Method Fish 1**, and preparation of agar and identification techniques are provided in **Method Fish 6**.

*Transforming Growth Factor Beta (TGF- ß)* **–** TGF- *ß* is a cytokine, or a molecule secreted by cells of the immune system for communication. Elevated concentrations have been demonstrated to suppress macrophage bactericidal activity in hybrid striped bass and white perch (Harms et al. 2000a, b; Chris Ottinger, personal communication, U.S. Geological Service, 11649 Leetown Road, Kearneysville, WV 25430). Methods for TGF-*ß* are provided as **Method Fish 7**.

*RNA:DNA Ratios* **–** The ratio of ribonucleic acid (RNA) concentration to deoxyribonucleic acid (DNA) concentration in body tissue is a useful indicator of nutritional status and somatic growth in fish (Bulow 1987). The quantity of DNA within a cell is constant within a species, while the quantity of RNA varies with protein synthesis. Since growth is a function of protein synthesis, RNA:DNA ratios provide a sensitive indicator of growth potential at any given time. Methods for RNA:DNA are provided as **Method Fish 8**.

*Endocrine Disruption* **–** Endocrine disruption has been linked to a variety of pesticides and commercial chemicals. In combination with histological observation, measurement of concentrations, or the ratio of vitellogenin and testosterone in blood, offers a further, and perhaps more sensitive, indicator of exposure (Sumpter and Jobling 1995). Methods are provided as **Method Fish 9**.

*Community Composition* - The Index of Biotic Integrity (IBI) is a well-established approach to examine changes in community structure associated with disturbance gradients (Karr 1981). Comparison of diversity indices, presence or absence of rare species, and trophic balance are all meaningful in identifying ecosystem change. Methods for field collection of organisms and calculation of diversity indices are provided as **Method Fish 10**.

*Nutritional Condition*- Measurement of tissue moisture content in fish muscle provides a relatively rapid metric for determining the nutritional status of fish. This metric takes advantage of the inverse relationship between tissue lipid and tissue moisture concentrations in fish; as lipid stores are reduced (i.e. starvation, poor feeding opportunities, reproduction), oil globules in the muscle are replaced by water (Love 1980). Use of tissue moisture alone as a metric precludes the need for more extensive laboratory analysis (i.e. proximate analysis) thereby lowering sample processing time and cost. Methods for measuring tissue moisture are included in **Method Fish 11.**

**Mummichog**

*Gross Pathology/Field and Observational Data* – External lesion prevalence, gross abnormalities, and internal and external parasite burden are common endpoints in both IBIs and biomarker studies, as elevated prevalence of any is broadly indicative of environmental stress. Additionally, the ratio of weight to length provides an estimate of the nutritional status of the organism. These observations are conducted in the field, and necropsy techniques and qualitative methods for ranking are included as **Method Fish 1**.

*Acetylcholinesterase (AChE)* – AChE is the enzyme responsible for the removal of acetylcholine, the primary neurotransmitter in fish. Considerable data exist concerning environmental toxicants (primarily pesticides) that are AChE inhibitors, most notably those containing high levels of organophosphates (Olson and Christensen 1980, Mineau 1991). Methods for AChE determination are included as **Method Fish 12**.

**Method Fish 1: Field Collection, Gross Pathology and Observation**

*White Perch*

1. Collection and Handling
    a. White perch are captured using either a 16-ft otter trawl, 100-ft 1/8in-mesh beach seine or hook and line.
    b. Fish are collected at random sites within each river mile segment until 12 fish are captured.

c. Trawls are pulled for no longer than 6min at 2 knots for minimal stress on catch.
d. Perch >160mm are immediately placed into a 50-gal container filled on location with river water.
e. Fish are immediately transported to the field processing area and maintained in separate 50-gal containers for each river mile segment.
f. A total of 12 fish per river mile segment are collected per season (36 total).

2. External Observation
   a. Examine behavioral abnormalities (spiral swimming, flashing, unusual gill opercula, prostration, etc.).
   b. Examine external abnormalities (hemorrhaging, cloudy cornea, body discoloration, ulceration, deformity, gill parasites, etc.).
   c. Record abnormalities on White Perch Data Sheet (Appendix I: Field Sheet - Fish).
   d. Fish are removed individually with a small aquarium net and handled with a wet rag covering the eyes and operculum.
   e. Length - All fish are measured to the nearest millimeter and data recorded.
3. Bleeding
   a. A peripheral blood sample (up to 3mL) is taken from the caudal vessels of un-anesthetized fish using a 22ga syringe.
   b. After removal of the needle and purging of air, a single microhematocrit capillary tube and HemaCue (HemoCue, Inc, Cypress, CA) cuvette are filled to determine hematocrit volume, plasma protein, and percent hemoglobin (**Method Fish 5**).
   c. The remainder of the blood (1-2mL) is transferred to heparinized microcentrifuge tubes, mixed gently, and placed on ice.
   d. Plasma is separated within an hour by centrifugation at 10,000 X *g* for 5min, then placed on dry ice and stored at -80°C for future analysis of endocrine disruption (**Method Fish 9**).
4. Euthanasia and Weight
   a. Following blood collection, fish are euthanized by severing the spinal cord posterior to the head.
   b. Fish are then weighed to the nearest gram and recorded on the data sheet (Appendix I: Field Sheet - Fish).

c. Fulton's condition factor (*K*) is calculated at ($Wt/Ln^3$) X 100000, and log weight-length regressions compared.
d. Rrelative weight (*Wr*) is calculated as (*Wt/Ws*) X 100 where Ws = $W_{standard}$ and is a "standard weight" for fish of the same length (Bister et al. 2000).

5. Skin Scrapes and Gill Biopsy
   a. Skin is scraped with the edge of a cover glass and placed in a drop of distilled water on a microscope slide and cover-slipped.
   b. Gill filaments are excised with a small pair of surgical scissors and placed in a drop of distilled water and cover-slipped.
   c. These are examined immediately at 40X and 200X since brachial epithelia rapidly deteriorate causing postmortem artifact.
   d. Bacteria, fungus and protozoan parasites such as *Ichthyobodo* and *Trichodina* are documented if present.
   e. Gas bubbles in capillaries, telangiectasia, hyperplasia, external parasites (bacterial, protozoan, fungal, metazoan), or other foreign bodies are noted.
   f. Parasite data are recorded on a separate data sheet (Appendix 1: Field Sheet - Fish).
6. Aseptic Necropsy
   a. The abdominal cavity is entered by first making a small anterior incision with sterile scissors through the anal opening.
   b. After first cut, tools are re-flamed to disinfect.
   c. Incision is continued from anal opening to throat, being careful not to cut the digestive tract; keeping one blade of scissors inside the body cavity and one blade on the outside of the fish.
   d. Flame the blade as necessary if crossed into or out of the fish.
   e. A second cut is made by again starting at the anal opening and moving upward to the lateral line and following the lateral line to underneath the operculum, using sterile tweezers to pull the body flap towards the head as the cut is made.
   f. Tools are again flamed as necessary to disinfect if blades crossed in or out of the fish.
   g. A final cut is made ventrally, behind the operculum to completely remove body flap.
   h. If swim bladder is full, deflate with scissors to better assess internal organs.
7. Bacteriology

a. A small incision is made in the liver and head kidney using sterile scissors.
b. A sterile applicator stick is then placed in the incision and streaked on tryptic soy agar plates **(Method Fish 6**).
c. Remaining pieces of spleen are aseptically removed and cut into three sections and processed accordingly:
    1. Place in a sterile, labeled sample bag packed on ice for culture of *Mycobacterium* spp. (**Method Fish 6**).
    2. Place in cryovial containing 500µL of RNAlater (AMBION, Inc., Austin, Texas) and store on wet ice for examination of TGF-ß (**Method Fish 7**).
    3. Leave in fish for histopathology (**Methods Fish 2, 3,** and **4**).

8. Gross Pathology
    1. After sterile procedures, the skin and internal organs of fish are examined visually and lesions on the viscera are noted for abnormalities such as: discoloration or mottled appearance; enlargement (hypertrophy); redness; abscesses or cysts; fluid in the abdominal cavity (ascites causing potbelly); tissue swelling, masses or foreign bodies such as fungus, or metazoan parasites
    2. Tissue Trimming
        a. Tissues are dissected from each fish for histopathology and condition indices.
        b. Liver is removed and weighed to the nearest 0.01gm to calculate the liver somatic index (Liver Wt/Whole Fish Wt) X 100).
        c. Liver is divided into six sections ≤0.5mm in any plane and placed in 10% neutral buffered formalin (**Method Fish 2**).
        d. Spleen, head kidney, and gills are preserved in a similar fashion as above.
        e. A body fat index is recorded as follows:
            1. 0 = no fat
            2. 1 = ~25% of visceral cavity consists of fat.
            3. 2 = ~50% of visceral cavity consists of fat.
            4. 3 = ~75% or greater of visceral cavity consists of fat.
    3. Muscle Sampling
        a. Moisture analysis

1. With a sharp filet knife make an incision on the dorsal side of the fish immediately posterior to the operculum.
2. Continue the incision down to the abdominal cavity/lateral line, removing the entire section of muscle extending to the tail fin.
3. Remove the skin portion and any bones from the fillet.
4. Wrap samples in a sheet of cellophane.
5. Place cellophane wrapped sample in freezer paper.
6. Freeze immediately on dry ice.
7. Store samples at -80° C for moisture assay (**Method 11**).

b. RNA:DNA analysis

1. Remove a section of white muscle from the left shoulder of each fish (skin removed).
2. Wrap tissue in freezer paper.
3. Freeze immediately on dry ice.
4. Store at -80°C until subsequent determination of RNA: DNA ratios (**Method Fish 8**).

*Mummichogs*

1. Collection and Handling
   a. Mummichogs are captured using either a 100ft 1/8in-mesh beach seine or baited minnow trap. Efforts are conducted randomly within each river mile segment until 12 fish are captured.
   b. GPS coordinates are recorded for each location where fish are captured.
   c. Fish are immediately placed into a 5gal container filled on location with river water.
   d. Fish are immediately transported to the field processing area and maintained in separate 5gal containers for each location within each river mile segment.
   e. Twelve fish per river mile segment are collected per season (36 total).
2. External Observation
   a. Fish are removed individually with a small aquaria net and are examined for sex and external lesions (hemorrhaging, cloudy cornea, body discoloration, ulceration, deformity, gill parasites, etc.).
   b. Information is recorded on Mummichog data sheet (Appendix I: Field Sheet - Fish).

c. Length of fish is measured to the nearest millimeter and data recorded.
d. Fish are euthanized by severance of the spinal cord posterior to the head.
e. Fish are then weighed to the nearest gram and recorded in the log book.

3. Brain and Histology Samples
   a. Sever head
   b. Immediately wrap in freezer paper, label, and place on dry ice for subsequent determination of acetylcholinesterase activity.
   c. Remove gut packet by cutting the length of the ventral side and place in 10% neutral buffered formalin for histological processing (**Method Fish 2**)
   d. Note any internal parasites.

Tissue Fixative Formula:

Neutral Buffered Formalin (NBF)

*37-40% formaldehyde stock*

Formaldehyde . . …… 1.0L **(potential carcinogen)**

Sodium phosphate dibasic anhydrous . . . . . 15.0g

Sodium hydroxide ……..……... 0.064g

*10% NBF working solution*

Stock . . . . . ……. 1 part

Filtered ambient or artificial seawater (see p. 144). . . . . 9 parts

Optimum fixation: 24-48hr (cytological preps, 5-15min) at 20°C

Buffer to pH 7.4-8.0.

Rinse to remove excess formalin before placing tissue in 70% ethanol.

Rinse cytological preps in tap water 5-30min before staining.

**Method Fish 2: Histopathology**

*White Perch and Mummichog*

1. Preserved tissues from each fish are trimmed and placed in several cassettes: spleen and head kidney are placed in one cassette, liver in one cassette, and each gonad are trimmed to a minimum of six pieces, when possible, and placed together in another cassette.

2. Mummichog - Process same except dissect spleen and liver from animal.
3. Tissues are processed overnight on a 15hr cycle in a tissue processor and embedded in paraplast; blocks are sectioned at 5µm and stained as follows:
    a. Spleen and head kidney – six slides are prepared from six disparate yet consecutive levels of spleen and head to provide tissue from throughout the organs. Slides # 1, 3, and 5 are stained with Mayer's hematoxylin-eosin-phloxine (MHE). Slides # 2, 4 and 6 are stained with Ziehl-Neelsen acid-fast bacteria (AFB).
    b. Liver – two slides with adjacent tissue are prepared per block. One is stained with MHE and the other is archived for possible AFB stain.
    c. Gonad - one slide per block are stained with MHE.

Tissue Stain Formula:

Mayer's Hematoxylin and Eosin (MHE) (1hr 10min) (Luna 1968)

1) Deparaffinize

2) Hydrate to water

3) Stain in aged Mayer's hematoxylin for 5min

4) Rinse in distilled water for 2min

5) Dip 2-3 times in 0.5% lithium carbonate or 30sec, or until sections turn blue (sections may turn blue in slightly alkaline tap water without lithium carbonate)

6) Rinse for 2min in distilled water to eliminate lithium carbonate

7) Soak in 70% ethanol for 3min

8) Prepis eosin Y-phloxine B stock and working solutions

9) Counterstain in eosin Y-phloxine B working solution for 3min

10) Dip 6 times in 95% ethanol

11) Dehydrate

12) Clear

13) Mount

Basophilic substances - blue; acidophilic substances - pink

***Reagents*:**

Mayer's hematoxylin

*Dissolve in order*

Distilled water .....1000.0mL

Aluminum ammonium sulfate ..... 50.0g

Hematoxylin .....1.0g

Citric acid .....1.0g

Chloral hydrate (see *Note*) ..... 50.0g

Sodium iodate ..... 0.2g

Note: Allow to age at least 1wk, filter before use, stores well for months. Chloral hydrate in the USA is a controlled substance that requires a special permit issued by the U.S. Department of Justice, Drug Enforcement Administration.

*20.5% lithium carbonate*

Lithium carbonate ..... 0.5g

Distilled water ..... 100.0mL

*Eosin Y-phloxine B stock solution*

95% ethanol .....877.5mL

1% eosin Y..... 112.5mL

1% phloxine B ..... 11.25mL

*1% eosin Y:*

Eosin Y ..... 1.0g

Distilled water ..... 100.0mL

*1% phloxine B:*

Phloxine B ..... 1.0g

Distilled water ..... 100.0mL

*Eosin Y-phloxine B working solution*

Stock solution ..... 200.0 mL

Glacial acetic acid ..... 0.9 mL

Ziehl-Neelsen and Harris' Hematoxylin (AFB) (1.75hr) (Farley 1965)

Specific for acid-fast substances (e.g., haplosporidan spores, microsporida, and *Cryptosporidium* oocysts). Use a positive control test slide.

***Procedure*:**

1) Deparaffinize
2) Hydrate to water
3) Stain in Ziehl-Neelsen fuchsin - 15min
4) Rinse in distilled water - 6 dips
5) Destain in 0.1 N sulfuric acid - 10-15min (time varies with specific tissue)
6) Ish in running tap water - 10min
7) Stain in Harris' hematoxylin - 2min
8) Rinse in distilled water - 6 dips
9) 0.5% lithium carbonate - 3 dips, 30s, or until sections turn blue (sections may turn blue in slightly alkaline tap water without lithium carbonate)
10) Rinse well in distilled water to eliminate lithium carbonate - 5min
11) Dehydrate
12) Clear
13) Mount

***Results***:

Basophilic substances – blue; Acid-fast substances, including mature haplosporidian, sporoplasms, lipofuscins, acid-fast bacteria, and *Cryptosporidium* oocysts - bright red.

***Reagents*:**

Ziehl-Neelsen fuchsin (Gray 1954)

Basic fuchsin (magenta) .....1.0g
Phenol (liquefied) ..... 5.0mL
90% ethanol .....10.0mL
Distilled water .....100.0mL

1) In fume hood, grind fuchsin with phenol in a mortar

2) When rinsed, add alcohol in 10 successive lots while grinding

3) Rinse stain from mortar 10 X 10mL each rinse with distilled water

4) Save and filter

Note: Basic fuchsin (pararosaniline) available from Fisher Scientifice cat. # F-98-10.

0.1N sulfuric acid ($H_2SO_4$)

Distilled water ..... 997.2mL

$H_2SO_4$ specific gravity =1.84 ..... 2.8mL

Harris' Hematoxylin

Note: Harris' hematoxylin available from Newcomer Supply cat. # 1201A.

0.5% lithium carbonate

Lithium carbonate .....0.5g

Distilled water ..... 100.0mL

**Method Fish 3: Histopathology Indicators and Scaling**

*Inflammation* –The primary eitiological agent for granulomatous inflammation in white perch may be bacterial infections associated with mycobacteriosis.

Tissue Preparation - Six tissue sections of liver, spleen, and anterior kidney tissue per fish is prepared as stated above in histological methods. Each MHE stained section is followed by a corresponding section stained with AFB.

a. Each of the six sections is observed at 10X, granulomas enumerated, and scored as follows:
    1. 0 if 0 granuloma.
    2. 1 if one granulomas.
    3. 2 if two to six granulomas.
    4. 3 if six or more granulomas is present per section.
b. Mean score is rounded to nearest whole number for each section and fish.

c. Fish tissues is considered positive if AFB+ bacteria is observed in any granuloma stained with AFB.

1. Parasite Burden – Equally characteristic of white perch is the presence of digenetic trematodes and other internal parasites. Numbers of encysted parasites are scored in spleen, liver, and anterior kidney using the same scale and process as for inflammation.
2. Liver Glycogen – Glycogen stores in the liver can be indicative of nutritional status and is readily visible, as the architecture of the vacuole remains after glycogen is removed during histological processing.
    a. Six sections of hematoxylin and eosin stained liver are examined and scored from 1-4 based on:
        1. No space present between hepatocytes
        2. Vacuoles present, but minimal or localized
        3. Vacuoles of equal size and distribution as hepatocytes
        4. Fatty liver, hepatocytes secondary to vacuoles
    b. Mean score is rounded to nearest whole number for each fish.

**Method Fish 4: Macrophage Aggregates**

1. Spleens are removed, fixed, and processed as described in Methods Fish 1 and Methods Fish 2.
2. At 10X magnification, a minimum of four random photos of spleen tissue is taken per fish.
3. Photos are then loaded into National Institutes of Health (NIH) ImageJ processing program.
4. Calibration of photo measurements is achieved using a stage micrometer and setting a 100µm known distance within ImageJ.
5. Total photo area equaled 560993.426µm$^2$. Four photos for one fish represent 2.24mm$^2$.
6. Using the image analysis software, total tissue area in each image is measured using color contrast (threshold function) area of each macrophage aggregate is determined by manual delineation using the freehand selection tool and measured individually for aggregate area.

7. Measurement data is transferred to a spreadsheet to correlate tissue area to total aggregate area and count of macrophage aggregate and analyzed using SAS software. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names is registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA

**Method Fish 5: Blood**

*Blood Withdrawal and Preparation*

1. Pre-label lithium heparin tubes and cryovials.
2. Withdraw 1.5mL blood through caudal vein (or as much as possible).
3. Euthanize fish by severing spinal cord behind head.
4. Carefully unscrew needle from syringe and dispose in a needle and sharps disposal container.
5. Fill one microhematocrit tube and place in clay in appropriate numbered space.
6. Label and fill one hemoglobin cuvette.
7. Fill two lithium heparin tubes, invert five times and place immediately on ice.

*Hematocrit*

1. Remove hematocrit tube from clay and gently push clay into tube to ensure it seats well.
2. Place four hematocrit tubes (clay ends first) into centrifuge.
3. Centrifuge at 3900 X *g* for 5min.
4. Remove from centrifuge, being careful to keep in order, and place on reader to record hematocrit volume.

*Protein*

1. After hematocrit reading, carefully snap tube in the plasma just above white cell line.
2. Gently tap plasma onto optical end of refractometer, using the unbroken end to tap against the optical end.

3. Hold up to light and take protein reading.
4. Dispose of broken tube in a needle and sharps disposal container.
5. Rinse optical lens with distilled water, dab with a delicate wipe, and repeat for remaining samples.

*Hemoglobin*

Following manufacturer's instructions for operation, insert cuvette into hemoglobin reader and record hemoglobin level.

*Plasma Collection*

1. Remove lithium heparin tubes from ice and place in centrifuge.
2. Centrifuge at 10,000 X *g* for 10min.
3. Using 1-mL pipette, remove plasma from lithium heparin tube and place in corresponding cryovial.
4. Repeat for duplicate lithium heparin tube, and once done place on dry ice.

*Supplies for Method Fish 5 - Blood*

| | | |
|---|---|---|
| 1½-in x 23ga x 3-mL syringe | Hemoglobin reader | Sharps container |
| Microhematocrit tubes | Hemoglobin cuvettes | Centrifuge |
| Hematocrit tube clay | Protein refractometer | 1mL pipette |
| Hematocrit reader | Lithium heparin tubes | 1mL pipette tips w/filter |
| Hematocrit tube centrifuge | Cryovials | Cooler with dry ice |

**Method Fish 6: White Perch Bacteriology**

*Plate Media*

1. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. Check pH and adjust if necessary. Media must be heated until agar dissolves before dispensing into bottles.
2. Pour into suitable screw-capped glassware.
3. Autoclave for 15min at 15psi or according to instructions of manufacturer. Be sure to leave lids loose.
4. Cool to approximately 50°C in a water bath before pouring.
5. Alternatively, cool media to room temperature and store. Label with type, date and initials. When media is needed, boil or microwave stored media bottles to melt agar and cool to 50°C before pouring.
6. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface.
7. Label the bottom of each plate with medium type and date prepared.
8. Remove bottle cap and pour into plates, lifting each petri dish lid as you go. Replace plate lids as soon as the medium is poured. Do not allow medium to run down the outside of the bottle. Hold the bottle as horizontal as possible while pouring.
9. Immediately rinse medium bottle and cap with hot water to remove agar and clean up any spilled agar.
10. Allow plates to sit overnight at 20°C or for a few hours at 37°C to reduce excess moisture. Store plates upside down in the refrigerator in a tightly sealed plastic bag. Label bag with medium type and date prepisd.

*Media Formulations*

1. Media for Tryptic Soy Agar (TSA)

| | |
|---|---|
| NaCl | 10g |
| Agar | 15g |
| Pancreatic digest of casein | 15g |
| Papaic digest of soybean meal | 5g |
| Distilled water | 1L |

2. Media for Middlebrook 7H10 agar (Becton, Dickenson & Co., Franklin Lakes, NJ)

| | |
|---|---|
| Middlebrook 7H10 Agar | 19g |
| Glycerol | 5mL |
| Middlebrook OAD Enrichment | 100mL |
| Distilled water | 1L |

3. Media for Phosphate Buffered Saline (PBS)

| | |
|---|---|
| NaCl | 7.650g |
| $Na_2HPO_4$, anhydrous | 0.724g |
| $KH_2PO_4$ | 0.210g |
| Distilled water | 1L |

*Enumeration of Mycobacterium spp. from spleen*

1. Pre-weigh a clear, sterile sample bag to be used for spleen collection.
2. Label Middlebrook 7H10 agar (Becton, Dickenson & Co., Franklin Lakes, NJ) plates with sample ID's (one plate per sample).
3. Spleens is removed from freezer and allowed to briefly thaw.
4. Re-weigh clear, sterile sample bag containing spleen and note spleen weight.
5. Using a flat, non-sharp tool gently mash spleen within clear, sterile sample bag to break apart tissue
6. Aseptically add 2mL of PBS to clear, sterile sample bag and stomach on high for 2min; up to 5 clear, sterile sample bags may be placed in stomacher.
7. Using autoclaved or new filtered pipette tips, add 200µL of spleen/PBS suspension to the center of a Middlebrook 7H10 agar (Becton, Dickenson & Co., Franklin Lakes, NJ) plate.
8. Dip spreading bar in 100% ethanol and pass through a flame to disinfect.
9. Allow bar to cool and spread spleen/PBS mix evenly around plate.
10. If organic matter accumulates on bar, scrape off before using on plate.
11. Replace plate lid and allow liquid to dry onto plate before inverting.
12. Once dry, seal plate with parafilm and incubate at 30ºC.

13. Check plates for growth after 2wk, and then 1/wk for up to 3mo.

**Method Fish 7: Transforming Growth Factor Beta**

*Sample Collection*

1. Remove a small (~1 by 1cm) section of white perch spleen and preserve in RNAlater (AMBION, Inc., Austin, Texas) as described in **Method Fish 1**.
2. Place on wet ice and store overnight at 4°C to allow buffer to penetrate.
3. Subsequently store all samples at -80°C until analyzed.

*RNA Extraction and Purification*

1. Total RNA is extracted from archived frozen spleen tissue samples using RNeasy kits (Qiagen, Gaithersburg, MD) according to manufacturer instructions.
2. All samples are treated with RNase-free DNase (Qiagen, Gaithersburg, MD).
3. RNA is quantified using a Nanodrop ND-8000 (Nanodrop/Thermo Fisher Scientific, Wilmington DE).
4. RNA quality is assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

*qPCR*

1. For each sample, 2µgm RNA is reverse transcribed in a 20µL reaction, using High Capacity Reverse Transcription kit (Applied Biosystems, Grand Island, NY) according to manufacturer's instructions.
2. Each reverse transcription reaction is then diluted tenfold in nuclease-free water.
3. Real-time PCR is performed on an ABI7900HT using Power SYBR Green (Applied Biosystems, Grand Island, NY).
4. All real-time PCR reactions (TGF-β and β-actin) are done in triplicate with 4µL diluted reverse transcription reaction as template in 20µL total volume qPCR reactions.
5. Primers for real-time PCR are designed using Primer Express (Applied Biosystems, Grand Island, NY).

6. Both TGF-β primers and β-actin primers span a predicted intron.
7. Mean expression of TGF-β is normalized to mean expression of β-actin for each sample.

*TGF-β Primers and β-actin Primers for Real-time PCR for Transforming Growth Factor Beta*

| Gene | Plasmid | Forward primer | Reverse primer |
|---|---|---|---|
| TGF-β | LRB-19 | LRO-196: CCACGGAGACGAAGGATGTC | LRO-197: AGCTCCGCACGCAACAG |
| β-actin | LRB-17 | LRO-198: ACCATCGGCAATGAGAGGTT | LRO-200: TGTAGGTGGTCTCGTGGATTCC |

**Method Fish 8: White Perch Growth Potential - RNA: DNA Analysis**

Formula for Digestion Reagents and Buffers

*Proteinase K Stock Solution Preparation*

1. Reconstitute 25mg of proteinase K in 15mL of DNase-free water.
2. Shake to dissolve (25mg/15mL= 1.7mg/mL).
3. Divide into 1mL aliquots and store at -20$^{o}$C.

*Tissue Digestion Buffer Preparation*

1. Add 25mL of nuclease-free water to 50-mL conical tube.
2. Add.

| | |
|---|---|
| Proteinase K | 2mL stock solution (33µg/mL per tube X 2 tubes = 66µg/mL) |
| 1% SDS | 0.5g sodium dodecyl sulfate |
| 0.1M NaCl | 0.2922g sodium chloride |

3. Add NaCl last to reduce bubbles before bringing to volume with 23.0mL of nuclease-free water.
4. This is enough for approximately 41 tubes at 1.2mL per tube.

5. Store at 4°C.

*TE Buffer Preparation*

1. Small batch: Add 525µL of 20X TE stock to 10mL volumetric and dilute with 9975µL of nuclease free water to create buffer. Mix thoroughly. Total volume = 10.5mL.
2. Large batch: Add 2.5mL of 20X TE stock to 47.5mL of nuclease-free water. Total volume = 50.0mL. Large batch will be enough for performing RNA and DNA analysis in same day.

Note: Need 22mL for 26 samples, PicoGreen or RiboGreen (Molecular Probes, Inc., Eugene, OR) and standard.

*Tissue Preparation*

1. Label a sterile RNase/DNase-free 2.0mL tube for each sample.
2. Transfer white muscle tissue archived at -80$^{o}$C to -20$^{o}$C until ready to cut.
3. In sterile aluminum tray, cut muscle while still frozen and firm with sterile blade (alcohol and flame) to obtain inner section with all outside surfaces removed. Remove excess tissue from tray and place back in freezer paper for further processing if necessary; hold at -20$^{o}$C until ready to archive again at -80$^{o}$C.
4. Using sterile tissue grinder, pulverize tissue sample into a thick paste leaving no un-homogenized chunks.
5. Using sterile scoopula (alcohol and flame), place small piece of tissue in labeled, tisd microcentrifuge tube to obtain 0.04g and 0.06g sample to keep similar sizes. Discard remaining homogenate. Keep samples on ice until ready to add digestion buffer.
6. Add 1.2mL of digestion buffer and place in heating block at 55$^{o}$C. Vortex each sample and mince with sterile forceps (alcohol and flame) several times to aid digestion buffer.
7. Vortex periodically and let sit until digested (3-16hr or overnight).

8. Spin tubes at 12,851g for 10min. Remove supernatant and place in new labeled sterile nuclease-free tube.

Tissue Analysis: DNA

*DNA Reagent Preparation (PicoGreen)*

1. For 25 wells, 100-µL each, add 12.5µL of PicoGreen to 2.35mL TE in 10mL centrifuge tube covered with aluminum foil. Mix thoroughly.
2. For 48 wells (half plate), add 25µL of PicoGreen to 4.975mL TE in 10mL tube covered with aluminum foil. Mix thoroughly.
3. For 96 wells, 100µL each, add 50µL of PicoGreen to 9.95mL TE in 10mL tube covered with aluminum foil. Mix thoroughly.

Note: Do not use glass container for PicoGreen.

PicoGreen (Molecular Probes, Inc., Eugene, OR).

*DNA Super Stock Solution*

1. In a 1.5mL microcentrifuge tube, add 10µL of DNA standard to 990µL of TE buffer and mix (final super stock concentration = 1µg DNA/mL).
2. Determine concentration of DNA with spectrophotometer. $A_{260}$ = 0.02 corresponds to 1µg/mL double stranded (ds) DNA solution.
3. To avoid DNA degradation due to freeze-thaw, make numerous aliquots of super stock solution in sterile, nuclease-free tubes, and freeze at -20$^{o}$C.

*DNA Standard Curve*

1. Thaw one aliquot of super stock solution; keep thawed solutions on ice.
2. Prepare and label five sterile, nuclease-free microcentrifuge tubes, each with 400µL of TE buffer.
3. Place 400µL of super stock into first 500ng/mL tube. Mix and remove 400µL of dilution and place into next tube (250ng/mL). Continue with dilutions.

*DNA Serial Dilution Chart*

| Volume (µL) of DNA from serial dilution | Volume (µL) of TE buffer/dilution tube | Final DNA in dilution tube (ng/mL) | Additional tube identifier |
|---|---|---|---|
| 400 of super stock | 400 | 500 | DNA 500 |
| 400 of 500ng/mL | 400 | 250 | DNA 250 |
| 400 of 250ng/mL | 400 | 125 | DNA 125 |
| 400of 125ng/mL | 400 | 62.5 | DNA 62.5 |
| 0 | 400 | 0 | DNA 0 |

*DNA Well and Sample Concentration Chart*

| Tube identifier | Volume (µL) of diluted solut add to well | Volume (µL) of PicoGreen reagent mix | Final DNA in well after adding PicoGreen (ng/mL) | Final DNA in tube (ng/mL) |
|---|---|---|---|---|
| DNA 1000 | 100 | 100 | 500 | 1000 |
| DNA 500 | 100 | 100 | 250 | 500 |
| DNA 250 | 100 | 100 | 125 | 250 |
| DNA 125 | 100 | 100 | 62.5 | 125 |
| DNA 62.5 | 100 | 100 | 31.25 | 62.5 |
| DNA 0 | 100 | 100 | 0 | 0 |

PicoGreen (Molecular Probes, Inc., Eugene, OR)

*DNA Sample Analysis*

Notes: 1. Requires 20mL of TE buffer to run full plate. 2. Turn on fluorometer and allow bulb to warm prior to loading wells. 3. All samples and standards should be plated in triplicate.

1. Add 90µL of cold TE buffer to each sample well.

2. Add 10µL of sample to each well.
3. Add 100µL of each standard to each well without adding PicoGreen (Molecular Probes, Inc. Eugene, OR) working solution according to plate layout.
4. While protecting sample from ambient light exposure, add 100µL of PicoGreen (Molecular Probes, Inc., Eugene, OR) working solution to each well (standards and samples) using sterile trough and multipipette.
5. Incubate 2-5min in fluorometer at room temperature protected from light.

*DNA Fluorometer Measurements*

1. Read fluorescence at ~480nm excitation and ~520nm emission.
2. Multiply DNA concentration by 10 to get actual DNA concentration in each tube.

Sample Analysis: RNA

Formulas for RNA Analysis Reagents and Buffers

*CaCl 1M Stock Solution* Store in refrigerator for up to one week.

In 10mL centrifuge tube:

| | |
|---|---|
| CaCl | 1.47g |
| Nuclease free water | 10ml |

*10X DNase digestion buffer*

In a 15mL Falcon (Becton, Dickenson & Co., Franklin Lakes, NJ) tube:

| | |
|---|---|
| 1M Tris-HCl ph 7.5 | 2ml |
| 1M MgCl | 1mL– empty vial and ish with distilled water |
| CaCl stock solution | 200µL |

Bring to volume with 6.8mL of nuclease-free water. Store at 4°C for up to 1wk.

*RNA Preparation/DNA Removal*

1. Add 150µL of 10X DNase digestion buffer to RNA tubes. {Will need 3.9mL for 26 tubes/samples = one full plate minus standards.}
2. Add 450µL of supernatant to a 1.5-mL microcentrifuge tube.
3. Add 10µL of DNase I (RNnase-free) to each tube and vortex briefly. {Will need 260µL total for 26 tubes = 1 full plate of samples.}
4. Incubate at 37$^{o}$C for 45min in dry bath.
5. Centrifuge at 6,797*g* for 5min. Remove supernatant and put in new tube.

*RNA Reagent Preparation (RiboGreen)*

1. For 96 wells, 100µL each, add 50.25µL of RiboGreen to 9.95mL of TE in plastic vial shielded from light with aluminum foil.
2. For 48 wells (1/2 plate), 100µL each, add 25.12µL of RiboGreen to 4.975mL of TE in 10mL plastic tube shielded from light with aluminum foil.
3. For 25 wells, 100µL each, add 25.1µL of RiboGreen to 4.9mL of TE in plastic vial shielded from light

RiboGreen (Molecular Probes, Inc., Eugene, OR).

*RNA Super Stock Solution*

1. In a 1.5mL microcentrifuge tube, add 10µL of RNA standard to 990µL of TE buffer and mix (final super stock conc. = 1µg RNA/mL).
2. Determine concentration of RNA with a spectrophotometer. $A_{260}$ = 0.025 corresponds to 1µg/mL of RNA solution.

*RNA Standard Curve Preparation*

1. Thaw RNA super stock solution. Once thawed, keep on ice.
2. Prepare and label five sterile, nuclease-free, microcentrifuge tubes with 400µL of cold TE buffer in each.
3. Place 400µL of super stock into first (500ng/mL) tube.
4. Mix and take 400µL of dilution and place into next tube (250ng/mL).
5. Continue with dilutions.

*RNA Serial Dilution Chart*

| Volume (µL) of RNA from serial dilution | Volume (µL) of TE buffer per dilution tube | Final RNA concentration per dilution tube (ng/mL) | Additional tube identifier |
|---|---|---|---|
| 400 of super stock | 400 | 500 | RNA 500 |
| 400 of 500ng/ml | 400 | 250 | RNA 250 |
| 400 of 250ng/ml | 400 | 125 | RNA 125 |
| 400of 125ng/ml | 400 | 62.5 | RNA 62.5 |
| 0 | 400 | 0 | RNA 0 |

*RNA Well and Sample Concentration Chart*

| Tube identifier | Volume (µL) of diluted solution add to well | Volume (µL) of RiboGreen reagent mix per well | RNA concentration per well after RiboGreen (ng/mL) | Final RNA concentration/ Tube (ng/mL) |
|---|---|---|---|---|
| RNA 1000 | 100 | 100 | 500 | 1000 |
| RNA 500 | 100 | 100 | 250 | 500 |
| RNA 250 | 100 | 100 | 125 | 250 |
| RNA 125 | 100 | 100 | 62.5 | 125 |
| RNA 62.5 | 100 | 100 | 31.25 | 62.5 |
| RNA 0 | 100 | 100 | 0 | 0 |

RiboGreen (Molecular Probes, Inc., Eugene, OR)

*RNA Sample Analysis*

1. Add 90µL of cold TE buffer to each well.
2. Add 10µL of sample to each well.

3. Add 100µL of each standard to each well without adding RiboGreen (Molecular Probes, Inc., Eugene, OR) working solution according to plate layout.
4. Add 100µL of RiboGreen (Molecular Probes, Inc., Eugene, OR) working solution to each well (standards and samples) using sterile trough and multipipette.
5. Incubate 2-5min in fluorometer at 20°C protected from light.

   Notes:

   a. Requires 20mL of TE buffer to run full plate.
   b. Turn on fluorometer and allow bulb to warm prior to loading wells.
   c. All samples and standards should be plated in triplicate.

*RNA Fluorometer Measurements*

1. Read fluorescence at ~480nm excitation and ~520nm emission.
2. Multiply RNA concentration measurements by 10.2623 to get actual RNA concentration in each tube.

*RNA:DNA Analysis - Chemical Vendors and Catalog Numbers*

| Chemical name | Vendor and catalog # |
|---|---|
| Calcium chloride dehydrate | Sigma #C3306-250G |
| DNA standard | Sold as part of RiboGreen kit |
| DNase I, amplification grade | Sigma #AMPD1-1KT |
| Magnesium chloride 1M | Sigma #M1028-10X1ML |
| Proteinase K | Invitrogen #25530-015 |
| Quant-it PicoGreen dsDNA assay kit | Invitrogen #P7589 |
| Quant-it RiboGreen RNA reagent only | Invitrogen #R11491 |
| Quant-it RiboGreen RNA Kit | Invitrogen #R11490 |
| RNA Standard | Sold as part of RiboGreen kit |
| RNase, DNase-free water | Fisher #FLBP561 1 |
| Sodium chloride | Sigma # S7653-250G |

| | |
|---|---|
| Sodium dodecyl sulfate | Sigma #4509-25G |
| TE buffer | sold as part of Pico and RiboGreen kits |
| Tris HCl ph 7.5 | Fisher #S4756 |

Fisher Scientific, Pittsburg, PA; Pico and RiboGreen, Molecular Probes, Inc., Eugene, OR Invitrogen Corporation, Carlsbad, CA; Sigma, St. Louis, MO

**Method Fish 9: Steroids**

*Sample preservation*

1. Draw blood from live fish from a venous sinus located in the caudal peduncle with a 1mL syringe and 23 or 25ga needle.
2. Transfer to a blood collection tube containing lithium heparin. Centrifuge at 410000 X *g* for 5min.
3. Decant plasma.
4. Archive in cryovials at -80°C.

*Estradiol and Testosterone Analysis*

Estradiol and Testosterone analysis is conducted with commercially available test kits according to manufacturer's protocols. A basic description is provided below. For full details please consult the following:

Testosterone EIA Kit (582701) – Cayman Chemical Company, Ann Arbor, MI - www.caymanchem.com/pdfs/582701.pdf

Estradiol EIA Kit (582251) – Cayman Chemical Company, Ann Arbor, MI – www.caymanchem.com/pdfs/582251.pdf

1. Transfer plasma to glass tube and extract steroids in 5mL diethyl-ether per manufacturer's instructions. 200ul of plasma may be used instead of the recommended 500ul.
2. Remove steroid-containing supernatant, place in a 30ºC water bath and evaporate under nitrogen. Reconstitute in 0.5mL EIA buffer.

3. Remove 10% of unknown sample and split into two subsamples after extraction.
4. Spike one subsample with a known concentration of either T or E2.
5. Incubate spiked and unspiked samples and steroid standards prepared in EIA buffer at 4ºC for 1hr (E2) or 2hr (T) with antiserum and AChE tracer in 96-well microtiter plates bound with mouse anti-rabbit antibody.
6. Wash plates to remove unbound antigen.
7. Activate the AChE of the hormone tracer complex with Ellman's reagent and read the absorbance values using a spectrophotometer at a wavelength of 410nm.
8. Determine concentrations of T and E2 in samples from standard curve of known steroid concentrations plotted against corresponding absorbance values.
9. Correct each sample for extraction efficiency using the average recovery determined from the cold-spiked samples (average recovered = 90.3%).

**Method Fish 10: Community Assessment**

Protocols established by the Maryland Department of Natural Resources (DNR), Tidal Fish Program are followed for this study. The DNR study routinely sampled several systems in Chesapeake Bay since 1980, however, only the Corsica is being sampled by DNR today. The Magothy and Rhode are sampled by DNR in the 1980s for several years and data is available for comparison. All historic DNR sites are included in the same framework to allow for analysis of change over time.

1. In each system, six seine sites are selected, one in each salinity/nearshore block (Appendix I: Field Sheet – Fish Community Composition).
2. A 100ft beach seine with 1/8in-mesh size is deployed fully at each station.
3. If the seine cannot be fully deployed to 100ft distance from the beach, the distance it can be deployed is documented.
4. All species collected in the seine are identified, counted, and examined for gross abnormalities. Depth is recorded and area seined calculated.
5. Physical water quality parameters are taken at each site.
6. Crabs are commonly captured in the seines, and are classified by size, sex, and presence/absence of shell disease.

7. During intensive sampling periods, six mummichogs are taken from each site for metrics described above. If they are not obtained during the routine sampling effort, minnow traps are deployed and additional seining is conducted within the block until numbers are reached.
8. Deepwater sampling of fish is conducted with a 16ft benthic otter trawl, towed at 2kn for 6min. Two stations are trawled: one station per block is fixed and represents historic Maryland DNR stations, and the other is random.
9. All fish are identified to species, counted and examined for gross abnormalities.
10. During intensive sampling intervals, six white perch greater than 175mm total length are sampled per site for the above listed metrics.
11. If numbers are not obtained within this framework, continued random trawls within the block are conducted to reach required sample size.
12. Thirty-six white perch are collected from each sub-watershed during each sampling period (108 annually).

**Method Fish 11: White Perch Tissue Moisture**

1. Place labeled small aluminum dishes in a 105° C oven and bake for at least 30min.
2. Place dishes in a desiccator and cool to 20°C, or at least 15min.
3. Remove filets from -80°C storage and hold at 20°C to allow tissue to partially melt for up to 30min.
4. Homogenize both filets from individual fish in a laboratory-grade blender.
5. Measure the mass of aluminum dish to 0.0001g.
6. Add a 4-5g aliquot of tissue homogenate to the dish record the mass of the dish and tissue. Analyze each homogenate in duplicate.
7. Dry tissue samples overnight, or at least 12h in a 105°C oven.
8. Remove aluminum dishes containing samples and place in a desiccator for at least 30min. to allow the samples to cool to 20°C.
9. Again weigh and record samples to obtain the final weight.
10. Calculate the mass of moisture in the sample by subtracting the final weight of the dish and tissue from the initial weight of the dish and tissue.
11. Determine percent moisture by dividing the mass of moisture by the mass of the sample

analyzed (mass initial dish and tissue – mass of dish) and multiply by 100.

12. Average the duplicate samples to determine the percent moisture content for the individual fish.

**Method Fish 12: Acetylcholinesterase (AChE) Assay for Mummichogs**

AChE Activity Buffer Formulas

*Tris Buffer 0.05M at 25°C*

4.02g Trizma HCl
2.97g Trizma base
1L nuclease-free water

1. Yields a pH of 8.1 at 25$^{o}$C.
2. Adjust pH of buffer according to temperature.
3. Subtract the actual temperature of the buffer from 25°C and multiply the difference by 0.03. Add this number to 8.1. Example (actual temperature is 22.6$^{o}$C): 25.0$^{o}$C – 22.6$^{o}$C = 2.4$^{o}$C; 2.4 X 0.03 = 0.072; 8.1 + 0.072 = 8.172.

For AChE and protein analysis 23.5mL is needed per plate – this does NOT include homogenization step. Solution is good for 1wk at 4°C. Since solution naturally becomes basic with time, check pH before each use and adjust pH with 10% HCl solution if necessary.

*Phosphate Buffer* (0.1M)

100mL distilled water,
2.68g sodium hydrogen phosphate hepthydrate,
Adjust pH to 7.0 with potassium phosphate monobasic (1.36g/100mL)

*Acetate Buffer* (0.2M)

100mL distilled water
2.72g sodium acetate
Add up to 400mL of acetic acid (6.0mL/500mL) until pH reaches 4.5; Yields ~500mL.

AChE Activity Reagent Formula

*Acetylthiocholine Solution* (0.0237M) MW = 289.18g

1. Under a chemical hood, weigh 0.137g ATCh (stored in -20$^{o}$C freezer)
2. Transfer to 50mL Falcon (BD, Becton, Dickinson and Company) tube. Save weigh boat.
3. Measure 20mL of acetate buffer.
4. Rinse the weigh boat with small volume of acetate buffer.
5. Use the remainder to fill tube to 20mL.
6. Transfer solution to a brown bottle on ice.

Note: For a full plate, 3.20mL is needed. Solution is good for 10-15d at 4°C.

*5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) Dye Reagent*

1. Dissolve 0.068g of sodium bicarbonate in 10mL of phosphate buffer in a 50mL Falcon (Becton, Dickenson & Co., Franklin Lakes, NJ) tube.
2. Under a chemical hood, add: 0.174g DTNB to Falcon tube (Becton, Dickenson & Co., Franklin Lakes, NJ).
3. Using additional 10mL of phosphate buffer, rinse weigh boat and bring volume to 20mL total. Transfer to an amber bottle.

Note: For full plate, 1.1mL is needed (with standards only in first three columns). A glass or aluminum weigh boat is used to avoid DTNB sticking to plastic. Solution is stored at 20°C.

*Eserine Stock Solution* (1 X 10$^{-1}$M)

1. Under a chemical hood, add 0.069g of eserine powder (kept at -20$^{o}$C) to 10-mL Falcon tube (Becton, Dickenson & Co., Franklin Lakes, NJ) wrapped in aluminum foil.
2. Add 2.5mL of 100% ethanol in small increments and rinse the weigh boat.
3. Transfer to small amber bottle.
4. Keep stock solution tightly capped and store at 4$^{o}$C.

*Eserine Working Solution* (1 X 10$^{-3}$M)

1. Under a chemical hood, add 100µL of 1 X $10^{-1}$M of eserine stock solution to 9.9mL of 100% ethanol in a 10mL tube wrapped in aluminum foil.
2. Keep stock solution tightly capped and stored at 4$^{o}$C.
3. A total of 56µL of working solution is needed for a full plate.
4. If eserine (ESE) values seem too high, prepare a new working solution.

*AChE Standard Stock Solution*

1. Reconstitute, in manufacturers bottle, acetylcholinesterase lyophilized powder to convenient working dilution (units/mL) with pH 8.0 buffer; 1.0mL + vial contents = 500U/mL.
2. Dilute to 5U/mL by adding contents to 100mL volumetric flask.
3. Rinse with pH 8.0 buffer, and fill to line.
4. Fill 90 1.5mL microcentrifuge tubes with 1.1mL each
5. Freeze at -20$^{o}$C.

Note: Standard aliquots are good for 6mo but can only be thawed once. Once thawed, standard will last 1d.

*Brain Extraction and Homogenization*

1. Keep mummichog heads frozen until ready to process.
2. Label and pre-weigh mortar microtubes (16).
3. Record tube weights on assay preparation chart.
4. Remove mummichog head from wrap. Grip head with sterile pronged forceps so that top of cranium is easily accessible with scalpel.
5. Using sterile scalpel and beginning between the eyes and moving towards the back of the head, shave the top of the head including bone until top of brain is exposed.
6. Excise brain using sterile scalpel or forceps, and place in appropriate micro-mortar. Any material from scalpel and forceps is removed before returning to alcohol.

7. Weigh brain on balance and record. Volume of Tris buffer to add is determined by dividing weight of brain by 0.02 (dilution = 20mg of brain/1mL of buffer), providing units of buffer in milliliter.
8. Keep brain on ice at all times.
9. Add 100µL of Tris buffer to a labeled microtube.
10. Using sterile pestle rod, homogenize the brain by moving pestle rod up and down 20 X or until completely homogenized.
11. Add remaining Tris buffer to tube (calculated amount of Tris buffer – 100µL = remaining amount to be add to tube). If amount of buffer exceeds 1.5mL (volume of microtube), transfer homogenate to larger, sterile, labeled tube.
12. Use Tris buffer to rinse microtube
13. Add remaining buffer to complete the dilution.
14. Vortex tube lightly and keep tube on ice.
15. Remove 80µL of each homogenate
16. Place in two pre-labeled tubes (40µL in each tube) for protein analysis.
17. Keep on ice until ready to store at -80°C.

*AChE Standard Dilution Preparation*

Standard concentrations: 1 unit/mL hydrolyzed 1µM acetylcholine per minute.

| Standard label | Standard concentration | Tris buffer (µL) | Eel standard (µL) |
|---|---|---|---|
| 1 | 1 unit/ml | 200 | 50 |
| 2 | 2 unit/ml | 150 | 100 |
| 3 | 3 unit/ml | 100 | 150 |
| 4 | 4 unit/ml | 50 | 200 |

1. Add ice cold Tris buffer to each tube.
2. Add eel standard stock solution to each tube and vortex after each addition.

3. Keep on ice.

*Microplate Reader and Software Preparation*

1. Turn on microplate reader prior to loading wells.
2. Within the Gen5™ software program, label wells with sample names.
3. Run protocol with no plate in the reader to allow the reader to come up to temperature.
4. Monitor progress to prompt program to continue through steps.

This protocol uses a BioTek Synergy 2 Microplate Reader and Gen 5 Software™ package (BioTek, Winooski, VT). Instructions may differ for other plate readers and software packages.

*AChE Analysis*

1. Load 287.2µL of ice-cold Tris buffer into each sample and standard well.
2. Load 304µL into blank (287.2 +16.8).
3. Load 283.7µL of Tris buffer into each eserine well.
4. Load 16.8µL of AChE standard onto plate in first three columns on microplate.
5. Load 16.8µL of homogenate into each well with four replicates for each sample. (i.e. each sample has four wells associated with it)
6. Make the room as dark as safely possible.
7. Load 11.5µL of DTNB 5,5'-dithiobis-(2-nitrobenzoic acid) dye reagent into each well.
8. Load three sections of plate (standards, left samples, right samples) with DTNB using three separate sets of tips (one for each section).
9. Load DTNB above wells in one section. Move right to left, mixing the wells by depressing the plunger several times so that tips go into eserine wells last.
10. Continue to work in the dark, load 3.5µL of eserine into each well of first column for each sample (see diagram below). Depress plunger several times to ensure mixing.
11. Allow to incubate in the microplate reader at 28$^{o}$C for 5min.
12. While standing next to reader and still in the dark, load 34.5µL of ice-cold ATCh reagent into each well, including eserine wells as quickly as possible.
13. Place microplate immediately into reader and run AChE computer protocol. The protocol is pre-set to quantify activity after a brief delay (0:20) by measuring absorbance (412nm) at 31s intervals for 5:45min (12 reads) at 28$^{o}$C.

14. Cholinesterase activity is expressed as nanomoles of substrate (ATCh) hydrolyzed per minute per gram of tissue.
15. Rerun samples with a coefficient of variance >10% unless the standard deviation is less than 3mOD per minute.
16. It is usually possible to thaw samples at least one more time and get similar results.
17. Refer to microplate layout below for buffer and reagent placement.

Microplate layout

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blk/ std 0 | Blk/ std 0 | Blk/ std 0 | ESE | Smpl 1 | Smpl 1 | Smpl 1 | ESE | Smpl 9 | Smpl 9 | Smp l 9 | Empty |
| B | STD 0.25 | STD 0.25 | STD 0.25 | ESE | Smpl 2 | Smpl 2 | Smpl 2 | ESE | Smpl 10 | Smpl 10 | Smp l 10 | Empty |
| C | STD 0.5 | STD 0.5 | STD 0.5 | ESE | Smpl 3 | Smpl 3 | Smpl 3 | ESE | Smpl 11 | Smpl 11 | Smp l 11 | Empty |
| D | STD 1 | STD 1 | STD 1 | ESE | Smpl 4 | Smpl 4 | Smpl 4 | ESE | Smpl 12 | Smpl 12 | Smp l 12 | Empty |
| E | STD 1.5 | STD 1.5 | STD 1.5 | ESE | Smpl 5 | Smpl 5 | Smpl 5 | ESE | Smpl 13 | Smpl 13 | Smp l 13 | Empty |
| F | STD 2 | STD 2 | STD 2 | ESE | Smpl 6 | Smpl 6 | Smpl 6 | ESE | Smpl 14 | Smpl 14 | Smp l 14 | Empty |
| G | Empty | Empty | Empty | ESE | Smpl 7 | Smpl 7 | Smpl 7 | ESE | Smpl 15 | Smpl 15 | Smp l 15 | Empty |
| H | Empty | Empty | Empty | ESE | Smpl 8 | Smpl 8 | Smpl 8 | ESE | Smpl 16 | Smpl 16 | Smp l 16 | Empty |

Note: 96µL of eserine working solution is used per full plate. Total concentration of eserine is 1.1 X $10^{-6}$g eserine/mL before rinsing and 2.25 X $10^{-8}$g eserine/mL after rinsing with 48mL of water (each well filled with water twice).

*Reagent Volume Synopsis*

| | Tris Buffer (µL) | AChE Standard (µL) | Homogenate (µL) | DTNB (µL) | ATCh (µL) |
|---|---|---|---|---|---|
| Blank | 304 | 0 | 0 | 11.5 | 34.5 |
| Eserine | 283.7 | 0 | 16.8 | 11.5 | 34.5 |
| Standard | 287.2 | 16.8 | 0 | 11.5 | 34.5 |
| Sample | 287.2 | 0 | 16.8 | 11.5 | 34.5 |

*Data Analysis*

Results are converted to international units of enzyme activity using the following equation.

1. {[(mean V) – (mean V of eserine blank)]/1000} X 76.59 = (nanomoles ATCh hydrolyzed/min)/mg tissue (Ellman et al. 1961).
2. Constant (76.59) must be calculated according to optimized conditions applied for each species and chemistry used.

Protein Analysis

This protocol makes use of the Lowry Assay based on the Bradford method of quantifying proteins used at the Center for Coastal Environmental Health and Biomolecular Research Laboratory in Charleston, SC and modified for 96-well microplates.

*Protein Standard Preparation*

1. Add nuclease-free water to tubes.
2. Add protein standard to each tube according to specifications in chart below.

| Standard (µg/mL) | Final protein in tube (µg/mL) | DI Water (µL) | Volume protein standard (µL) from serial dilution |
|---|---|---|---|
| 200 | 200 | 400 | 400µL of stock |

| | | | |
|---|---|---|---|
| 100 | 100 | 400 | 400µL of 200µg/mL |
| 50 | 50 | 400 | 400µL of 100µg/mL |

*Microplate Reader and Softwis Preparation*

1. Turn on microplate reader prior to loading wells to increase temperature.
2. Using the Gen5™ software program, label wells with sample names.
3. The set point for the assay is ≥ 21$^{o}$C; may need a "blank" run if the ambient temperature is below this.

*Protein Analysis*

Formula for Protein Component

*Lowry Reagent Solution*

1. Under a chemical hood,
2. Add 40mL nuclease-free water to the Lowery reagent bottle.
3. Mix with magnetic stirrer for ~30min.
4. Store at 20°C, do not refrigerate.
5. Need 9.6mL for full plate.

*Folin & Ciocalteu phenol reagent*

1. Use a hood.
2. Transfer 9mL of Folin & Ciocalteu phenol reagent from large amber bottle
3. Add 45mL of nuclease-free water.
4. Store at 20°C, do not refrigerate.
5. Need 4.8mL for full plate.

*Protein Standard Stock Solution (400µg/mL)*

Add 0.008g of ovalbumin to 20mL nuclease-free water. Aliquot 500µL into each sterile tube and store at -20$^{o}$C. This product has not been tested for long term frozen storage.

*Protein Analysis*

1. Thaw samples. Once thawed, keep on ice until ready to proceed.
2. Make protein standard dilutions according to chart above.
3. Add 90µL of nuclease-free water to blank and sample wells in triplicate.
4. Add 10µL of Tris buffer to each blank.
5. Add 10µL of each sample to each well.
6. Pipette 100µL of each standard to wells in triplicate.
7. Add 100µL of Lowry reagent to each well using multipipette. Add reagent to each set of samples by holding multipipette above wells. Then using same tips, slowly depress the plunger repeatedly to mix the sample and reagent in the wells. Avoid making bubbles in the well by not fully depressing the plunger. Replace with clean tips and add reagent to next set of sample wells.
8. Allow plate to stand for 20min at room temperature.
9. While working in the dark, add 50µL Folin & Ciocalteu phenol reagent (FCR) to each well and cisfully mix thoroughly to avoid making bubbles after dispensing FCR into all three wells.
10. The Gen 5 protocol is set to incubate the plate for 30min at ≥21$^{o}$C and then read at 700nm.

Materials Catalog Numbers for AChE and Protein Components

| Chemical | Vendor and No. |
|---|---|
| 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB dye) | Sigma-Aldrich D8130-5G |
| Acetic acid | Sigma-Aldrich 338826 |
| Acetylthiocholine iodide | Sigma-Aldrich A5751-5G |
| Disodium hydrogen phosphate heptahydrate 250g *aka sodium hydrogen phosphate heptathydrate* | Sigma-Aldrich 431478 |

| | |
|---|---|
| acetylcholinesterase lyophilized powder, 500 units from *Electrophorus electricus* (electric eel) | Sigma-Aldrich C3389 |
| Eserine | Sigma-Aldrich E8375-1G |
| Folin & Ciocalteu's Phenol Reagent | Sigma-Aldrich F-9252 |
| Lowry's Reagent (case of 25 vials) | Sigma-Aldrich L-3540 |
| Ovalbumin protein standard | Sigma-Aldrich A5503 |
| Potassium phosphate monobasic *aka monopotassium phosphate* | Sigma-Aldrich P5655 |
| Sodium acetate | Sigma-Aldrich S2889 |
| Sodium bicarbonate | Sigma-Aldrich S6014 |
| Trizma base 100g | Sigma-Aldrich T1503 |
| Trizma HCl, 100g | Sigma-Aldrich T3253 |
| 96-well, clear microplates | Fisher 12-565-501 |

As this protocol is modified from cuvettes to a microplate reader, chemicals are used in small quantities. Eserine is a cholinesterase inhibitor that targets the central nervous system, but it is necessary to use this chemical in this assay. Other chemicals that may be used are also cholinesterate inhibitors and are just as toxic; appropriate safety precautions for handling and disposal are followed.

# Crab Indicators

**Introduction**

Blue crab *Callinectes sapidus* in Chesapeake Bay have a broad spatial and temporal distribution. This ubiquity makes them available in most river systems sample for variation in health parameters among varying land use. Blue crabs not only comprise the most valuable fishery in the Chesapeake Bay, but are major predators of benthic communities and are prey for many other fish species. The estimated 2010 total harvest of blue crabs from the Bay and tributaries was 91.6 million pounds. This was the highest harvest since 1994, and was 22% above the long-term (1990-2010) average of 75 million pounds (http://chesapeakebay.noaa.gov/fish-facts/blue-crab).

The blue crab is an essential component of the Chesapeake Bay ecosystem due to the economic and iconic value; it is considered a keystone species that influence many characteristics of the Bay's ecosystem. Various parameters that can be measured in blue crabs, including pathology, parasites, physiology, and shell condition can be indicators of environmental change or anthropogenic activities.

**Sampling Design**

During the first year of the developmental stage of this program, effort was put into collecting blue crabs from each of the three river mile segments during spring, summer and fall. Spring sampling however was too early or the water was too cold to catch sufficient numbers of blue crabs in all three river systems; and crabs are only abundant in the mouth segment of any river during any sampling period. During the second year, 30 blue crabs were collected from the mouth segments of each of three rivers during summer and fall with either a trawl or dredge. During the summer and autumn of the third year, 30 crabs per river were collected using a trotline, which was selected to reduce stress related to capture (Method Crab 1). Ultimately, this method has been selected as the ideal method to collect crabs for this project.

**Metrics**

*Gross Pathology/Field and Observational Data*

Blue crab community structure in estuaries changes seasonally in response to natural temperature and salinity gradients (Millikin and Williams 1984). Since our purpose was to assay how land use influences community structure, we conducted this survey to see if anthropogenic gradients result in community shifts (**Method Crab 1).**

*Shell Disease*

Crustacean shell disease (rust spot, black spot, brown spot) is a common syndrome among marine decapods. Chitinoclastic bacteria including *Vibrio* and *Pseudomonas* are often isolated from shell lesions (Johnson 1983). A study of the blue crab found a decrease in the antibacterial activity of crab hemolymph in crabs possessing visible signs of shell disease compared to blue crabs with healthy exoskeletons (Noga et al. 1994). Shell disease occurs naturally in all populations but is generally higher in stressed populations (Sindermann 1989). This effort will establish whether anthropogenic activities affecting the environment result in significant variation in prevalence of shell disease among distinct watersheds characterized with different land-use strategies. **Method Crab 1**

*Histopathology*

Research on the blue crab has shown that stress can increase prevalence and intensity of disease. Various pathogens such as including reolike (RLV) virus, rhabdolike (RhVA) virus, Chesapeake Bay virus (CBV), and bifacies virus (BFV) have been found in stressed crabs. Aside from viruses, other pathogens have included microsporidians, paramoeba, *Hematodinium* sp., fungus, rickettsia-like infections, and ciliates. Additionally, histological abnormalities observed have included scab-like gill lesions and an unusual strand-like structure in the lumen of the hepatopancreas. Some or all of these parasites or manifestations of disease may be latent in the crab until stress causes infections to become patent. This effort will assay whether land use affects significant variation in prevalence of parasites or disease in blue crabs. Methods for histopathology are included as **Method Crab 2.**

*Gill Epibionts*

Gill blackening, erosion and melanization have been observed in crabs collected from polluted waters (Sawyer 1982). Gill blackening caused by fungal infections (Lightner and Fontaine 1975), heavy metal exposure (Couch 1978) or exposure to certain pesticides (Doughtie and Rao 1983) have also been reported. This survey assayed whether land use results in significant variation of prevalence of gill epibionts in blue crabs. Methods for documenting gill epibionts and ciliates are provided in **Method Crab 2.**

*Stress Protein, Carbohydrate Levels: Glucose and Lactate*

Crustaceans respond to abnormal changes in the environment through physiological mechanisms that can be quantified by assaying levels of stress proteins or heat shock proteins (HSPs) and comparing levels of carbohydrates in tissues including hemolymph. This effort assays whether land use results in significant variation of measurable stress parameters in crab hemolymph, including proteins and carbohydrates. **Method Crab 3** assays glucose levels in crab hemolymph; **Method Crab 4** assays lactate levels in crab hemolymph; and **Method Crab 5** assays protein levels in crab hemolymph.

## Method Crab 1: Field Collection, Gross Pathology and Observation

*Crab Collection and Handling*

1. Near the mouth of the sub-estuary deploy a trotline baited with poultry necks.
2. Set the trotline parallel to the shore where the bottom drops off at 4 - 20ft depth.
3. If insufficient crabs are captured with a trotline, drag a dredge over nearby oyster shell bottom where crabs are likely to be found in cooler autumn weather.
4. Place crabs immediately in a cooler with blue ice and damp newspapers.
5. Once five crabs are collected, withdraw hemolymph from each crab.
6. Label bled crabs with permanent ink and place on blue ice.
7. Once 30 crabs are collected, dissect as soon as possible.

A. *Hemolymph* – Hemolymph is withdrawn from crabs within 5min of capture, the carapace is cleansed with 70% ethanol and a 20ga needle with a 3cc syringe is inserted into the articular membrane of a walking leg or directly into the heart via the juncture of the carapace to the ventral body. Up to 3mL of hemolymph is removed and decanted into microcentrifuge tubes that may contain fixatives or anticoagulants for further assays.
   1. Molecular assay for virus: 0.66mL of 95% ethanol plus 0.33mL of hemolymph, stored at -80°C for future molecular assays
   2. 1mL of 70% ethanol plus 0.25mL of hemolymph stored at 4°C for future molecular assays for protozoans
   3. 0.25mL of hemolymph placed in 0.25mL of anticoagulant and stored at -80°C for future glucose ( **Method Crab 3**), lactate (**Method Crab 4**) and protein assays (**Method Crab 5**).

*Anticoagulent Solution*: (Söderhåll and Smith 1983), modified

| | |
|---|---|
| 0.35M NaCl | 0.35(58.54) = 20.49g/L |
| 30mM trisodium citrate | 30(294.10)/1000 = 8.823g/L |
| 26mM citric acid | 26(192.930)/1000 = 4.99g/L |
| Use 1 or 2mM (final) of EDTA. | 1(372.24)/1000 = 0.372g/L |

pH 7.0; filter; can use at lower pH of 4.5; higher pH produces precipitate. Store at 4°C.

B. *External Observation* - Carapace width from point to point to the nearest millimeter is recorded; crabs are examined for external lesions (body discoloration, ulceration, deformity, parasites, etc.) and recorded on data sheet (Appendix I: Field Sheet - Crab).

1. Shell lesions are ranked on a scale of 0-5, with small darkened areas ranked as 1 to deeply pitted extensive areas ranked as 5 (Appendix II: Crab - Shell Disease Ranking).
2. The underside of each point of the carapace is scraped with a sterilized razor blade, placed in a labeled microcentrifuge tube, and archived at -80°C for possible future assays of bacterial community composition.
3. A walking leg is removed and placed in a 5cc test tube and frozen immediately on dry ice and archived at -80°C for molecular analysis for virus

*C. General Necropsy* - Each crab is sampled for routine histopathology and condition indices. Except for gills, tissue pieces of $5mm^3$ or less are preserved in 1% Glutaraldehyde/4% Formaldehyde (1G4F) to allow for future analysis with electron microscopy if desired.

1. Remove the carapace over the visceral cavity using heavy duty pointed scissors and a probe to separate the epidermis and connective tissue.
2. Remove and preserve a 5mm portion of pigmented epidermis overlying the heart.
3. Reveal visceral cavity, and locate heart, cardiac stomach, hepatopancreas, gonads and gills.
4. Remove and preserve a gill; cut in half if large.
5. Remove and preserve a portion of heart; beating if crab alive.
6. Remove and preserve hepatopancreas.
7. Remove the GI tract beginning at the esophagus, which is ventral and anterior to the cardiac stomach and continue with the entire stomach and intestine.
8. Remove and preserve portions of the pyloric stomach, midgut and hindgut.
9. Remove and preserve a section of the gonads, located posterior to the heart on both sides.
10. Gonads are intermixed with, and obstruct extraction of intestine.

11. Expose the thoracic ganglion, which lies beneath the heart on the floor of the body cavity, by removing the residual hepatopancreas.
12. Remove and preserve a portion of the thoracic ganglion. The correct organ has been obtained if severance of the radiating peripheral nerves causes twitching of the respective walking leg of the crab.
13. Place preserved tissues in tissue-processing cassettes, separating larger tissues from smaller tissues. Label each cassette with unique number to keep track of animals.
14. Place tissues on a 2hr cycle in an automated tissue processor.
15. Embed in paraplast.
16. Cut paraplast blocks at 5µ, one slide per block, and stain with (MHE), Mayer's hematoxylin and eosin-phloxine stain.

Note: See Johnson (1983) for illustrations.

Formulas:

1% Glutaraldehyde/4% Formaldehyde (1G4F) (McDowell and Trump 1976)

*37-40% buffered formaldehyde stock*

Formaldehyde ……. 5.0gal **(potential carcinogen)**

Disodium phosphate ……284.0g

Phenol red ……...0.5g

Sodium hydroxide ……..... 1.2g

*1G4F working solution*

37-40% buffered formaldehyde stock ……. 120.0mL **(potential carcinogen)**

50% glutaraldehyde …….. 20.0mL

Tap water ……. 360.0mL

Filtered ambient or artificial seawater ……..500.0mL

Optimum fixation: 24-48hr (cytological preps, 30min) at 20°C; pH 7.2-7.4. Immediately prior to embedment, wash approximately 4hr in ambient water.

Note: If not washed well, fixative interferes with some diagnostic stains.

**Method Crab 2: Histopathology**

Slides are observed under light microscopy and any lesions or parasites are documented. Ciliates are documented by observing 4µm thick sections of gills stained with MHE at 200X magnification and counting the number of ciliates on the entire section of gill lamellae. See Johnson (1980) for normal histology.

**Method Crab 3: Glucose Concentration in Hemolymph**

*Sample Deproteinization*

1. Thaw 50% hemolymph, 50% anticoagulant samples.
2. While samples are thawing, label 24 1.5mL tubes for sample deproteinization.
3. Label 48 0.5mL tubes for protein analysis (two tubes per sample).
4. Once thawed, vortex each sample lightly to ensure thorough mixing. Store samples on ice.
5. On bench top, put 40µL of ultrapure water into each deproteinization tube.
6. Add 50µL of sample to each tube. Store tubes on ice until assay complete.
7. Under fume hood, add 10µL of 50% nitric acid to each tube.
8. Vortex each sample for 30s.
9. Spin samples at 20817.16*g* for 5min.
10. Remove 50µL of supernatant and place into 1.5-mLtube with 450µL of 0.2M sodium phosphate buffer.
11. Before returning samples to -80°C freezer, remove two aliquots (40µL each) of hemolymph and transfer to two pre-labeled tubes for protein analysis.
12. Once deproteinized and placed in sodium phosphate buffer, samples can be stored at -20$^{o}$C.

*Glucose Standard Preparation*

1. Label seven 1.5mL tubes with appropriate standard concentrations.

2. Transfer 955µL of water into tube marked 45µg/mL. Put 500µL of water into all subsequent tubes including blank.
3. Add 45µL of glucose standard into tube containing 955µL of water. Vortex briefly.
4. Perform serial dilution by transferring 500µL of this mixture into second tube (22µg/mL) containing 500µL of water. Continue similar dilutions until final concentration (1.4µg/mL) was reached. Blank should contain 500µL of water.
5. Standards could be kept at room temperature for the day with enough standard to accommodate two plates.

Glucose Standard Serial Dilution Chart

| Water | Glucose standard | Concentration |
|---|---|---|
| µL | µL | µg/mL |
| 955 | 45 | 45 |
| 500 | 500 of 45 | 22.5 |
| 500 | 500 of 22.5 | 11.25 |
| 500 | 500 of 11.25 | 5.625 |
| 500 | 500 of 5.625 | 2.8125 |
| 500 | 500 of 2.8125 | 1.4 |
| 500 | 0 | 0 |

*Sample Analysis*

1. Transfer 200µL of buffered sample into labeled 1.5mL microcentrifuge tube.
2. While working in the dark, add 400µL of chromagen reagent at 30s intervals.
3. After adding chromagen reagent, vortex briefly and incubate for 30min at 37$^{o}$C.
4. After 30min and still working in the dark, add 400µL of stop solution. Vortex briefly and place tube in rack at 20°C.
5. Load samples in microplate in triplicate. Read absorbance at 540nm. Protocol was pre-set to subtract blank from readings and to produce a standard curve.

6. Multiply results by 40 to determine actual glucose concentration.
7. Rinse plate twice with distilled water, and dispose waste into appropriate waste container.

The original protocol called for perchloric acid which may spontaneously combust and requires a special hood; 50% nitric acid was chosen as an effective and less hazardous substitute.

*Sigma-o-Dianisidine Reagent*

Reconstitute contents of vial with 1.0mL ultrapure water.
Avoid exposing contents to light, stable for 3mo at 2-8°C.

*Glucose Oxidase/Peroxidase Solution*

Following directions in kit, dissolve contents of capsule in 39.2mL of ultrapure water to bottle of oxidase/peroxidase solution. Need 12.4mL for 24 samples and seven standards.
Good for 1mo stored at 4°C and at least 6mo stored at -20°C. Discard if turbidity develops.

*Assay Solution*

Add 0.8mL of o-Dianisidine reagent to amber bottle containing glucose oxidase/peroxidase solution. Invert bottle several times to mix. Stable up to 1mo stored at 2-8°C. Discard if turbidity develops.

*0.2M Sodium Phosphate Buffer, pH=7.4* (Dawson et al. 1986). Store at 20°C.

5.678g $Na_2HPO_4$ in 200mL ultrapure water
40.5mL of 0.2M dibasic Solution

4.799g NaH2PO4 in 200mL ultrapure water
9.5mL of 0.2M monobasic Solution

*Stop Solution:*

50mL of 12N sulfuric acid

150mL of ultrapure water.

Store at 20°C.

**Method Crab 4: Lactate Concentration in Hemolymph**

*Materials:*

| | |
|---|---|
| Trinity Biotech lactate kit #735 | Pipetter |
| Trinity Biotech lactate standard solution #826-10 | BioTek® Synergy™ 2 Microplate Reader, Gen5.107 software (BioTek) |
| 10mL pipette | Nitrile gloves |
| Laboratory coat | Safety glasses |
| 96-well clear microplates | |

BioTek® Synergy™ 2 microplate reader and Gen 5™, BioTek, Winooski, VT

*Lactate Standard Preparation*

1. Working in the dark, add 10.0mL of deionized water to amber reagent vial.
2. Invert several times to ensure full reconstitution.
3. Place reagent on ice or place in fridge.
4. Keep all standards on ice.
5. Pipette and label accordingly into a 2mL tube:

| Standard label | Standard concentrate | Lactate standard (µL) | Deionized water (µL)) |
|---|---|---|---|
| 1 | 40 | 200µL of standard concentrate 80 (provided) | 200 |
| 2 | 20 | Provided | |
| 3 | 10 | 200µL of standard concentrate 20 | 200 |
| 4 | 5 | 200µL of standard concentrate 10 | 200 |

| | | | |
|---|---|---|---|
| 5 | 0 | 0 | 200 |

*Microplate Reader*

1. Open new experiment in Gen5™ program (BioTek, Winooski, VT), choose appropriate protocol.
2. Label wells with sample names.

*Sample Analysis*

1. Keep samples and standards on ice.
2. While working in the dark, load a clear, 96-well microplate with 5µL of each standard in triplicate. Keep lights off until plate is loaded into microplate reader.
3. Load 5µL of sample into each sample well in triplicate.
4. Using multipipette, add 100µL of lactate reagent into each sample and standard well.
5. Set protocol to read at 540nm after incubation at 20°C in BioTek® Synergy™ 2 microplate reader (BioTek, Winooski, VT) for 6.5min followed by 30s shake period. Read again at 540nm.

*Results:* To determine final sample lactate concentration: lactate mg/dL = (absorbance test/absorbance std.) X 20

**Method Crab 5: Protein Concentration in Hemolymph**

This protocol makes use of the Lowry Assay based on the Bradford method of quantifying proteins and uses a BioTek® Synergy™ 2 microplate reader (BioTek Winooski, VT.).

*Protein Standard Preparation*

| Standard (µg/mL) | Final protein in tube (µg/mL) | Tris buffer (µL) | Volume of protein standard (µL) from serial dilution |
|---|---|---|---|
| 400 | 400 | 0 | 800 of stock |

| | | | |
|---|---|---|---|
| 200 | 200 | 400 | 400 of 400 µg /ml |
| 100 | 100 | 400 | 400 of 200 µg /ml |
| 50 | 50 | 400 | 400 of 100 µg /ml |

*Microplate Reader*

1. Turn on BioTek® Synergy™ 2 (BioTek, Winooski, VT) microplate reader.
2. Turn on computer and choose Gen5™ program (BioTek, Winooski, VT).
3. Open new experiment and choose protein analysis protocol.
4. Label wells with sample dilutions.
5. Set assay set point to ≥ 21°C; may need a "blank" run if the room temperature is below 21°C.

*Protein Analysis*

1. Thaw samples, once thawed, keep on ice until ready to proceed.
2. Make protein standard.
3. Add 99.25µL of nuclease-free water to blank and sample wells.
4. Add 0.75µL of each sample to each well.
5. Pipette 100µL of each standard to wells in triplicate.
6. Add 100µL of Lowry reagent to each well using multipipette. Add reagent to each set of samples by holding multipipette above wells. Then using same tips, slowly depress the plunger repeatedly to mix the sample and reagent in the wells. Do not depress plunger all the way to avoid making bubbles. Replace with clean tips and add reagent to next set of sample wells.
7. Allow plate to stand for 20min at 20°C.
8. Make room as dark as possible. Add 50µL Folin - Ciocalteu's Phenol Reagent to each well and carefully mix thoroughly with pipette to avoid making bubbles after dispensing into all three wells.
9. Gen5 (BioTek, Winooski, VT) protocol was set to incubate the plate for 30min at ≥21°C and then read at 700nm.

*Protein Analysis Buffer and Reagent Formulas*

*Lowry Reagent Solution*

While working under a hood, add 40mL of nuclease-free water to Lowry reagent bottle and mix with magnetic stirrer for ~30min. Store at 20°C. Do not refrigerate.
Need 9.6mL for full plate.

*Folin - Ciocalteu's Phenol Reagent*

Transfer 9mL of Folin & Ciocalteu's Phenol reagent from large amber bottle and 45mL of nuclease-free water. Store at 20°C. Do not refrigerate. Good for ~1mo or until see variability in results. Need 4.8mL for full plate.

*Protein Standard (400µg/mL)*

Reconstitute Bovine Serum Albumin (BSA) protein standard according to manufacturer instructions using ultrapure water. Final concentration is 400µg/mL. Store at 4°C in original bottle wrapped in foil. Good for ~1mo, discard if turbidity develops.

*Tris Buffer*: 0.05M at 25°C

4.02g Trizma HCl
2.97g Trizma base
1L Nuclease-free water

This yields a pH of 8.1 at 25°C. Check pH before using each time since tends to become basic. Adjust pH with 10% HCl solution, if necessary, to 7.9. Stable 1wk at 4 °C.

*Materials Catalog Numbers*

| Chemical | Vendor and No. |
|---|---|
| Folin - Ciocalteu's phenol reagent | Sigma-Aldrich F-9252 |

| | |
|---|---|
| Lowry's reagent (case of 25 vials) | Sigma-Aldrich L-3540 |
| Bovine serum albumin protein standard | Sigma-Aldrich P5619-25VL |
| 96-well, clear microplates | Fisher 12-565-501 |

# Clam Indicators

**Introduction**

The integrity of marine and estuarine ecosystems depends, to a great extent, on the health of its flora and fauna. As filter-feeders, clams, mussels, oysters and other bivalve mollusks safeguard water quality in estuaries and act as shields against environmental fluctuations. These benthic macroinvertebrates are reliable and sensitive indicators of habitat quality in Chesapeake Bay and ecologically important components of the Bay's food web.

Living on, in or near the benthic zone, bivalve mollusks are commonly used as sentinel organisms in monitoring programs to identify status and trends of chemical exposure and pollution gradients in a variety of environments. Bivalve mollusks serve as good sentinels since they concentrate both metals and organic contaminants yet have limited ability to metabolize accumulated contaminants. Further, bivalve mollusks are abundant and widely distributed

among habitats including those relatively heavily polluted, relatively immobile, persistent and accessible (Kim et al. 2008). Physiological responses to changes in the environment can also be measured, thereby facilitating the establishment of relationships between the concentration of chemicals in water, sediment and tissues, and associated biological effects (Salazar and Salazar 1995; Dame 1996).

The Chesapeake Bay supports a variety of bivalve mollusks and their distribution depends on salinity, temperature, substrate, and other factors. *Macoma balthica* and *Rangea cuneata* are selected in this study primarily based upon their presence in selected river systems and their feeding mechanisms. *Macoma balthica* inhabits mesohaline waters in Chesapeake Bay and are some of the most abundant clams found. Baltic clams live buried below the surface, maintaining contact with overlying water by means of the inhalant and exhalant siphons (Brafield and Newell 1961). This species is capable of deposit as well as suspension feeding (Brafield and Newell 1961, Wolff 1973) and is able to withstand low winter temperatures.

The brackish water clam *Rangea cuneata* inhabits oligohaline and lower mesohaline waters in Chesapeake Bay. These clams were introduced to Chesapeake Bay in the early 1960s and are now commonly found in the sandy and muddy substrates of subtidal and intertidal areas. The brackish water clam thrives in tidal freshwater and low salinity river systems but also survives in areas of higher salinities. Winter kills in the shallow waters of Chesapeake Bay suggest that this species cannot survive in temperatures at or below freezing.

**Clam Sampling Design**

Sample collection is conducted in shallow and deepwater sites in accordance with the stratified sampling framework. Clams are collected by hand digging or deployment of a Smith-McIntyre sediment grab or an oyster dredge lined with netting. Thirty clams are collected from the head, middle, and mouth of each river system, for a total of 90 clams collected from each sub-watershed during each spring, summer and fall sampling period. See **Method Clam 1** for more details.

**Metrics**

*Field Collection and Processing.* Clams are collected by shovel, grab, or dredge and then counted and rinsed with ambient seawater to remove sediments. Individuals are processed immediately for specific assays or stored in a container on ice for transport to the laboratory for immediate processing or storage in freezers. An indirect measure of population density or abundance is determined at each site by calculating catch per unit effort (CPUE), which is number of tows or time passed to collect clam samples (**Method Clam 1)**.

*Histology/Histocytology*. Histopathology surveys of parasites and pathologies are important components for biomonitoring studies of environmental pollution. A number of studies including NOAA Mussel Watch have examined relationships among parasites, pathologies, and selected morphological conditions (Kim and Powell 2007). Fixed tissues of clams could be examined by light microscopy to assess their general health status, specific parasites (e.g., *Perkinsus* spp.), diseases (e.g., sarcomas), and other conditions (e.g., increase in mucous cells). Methods for assaying disease prevalence are provided in **Method Clam 2**

*Metalliothionein Proteins*. The metallothioneins (MT) have been extensively used as a tool for biomonitoring metal contamination. These proteins have a role in protecting cells from deleterious effects of high concentration of metal ions and become upregulated upon heavy metal exposure (Bernal-Hernandez et al. 2010). Methods for assaying MT proteins in clams are provided in **Method Clam 3.**

*Acetylcholinesterase*. Measurement of acetylcholinesterase (AChE) activity is widely used for evaluating the effects of exposure to neurotoxic compounds in vertebrates and invertebrates, including aquatic species (Bernal-Hernandez et al. 2010). Certain chemical classes of pesticides such as organophosphates and carbamates act by interfering with, or inhibiting, AChE. Possible exposure to neurotoxic compounds may be evaluated in clams by measuring cholinesterase activity in tissues. Methods for assaying for AChE in clams are provided in **Method Clam 4.**

*RNA:DNA Ratio*. Condition indices are used as indicators of general health. The ratio of ribonucleic acid (RNA) concentration to deoxyribonucleic acid (DNA) concentration is a measure of soft tissue growth in bivalve mollusks. This measure responds quickly to changes in

the environment and is also used as an indicator for growth potential (Mayrand et al. 1995). Methods for performing RNA:DNA ratio are provided in **Method Clam 5.**

*Bacteria*. *Vibrio* spp. bacteria are found in the normal intestinal flora of healthy organisms and are ubiquitous in marine and estuarine environments. *Vibrio* spp. may also cause mortalities of aquatic animals especially under conditions of stress. Clam hemolymph streaked on selective media plates permits the isolation and identification of *Vibrio* spp. Methods for assaying *Vibrio* spp. in clams are provided in **Method Clam 6.**

*Contaminants.* Direct measures of contaminants can be used as diagnostic indicators when related to biological indicators, e.g., measures of the sediment content of contaminants such as heavy metals, chlorinated organic compounds, and polycyclic aromatic hydrocarbons. Bivalve mollusks have the ability to accumulate anthropogenically derived chemicals, and their tissues can be used as an indirect measure of a particular chemical. Methods for preparing clams for measurement of chemical contaminants in clam tissues are provided in **Method Clam 1**. Methods for measuring chemical contaminants in sediments and sediment toxicity are provided in **Method Benthic Contaminants 1, 2** and **4**.

**Method Clam 1: Field Collection, Gross Pathology and Observation**

Sediment Grab

1. Clams are collected from sediments by lowering a modified Smith McIntyre sediment grab vertically into the water from a stationary boat.
2. Suspend grab from hydraulic winch on an A-frame of vessel if available.
3. In shoal waters, suspend grab from a side-mounted steel frame on a smaller boat.
4. Lower grab at a steady speed to the seafloor.
5. Contact with the seafloor triggers the buckets to close.
6. Raise the grab slowly and position on top of a stand fabricated to hold the grab.
7. Ensure stand is equipped with a bucket and drain and supports a wooden-framed sieve with a 0.5mm-wire mesh bottom.

8. Release sediments from the grab onto the sieve and wash with ambient seawater.
9. Retrieve clams from top of sieve, rinse sediment with ambient water, place in labeled plastic containers, and hold on ice in a cooler.
10. Wear plastic gloves to protect hands.
11. Record observations on abundances and diversity of species and sizes, and quantities as well as frequency of boxes (empty shells).
12. Collect sediments for chemical contaminant and sediment toxicity analysis from the first sediment sample collected from each site. (See **Method Benthic Habitat 1**.)
13. Record water quality measurements at the surface and bottom on a datasonde.
14. Record latitude and longitude, date, time at start and end of sampling, depth, gear, number of grabs, number of clams, tide, weather conditions, and ambient parameters on a field sheet. (Appendix I: Field Sheet - Clams)

Modified Oyster Dredge: When clams are small (<0.5mm), it is necessary to collect samples using an oyster dredge lined with fine mesh netting. All steps are the same except:

1. The dredge is lowered slowly, using an A-frame and hydraulic winch, into the water until it touches the bottom, then tow it briefly behind the boat to collect sediments and clams.
2. The dredge is retrieved and emptied onto a sorting table positioned near the edge of the boat. The table is outfitted with a removable wall with holes sized to allow water to drain but retain animals.

Hand Digging with Shovel: Clams are collected by hand in areas where boat access is limited or clams are located near shore. All steps are the same as above except:

1. After carefully wading into water wearing knee or chest waders, a rounded or square-edged shovel is lowered to the bottom and sediment scooped.
2. Contents of shovel are unloaded into a wooden framed sieve outfitted with a 0.5mm-wire mesh base being held by a teammate.
3. Teammate slowly swings the sieve side to side just under the surface of the water to gently wash away sediments.

Gross Pathology: Clams collected in the field are chilled and transported to a lab and prepared for evaluation by selected methods such as condition, histopathology, physiological and biochemical assays, and molecular biology. Samples may be stored overnight at 4°C for routine histology; however, some assays (as noted below) require immediate processing.

1. Rinse clams in artificial seawater diluted to the salinity of the collection site to remove excess sand and mud.
2. Record length across the shell from anterior to posterior.
3. Record shell lesions or discolorations.
4. Open shells of clam by inserting a sharp knife into the valve near the umbo.
5. Carefully slice posterior and anterior adductor muscles to remove the shell.
6. Rate the gross physiological condition of the body as fat, medium, or watery. Clams in good condition have plump, creamy bodies, brown digestive glands, and beige gills.
7. Record obvious abnormalities or parasites.
8. Record obvious dark, thickened mantle areas along the edge of the shell.
9. For chemical contaminant assays place clam tissues in labeled, sterile plastic centrifuge tubes, seal, and freeze at -20°C or -80° C for additional assays.
10. Continue to next step for histopathology or fluid thioglycolate (RFTM) assay for *Perkinsus sp.*

**Method Clam 2: Histopathology and Fluid Thioglycolate Assay for *Perkinsus* spp.**

Diagnostic methods used for the detection of the parasite *Perkinsus* spp. in mollusks vary in complexity and efficacy; however, the relatively inexpensive, semi-quantitative assay developed by Ray (1952) is preferred. In this technique, pieces of gill, palps, rectum or other tissues are incubated separately in Ray's fluid thioglycolate medium (RFTM) for several days. *Perkinsus* spp. cells become enlarged in RFTM and form hypnospores which stain with Lugol's iodine solution (Ray 1952). Procedures and solution preparation are described in Howard et al. 2004 and in Oyster Method 3.

1. When clams are sufficiently large, excise anus or pieces of gill and palps may be excised and incubated in RFTM if clams are moderately small.
2. Whole bodies of clams may be incubated in RFTM when clams are too small to yield tissue for both RFTM and histology. In these circumstances, whole bodies of 30 clams

are incubated in RFTM and an additional 30 clams are processed for histology.

3. Cut a transverse section of the entire clam so it includes gill and a portion of most internal organs, tissue section should be no more that 4-5 mm thick.
4. Place section in Davidson's 24-48hr; can be stored 4°C for several days.
5. Place in 50% ethanol, 2hr minimum.
6. Place in 70% ethanol, 2hr minimum.
7. Process fixed specimens on a 15hr overnight cycle in an automated tissue processor.
8. Embed tissues in paraplast blocks.
9. Section blocks at 4-6µm thickness.
10. Stain sections with Mayer's hematoxylin and eosin (MHE) or special stains as described in Howard et al. (2004).

Tissues may be archived in 70% ethanol or stock Davidson's without acetic acid.

Tissues may also be preserved in 1% glutaraldehyde-4% formaldehyde (1G4F) in seawater of half ambient salinity (McDowell and Trump 1976, Farley et al. 1986). In this case steps 5-6 can be omitted.

Formula:

*Davidson's* (Shaw and Battle 1957) 1gal stock (3600mL)

Glycerin . . . . . ……..400.0ml

37-40% formaldehyde . . . ……..800.0mL **(**potential carcinogen**)**

95% ethanol . . . ……1200.0ml

Filtered ambient or artificial seawater. ..1200.0ml

For best results, follow order of ingredients when preparing; shake well before use.

Store at 20°C

Working Davidson's solution (10% acetic acid)

Davidson's stock . . . . . …… 9 parts

Glacial acetic acid . . . . . …… . . 1 part

For DNA extractions, fix with stock solution since acetic acid interferes with DNA recovery.

**Method Clam 3: Metallothionein** (Viarengo et al. 1997)

1. Once sample number of clams has been collected, immediately dissect gill and digestive gland, damp dry, and store at 70$^{o}$C.
2. Homogenize tissues in a volume of 0.5M sucrose, 20 mM of Tris-HCl buffer, pH 8.6 with added 0.006mM of leupeptine, 0.5mM of PMSF (phenylmethylsulphonylflouride) and 0.01% of ß-mercaptoethanol. Amount should be three times the volume of clam tissue to be homogenized.
3. Centrifuge homogenized tissues 100,000 X *g* for 90min at 0-4$^{o}$C.
4. Add 1.05mL of cold absolute ethanol and 80µL to 1mL aliquots of supernatant.
5. Centrifuge at 6000 X *g* for 10min at 0-4$^{o}$C.
6. Combine supernatant with 1mg of RNA and 40µL of 37% HCl and three volumes of cold ethanol to a final concentration of 87%.
7. Maintain sample for 1hr at -20$^{o}$C and centrifuge at 6000 X *g* for 10min.
8. Wash pellet with 87% ethanol and 1% chloroform in homogenizing buffer.
9. Centrifuge at 6000 X *g* for 10min and dry under nitrogen gas stream.
10. Resuspend pellet in 150µL of 0.25M NaCl.
11. Add 150µL of 1N HCl containing 4mM of EDTA to the sample.
12. At 20°C, add 4.2mL of 2M NaCl containing 0.43mM of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) buffered with 0.2M of sodium phosphate, pH 8.0 to the sample.
13. Centrifuge at 3000 X *g* for 5min.
14. Evaluate absorbance at 412nm using a spectrophotometer and estimate metallothionein (MT) concentration using reduced glutathione (GSH) as reference standard.
15. Calculate amount of MT assuming a cysteine content in mussel MT of 29% (Mackay et al. 1993).

**Method Clam 4: Acetylcholinesterase (AChE) Activity**

1. Dissect gills and digestive gland from clam within 1-2hr after collection.
2. Damp dry and pool tissues from 10 animals in triplicate. Store at -80$^{o}$C.
3. Thaw tissues to 23$^{o}$C and homogenize in 1:5 (tissue weight: buffer volume) ratio in ice-cold 100mM of Tris-HCl buffer saline solution, pH 8.0, containing 0.1% Triton X-100.
4. Centrifuge homogenates at 10,000 X *g* for 15min at 4$^{o}$C. Repeat 2 X and store overnight,

if needed, at 4°C.

5. Thaw tissues to 23°C (15min) and react 50µL of supernatant with 0.43mM of (DTNB), phosphate buffer (pH 7.0) in the presence of 1mM acetylthiocholine (ASCh).
6. Measure absorption of 2-nitro-5-thiobenzoate anion formed from reaction using a spectrophotometer at 412nm absorbance every 60s for 7min at 20°C.
7. Calculate kinetics in linear range after subtraction of blank activities due to substrate autohydrolysis.
8. Express AChE activity as nMoles of product developed per minute per milligram of proteins.
9. See Binelli et al. 2006, Ellman et al. 1961 and Lionetto et al. 2003 for additional information on methods.

**Method Clam 5: RNA/DNA Ratio:** Please see Method Fish 8 – White Perch RNA: DNA Analysis for protocol, substituting clam foot material for fish muscle material.

**Method Clam 6: Isolation of Bacteria (*Vibrio* spp.)**

1. Swab the surface of the shell and the adductor muscle with 70% ethanol.
2. Withdraw a few drops of hemolymph from posterior or anterior adductor muscle by inserting a sterile hypodermic syringe and gently pulling back on the plunger. Select appropriate gauge of the syringe and needle based upon size of clam.
3. Using aseptic techniques lift lid of thiosulfate citrate bile salts sucrose (TCBS) agar plate while working in a laminar flow or dead air space hood, or beneath a flame of a Bunsen burner.
4. Gently expel 2-3 drops of hemolymph onto the surface of the TCBS plate near an edge.
5. Close lid.
6. Sterilize an inoculating loop by placing it at an angle over a flame.
7. Open lid of TCBS plate and touch tip of inoculating loop on an edge away from hemolymph.
8. Streak the loop through the drops of hemolymph in a zig-zag pattern until one-third of the plate is covered.

9. Sterilize the loop again in the flame; cool it at the edge of the agar away from the inoculums.
10. Rotate the plate about 60° and spread the bacteria from the first streak into a second area.
11. Sterilize the loop again, cool in agar away from inoculums, and spread inoculums from the second streak into a new area.
12. Sterilize the loop again.
13. Replace lid and invert the plate. Incubate the plate aerobically for 18-24hr at 35-37$^{o}$C.
14. Examine plates for isolated colonies.
15. Isolated colonies could be identified using the API-20E Bacterial Identification Test Strip (bioMerieux, Marcy l'Etoile, France).

# Oyster Indicators

**Introduction**

Historically, the harvest of eastern oysters *Crassostrea virginica* along the east coast of the United States has been from natural set wild populations. Early settlers often reported oyster reefs or bars so abundant and expansive that they posed a threat to navigation in many coastal bays and waterways (Wharton 1957). At the beginning of the 20th century, the Chesapeake Bay oyster fishery was considered one of the most important in the United States (Kennedy 1989). However, by the late 20th century, overexploitation and oyster disease led to a severe decline of the resource in many states. Today wild and cultured oysters play an important economic and ecological role in many coastal areas, but the loss of habitat from overharvesting, siltation degradation of water quality, disease, parasitism, and stressors related to extreme weather events, land use and associated stressors have resulted in a less abundant resource (Andrews 1984, Bushek and Allen 1996, Burreson and Ragone-Calvo 1996, Funderburk et al. 1991, Kennedy 1996, MacKenzie 1996**)**.

The eastern oyster is well known for its ability to filter phytoplankton and suspended detritus from the water column. It has been proposed that the decline of oysters in Chesapeake Bay has led to a shift in food webs and reduced water quality. At pre-1880 oyster population levels it took only 3-6d to filter the entire water system of Chesapeake Bay. At 1988 oyster population levels it took 325d (Haven and Morales-Alamo1970, Newell 1988). This dramatic increase is associated with decreased water quality. While the bay has other filter feeders, oysters shell is the attachment substrate for many (Kennedy 1992) and the loss of filtering capacity and quality from oysters compared to the total filtering capacity of the bay is less certain. Loss of healthy oyster reefs also affect availability of food, shelter, and habitat, for other species (Wells 1961, MacKenzie 1996, Lippson and Lippson 1997, Coen et al., 1999), It has been estimated that healthy oyster beds may provide 50 times greater habitat surface area than the same area of flat river bottom (Lippson and Lippson 1997). Thus, oysters provide a valuable and complex community ecosystem in waters where the species may still be found.

As documented in the previous chapter, macroinvertebrates, especially oysters, are reliable and sensitive indicators or biomonitors of marine and estuarine environments. For example, the NOAA's Mussel Watch program, which includes oyster assays, is one of the longest running continuous monitoring programs in U.S. coastal and Great Lakes areas (http://ccma.nos.noaa.gov/about/coast/nsandt/musselwatch.aspx). The oyster's filter-feeding habit, it's benthic, sessile, stationary nature; and its integral role in the food chain make it a valuable indicator. The oyster serves as an excellent sentinel organism that can provide valuable baseline and new information of anthropogenic effects on estuaries (http://www.epa.gov/bioiweb1/html/marinetidal.html, Jenny et al. 2002, Volety et al. 2009, Coen and Luckenback 2000, Coen et al. 1999).

**Sampling Design**

Our goal is to assess community diversity on oyster bars in an attempt to identify the effect of land use on the abundance and health of oyster bars from different river systems that have predominant land use. An Index of Biotic Integrity (IBI) is a well-established approach to examine changes in community structure associated with disturbance gradients (Karr

1981). Benthic communities serve as good indicators due to their role in maintaining sediment and water quality and their sensitivity to exposure to pollutants (Holland et al., 1987) and to accumulate biocontaminants over time (Sanders et al. 1980; Nixon et al. 1986).

**Metrics**

*Community Assessment* – Oyster reefs provide structure and habitat for a variety of organisms. As in sediments and fish communities, the diversity of organisms, number of specific species, age structure, and growth characteristics can be used to determine the overall health and function of this critical habitat. Comparison of diversity indices, presence or absence of rare species, and trophic balance are all meaningful in identifying ecosystem change http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=190806. Method Oyster 1

*Disease Prevalence* - Disease has played a significant role in the decline in abundance of the native eastern oyster along the US east coast. In particular protozoan parasites, *Haplosporidium nelsoni* and *Perkinsus marinus*, have been problematic. A number of studies have examined the relationships among parasites, pathology, and selected morphological conditions (NOAA Mussel Watch). Surveys of parasites and general animal health, including the use of histopathology, can be an important component of biomonitoring studies of environmental pollution. Monitoring the prevalence and severity of pathology and parasites, Method Oyster 2 including, *Perkinsus marinus* Method Oyster 3, provides data from which an IBI may be developed that reflects the influence of habitat quality.

**Methods**

Although oyster reef ecosystems are valuable biological indicators (Jenny et al. 2002, Volety et al. 2009, Coen and Luckenback 2000, Coen et al. 1999 and (http://www.epa.gov/bioiweb1/html/marinetidal.html), wild oysters often are not available from the representative rivers chosen for this integrated assessment to provide statistically significant numbers of specimens to conduct viable assays. This is due to several reasons: 1) the absence of viable oyster bars in some river systems, 2) private ownership of viable oyster bars prohibiting sampling, 3) salinity of some rivers is not conducive to oyster growth.

For the purposes of the Oxford Integrated Environmental Assessment, an abbreviated oyster community assessment and disease survey is conducted in areas where oysters are available to obtain baseline information and evaluate the effect of land use on the abundance and health of oysters and their communities.

**Method Oyster 1: Community Assessment**

1. Tow oyster dredge over oyster bar in river chosen for predominant land use. Repeat tows to obtain sufficient oysters to take a random sample of 30 live individuals. Record start and end GPS coordinates for each dredge tow on Oyster Community Assessment Data Sheet, and track number of tows needed to obtain 30 oysters for evaluation.
2. Record water quality information obtained and logged using a datasonde
3. Separate live and dead (boxes – shells attached by adductor muscle, but no oyster meat present) oysters from other material and record number of each. Leave fowling organisms on live oysters.
4. Record weight and volume of miscellaneous shell hash, mussels, miscellaneous shells, etc. in each dredge tow that is not attached to live oysters.
5. Collectively weigh the 30 live oysters in their shell including attached mussels and other fowling organisms.
6. Carefully remove and save fowling organisms (mussels, barnacles, anemones, *Molgula* (sea squirts), etc. from the sample of live oysters.
7. Record number and length of spat on the 30 live oysters.
8. Measure length of a representative sample of dead oysters (boxes) from hinge to bill. Record individual and collective weight of the dead oysters. Calculate mean lengths and weights.
9. Count, weigh, and obtain wet volume of each type of fouling organism removed from the 30 live oysters. Wet volume is obtained by filling a volumetric container with water to a level above the contents being measured and gently taping and swirling contents to obtain a compacted volume. Where appropriate, measure length of a random subsample of fouling organisms.
10. Count, weigh, measure and obtain sex of individual crabs from each dredge tow. This includes all crabs (blue, mud, horseshoe, etc.). Obtain wet volume of mud crabs.

11. Count, measure, and weigh fish from each dredge tow and quickly return to the water.
12. Count, measure, weigh, and obtain wet volume of other live sea life and biological and non-biotic components associated with combined dredge tows.
13. Document, photograph, count and weigh combined trash and marine debris from tows.
14. Record and photograph evidence of sea grasses from combined dredge tows.
15. Note presence or abundance of jellyfish or comb jellies from combined dredge tows.
16. If there is uncertainty of species identity, place examples in preservative for identification later.
17. Arrange and assign oyster number (1-30). Weigh and measure length of each of the 30 live oysters from hinge to bill. Return each oyster to assigned position for processing. Calculate mean lengths and weights.
18. Process oysters for histology and detection of *Perkinsus marinus* onboard the vessel when possible (See Oyster Method 2 and 3) taking care to return shells to the same oyster bar from which they are collected. If processing occurred onshore in the laboratory, oysters are placed in chilled cooler for transport. Do not store live oysters in unventilated bags.
19. Return all non-trash dredged material to area collected after documentation and quantification.

**Method Oyster 2: Oyster Histology**

1. Within 24hr of collection process individual oysters for histology.
2. Record shell length and any remarkable shell epibionts or abnormalities.
3. Rinse well and scrub to remove sediments.
4. Weigh and record weight of each oyster in shell.
5. Remove oyster meat from shell and put aside.
6. Weigh and record weight of shells only.
7. Weigh and record weight of oyster meat.
8. Calculate and record mean values of measurements and weights.
9. Review Howard et al. (2004) for precise processing methods.
10. Record gross condition of oyster meat

11. Excise a section of rectal tissue and place in Ray's Fluid Thioglycollate Medium (RFTM) (Ray 1966).
12. RFTM medium (see **Method Oyster 3**)
13. Remove cross section of oyster
14. Place dissected tissue in labeled cassette
15. Place labeled cassette in Davidson's fixative
16. Repeat 2-11 for each oyster until all 30 oysters placed in Davidson's
17. Place in refrigerator and allow to fix
18. Process oyster on 15hr overnight cycle on automated tissue processor
19. Embed processed tissues in paraplast
20. Section blocks at 5µm, place one section on a slide
21. Stain with Mayer's hematoxylin-eosin-phloxine stain.

**Method Oyster 3: Dermo *Perkinsus* sp. (RFTM)** (Howard et al. 2004)

1) With scissors, excise target tissue, rectal, gill or palp of each animal during initial necropsy.
2) Place tissue in separate labeled tube containing Ray's fluid thioglycolate medium (RFTM).
3) Rinse scissors in distilled water 5-10s to avoid cross contamination.
4) Once all animals are processed, place tubes in dark to incubate for at least 72hr.
5) Post incubation, remove tissue from tube with hooked end of glass rod and place on labeled slide.
6) Add a drop of Lugol's iodine working solution.
7) Tease tissue apart with probes, clean probes between specimens.
8) Add another drop of Lugol's iodine working solution and place coverslip over tissue.
9) Examine slides for presence of *P. marinus* which appear as blue/black smooth spheres.
10) Rate infection intensity.

*RFTM Ray's Fluid Thioglycollate Medium (+dextrose)(Ray 1966)*

Thioglycollate medium……..14.6g

NaCl…….10.0g

Distilled water…….485.0mL

*Chloramphenicol*

Chloramphenicol…….0.25g

Distilled water……10.0mL

*Nystatin*

Nystatin……..500,000 usp units

Sterile distilled water……..125.0mL

*Lugol's iodine stock*

Potassium iodide……6.0g

Iodine……..4.0g

Distilled water……100.0mL

*Lugol's iodine working solution*

Distilled water…….30.0mL

Lugol's stock……..15.0mL

## Appendix I: Field Data Sheet - Water Quality

| Sampling Station | | Date | Time |
|---|---|---|---|
| | | mm/dd/yy | 00:00H |

| Air temp (°C) | Tide state | Weather code today |
|---|---|---|
| | ebb, flood, high, low | |

| Cloud cover (%) | Weather code yesterday |
|---|---|
| | |

| Number of samples | Wind direction | Wind velocity | | Chlorophyll (mg/L) |
|---|---|---|---|---|
| | | min | max | |

| DO method | Total depth | Secchi depth (m) | Wave Height (m) | DNA (mg/L) |
|---|---|---|---|---|
| YSI | | | | 1) |
| | | | | 2) |

| Depth (m) | Water temp (°C) | Specific Conductance (µmhos/cm) | Salinity (ppt) | pH | Turbidity (NTU) | Chlorophyll (ug/L) | DO (%) | DO (mg/L) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
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**KEY**

| Weather codes | |
|---|---|
| | * 10 – no precipitation |
| | * 11 – drizzle |
| | * 12 – rain |
| | * 13 – heavy rain |
| | * 14 – squally (fast moving storms) |
| | * 15 – Frozen precipitation |

| Wind velocity | What to record | How the water appears |
|---|---|---|
| | * 00-01 knots | calm |
| | * 01-03 knots | light & variable |
| | * 04-06 knots | small waves |
| | * 07-10 knots | scattered white caps |
| | * 11-16 knots | numerous white caps |
| | * 17-21 knots | moderate waves |
| | * 22-27 knots | large waves 2.5 to 4 meters |
| | * 28-33 knots | resistance against walking |

| Wave height | What to record | How the water appears |
|---|---|---|
| | * 0.00m | flat calm |
| | * 0.09m | slight ripple |
| | * 0.20m | ripple to 1ft |
| | * 0.40m | 1 – 2 ft |
| | * 1.00m | 2 – 4 ft |
| | * 1.50m | 4 – 6 ft |

**Depth Conversion**

| Meters | Feet | Meters | Feet |
|---|---|---|---|
| 0.5 | 1.64 | 5.5 | 18.04 |
| 1 | 3.28 | 6 | 19.69 |
| 1.5 | 4.92 | 6.5 | 21.33 |
| 2 | 6.56 | 7 | 22.97 |
| 2.5 | 8.20 | 7.5 | 24.61 |
| 3 | 9.84 | 8 | 26.25 |
| 3.5 | 11.48 | 8.5 | 27.89 |
| 4 | 13.12 | 9 | 29.53 |
| 4.5 | 14.76 | 9.5 | 31.17 |
| 5 | 16.40 | 10 | 32.81 |

**Appendix I: Field Data Sheet - White Perch Necropsy**

IA White Perch Data Date_____ Accession#_____

Weight _____

Length _____

BFI _____

Histo Y / N

Micro K/L TSA Spleen

Photos _____

Blood Y / N

Liver Wt _____

Gross Ext

Gross Int

IA White Perch Data Date_____ Accession#_____

Weight _____

Length _____

BFI _____

Histo Y / N

Micro K/L TSA Spleen

Photos _____

Blood Y / N

Liver Wt _____

Gross Ext

Gross Int

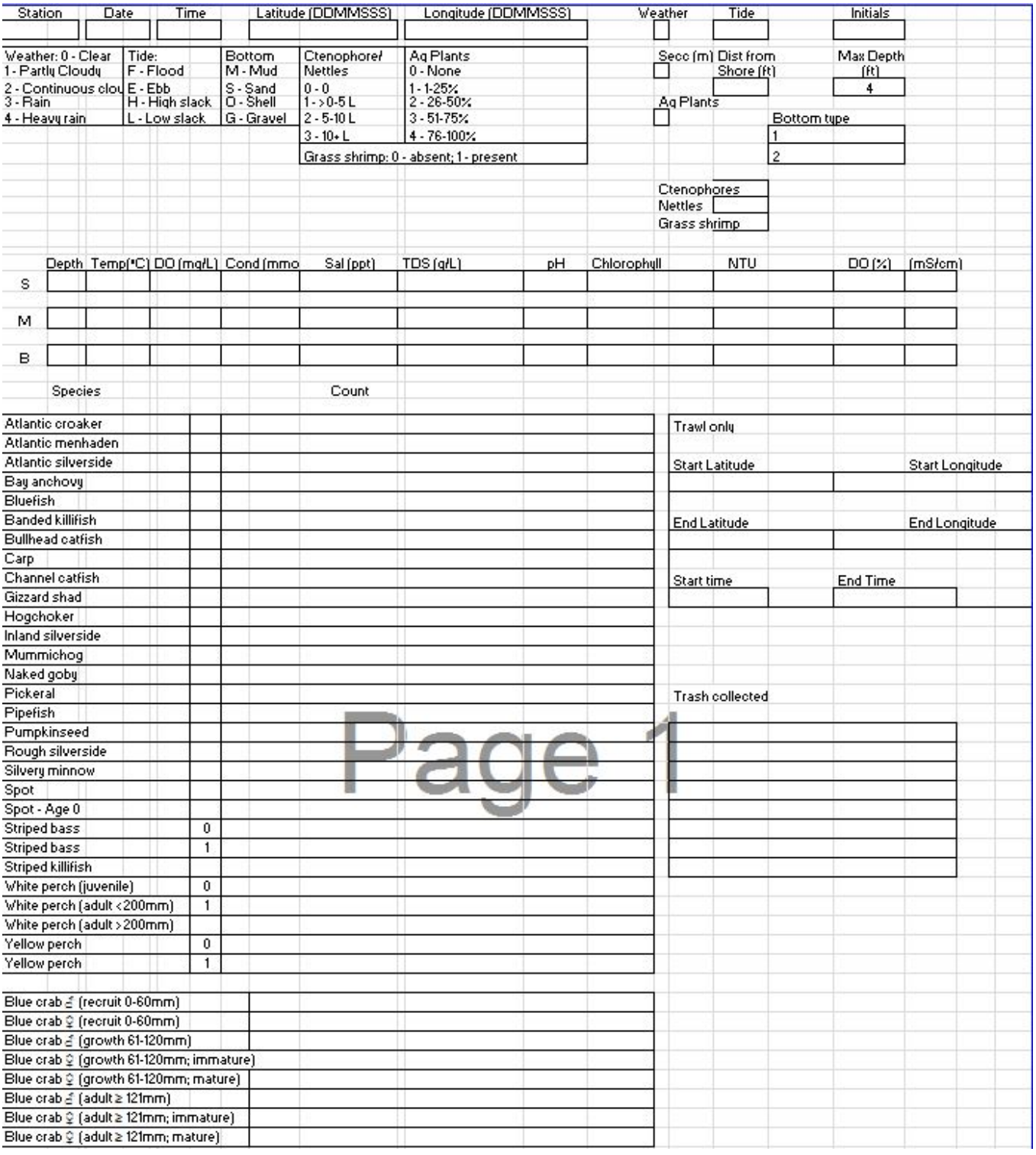
## Appendix I. Field Data Sheet - White Perch Parasites

| NOAA White perch (rank scale 0-5) | | | | | | | | Date | | | River | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | rank | rank | rank | rank | rank | rank | count | count | rank | rank | count | rank | |
| # | skin trichodina | skin sessile peritrich | skin costia (ichthyo-bodo) | gill trichodina | gill sessile peritrich | gill costia (ichthyo-bodo) | gill digene | fluke Dactylogyrus | laminar pockets | copepod | isopod | acantho-cephala | Obs |
| T1 | | | | | | | | | | | | | |
| T2 | | | | | | | | | | | | | |
| T3 | | | | | | | | | | | | | |
| T4 | | | | | | | | | | | | | |
| T5 | | | | | | | | | | | | | |
| T6 | | | | | | | | | | | | | |
| T7 | | | | | | | | | | | | | |
| T8 | | | | | | | | | | | | | |
| T9 | | | | | | | | | | | | | |
| T10 | | | | | | | | | | | | | |
| T11 | | | | | | | | | | | | | |
| T12 | | | | | | | | | | | | | |
| T13 | | | | | | | | | | | | | |
| T14 | | | | | | | | | | | | | |
| T15 | | | | | | | | | | | | | |
| T16 | | | | | | | | | | | | | |
| T17 | | | | | | | | | | | | | |
| T18 | | | | | | | | | | | | | |
| T19 | | | | | | | | | | | | | |
| T20 | | | | | | | | | | | | | |
| T21 | | | | | | | | | | | | | |
| T22 | | | | | | | | | | | | | |
| T23 | | | | | | | | | | | | | |
| T24 | | | | | | | | | | | | | |
| T25 | | | | | | | | | | | | | |
| T26 | | | | | | | | | | | | | |
| T27 | | | | | | | | | | | | | |
| T28 | | | | | | | | | | | | | |
| T29 | | | | | | | | | | | | | |
| T30 | | | | | | | | | | | | | |

**Appendix I: Field Data Sheet - Mummichog**

| River: | Section: | Date: | Latitude: | Longitude: |
|---|---|---|---|---|
| Fish # | Sex | Length (mm) | Weight | Parasites? |
| 1 | | | | |
| 2 | | | | |
| 3 | | | | |
| 4 | | | | |

## Appendix I: Field Data Sheet: Fish Community Composition, Page 1



| Station | Date | Time | Latitude (DDMMSSS) | Longitude (DDMMSSS) | Weather | Tide | Initials |
|---|---|---|---|---|---|---|---|
| | | | | | | | |

| Weather: 0 - Clear | Tide: | Bottom | Ctenophore/ | Aq Plants |
|---|---|---|---|---|
| 1 - Partly Cloudy | F - Flood | M - Mud | Nettles | 0 - None |
| 2 - Continuous clou | E - Ebb | S - Sand | 0 - 0 | 1 - 1-25% |
| 3 - Rain | H - High slack | O - Shell | 1 - >0-5 L | 2 - 26-50% |
| 4 - Heavy rain | L - Low slack | G - Gravel | 2 - 5-10 L | 3 - 51-75% |
| | | | 3 - 10+ L | 4 - 76-100% |
| | | | Grass shrimp: 0 - absent; 1 - present | |

| Secc (m) | Dist from Shore (ft) | Max Depth (ft) |
|---|---|---|
| | | 4 |

Aq Plants

| Bottom type |
|---|
| 1 |
| 2 |

| | |
|---|---|
| Ctenophores | |
| Nettles | |
| Grass shrimp | |

| | Depth | Temp(°C) | DO (mg/L) | Cond (mmo | Sal (ppt) | TDS (g/L) | pH | Chlorophyll | NTU | DO (%) | (mS/cm) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S | | | | | | | | | | | |
| M | | | | | | | | | | | |
| B | | | | | | | | | | | |

| Species | Count |
|---|---|
| Atlantic croaker | |
| Atlantic menhaden | |
| Atlantic silverside | |
| Bay anchovy | |
| Bluefish | |
| Banded killifish | |
| Bullhead catfish | |
| Carp | |
| Channel catfish | |
| Gizzard shad | |
| Hogchoker | |
| Inland silverside | |
| Mummichog | |
| Naked goby | |
| Pickeral | |
| Pipefish | |
| Pumpkinseed | |
| Rough silverside | |
| Silvery minnow | |
| Spot | |
| Spot - Age 0 | |
| Striped bass | 0 |
| Striped bass | 1 |
| Striped killifish | |
| White perch (juvenile) | 0 |
| White perch (adult <200mm) | 1 |
| White perch (adult >200mm) | |
| Yellow perch | 0 |
| Yellow perch | 1 |

| | |
|---|---|
| Blue crab ♂ (recruit 0-60mm) | |
| Blue crab ♀ (recruit 0-60mm) | |
| Blue crab ♂ (growth 61-120mm) | |
| Blue crab ♀ (growth 61-120mm; immature) | |
| Blue crab ♀ (growth 61-120mm; mature) | |
| Blue crab ♂ (adult ≥ 121mm) | |
| Blue crab ♀ (adult ≥ 121mm; immature) | |
| Blue crab ♀ (adult ≥ 121mm; mature) | |

Trawl only

| Start Latitude | Start Longitude |
|---|---|
| | |

| End Latitude | End Longitude |
|---|---|
| | |

| Start time | End Time |
|---|---|
| | |

Trash collected

| |
|---|
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## Appendix I: Field Data Sheet: Fish Community Composition, Page 2

Anomalies (1st 30 individuals)

| Species | Anomaly description | Anomaly count added into above (Y/N) |
|---|---|---|
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## Appendix I: Field Data Sheet - Crab

| Integrated Assessment - Blue Crab | | | | | | | | **Collection Date:** | | **River:** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | |
| Crab # | Code | CW | Sex M/F | Adult Juvenile | Shell Disease (0-5) | Molt: pre,post, inter | Gear | Sample for Assay: Histo | Sample for Assay: Carbo-hydrates | Sample for Assay: _____ | Sample for Assay: _____ | Notes: |
| 1 | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | |
| 5 | | | | | | | | | | | | |
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| 9 | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | |
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| 25 | | | | | | | | | | | | |
| 26 | | | | | | | | | | | | |
| 27 | | | | | | | | | | | | |
| 28 | | | | | | | | | | | | |
| 29 | | | | | | | | | | | | |
| 30 | | | | | | | | | | | | |

**Appendix 1: Field Data Sheet: Clams**

**COL Integrated Ecological Assessment**
**Clam Field Collection**

Date: _____ Time: Onsite _____ Depart _____

Crew: _____

River: Corsica Rhode Magothy Middle Nanjemoy Sassafras

Segment: 1 (Head) 2 (Middle) 3 (Mouth)

Site Code: _____
Latitude: _____ Longitude: _____

Gear: SM Grab Dredge Shovel Other: _____

Sediments: Microtox (glass/refrig) Organic (glass/freeze) Metals (plastic/freeze) Benthic (ziploc/refrig)

| Species collected: | a. | Adults (n= ) | Juveniles (n= ) |
|---|---|---|---|
| | b. | | |
| Other species: | | | |
| Relative abundance | | | |

# Grabs/Tows/Digs: _____ Start time: _____End: _____ Total min: _____

Datasonde ( _____)

| | **Depth** | **Salinity** | **Temp C° or F°** | **Conductivity mS/cm** | **pH** | **DO** |
|---|---|---|---|---|---|---|
| Surface | | | | | | |
| Bottom | | | | | | |

Notes:
_____
_____
_____
_____
_____
_____
_____
_____

## Appendix I. Field Data Sheet - Oyster

**OYSTER COMMUNITY ASSESSMENT DATA SHEET**

**Station** _____ **River System** _____

**Date** _____ **Histological Code** _____

Water Temperature _____ Salinity _____

Water Depth _____ Specimens Fixed - _____

Abundance of Jelly Fish of Comb Jellies Heavy, Moderate, Light, None (Circle most appropriate)

GPS Coordinates-beginning _____ _____ _____

GPS Coordinates - End _____ _____ _____

Number of dredge tows

Total Number of Live Oysters _____ Weight (30 oyster sample) _____

Mean Size Live (30 oyster sample) Number Spat (30 oysters)

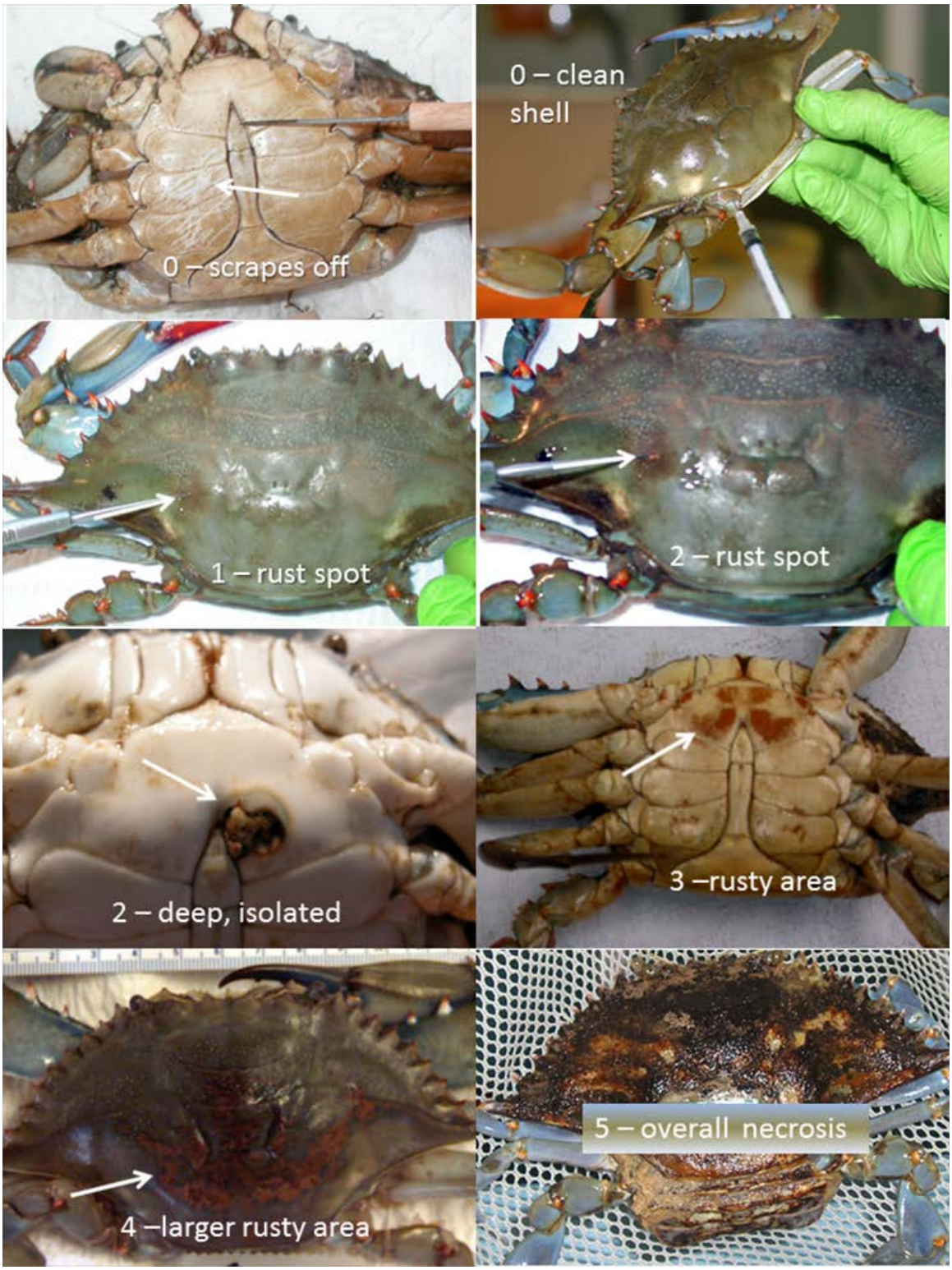
Total Number of Dead Oysters _____ Weight (30 oyster sample) _____

Mean Size of Dead Oysters Wt. of Remaining Shell Hash

Community Assessment

| Species | Number | Weight | Wet Volume | |
|---|---|---|---|---|
| Invertebrates | | | | |
| Anemomes | | | | |
| Molgula | | | | |
| Barnacles | | | | |
| Mussels | | | | |
| Crabs | | | | Count Male & Female |
| Mud Crabs | | | | |
| Horseshoe Crabs | | | | |
| Blue Crabs | | | | |
| Other | | | | |
| | | | | |
| | | | | |
| Vertebrates | | | | |
| Fish - Gobbies | | | | |
| Blennies | | | | |
| Other | | | | |
| Other | | | | |
| Trash Items In Tow(s) | | | | |

**Appendix II: Crab Shell Disease Rankings**




Rankings are from 0-5, with 5 being the worst. A discoloration that scrapes off with a fingernail is not shell disease.

***Environmental Management System Statement***

These protocols fully incorporate the NOAA/NCCOS Environmental Management System (EMS) requirements and conform to E.O. 13423 and E.O. 13514. This document includes appropriate considerations regarding evaluating and minimizing an environmental footprint and implementing energy and water conservation directives. Specific considerations have been made to reduce the number of animals sampled and to use or substitute protocols and chemicals to be environmentally friendly.

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