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WORK PLAN FOR TRACE LEVEL INORGANIC ANALYSIS OF MARINE AND ESTUARINE SAMPLES NSY PORTSMOUTH ME

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UNIVERSITY OF RHODE ISLAND WORK PLAN

Trace Level Inorganic Analysis of Marine and Estuarine Samples

August 1993

Prepared By:

Center for Atmospheric Chemistry Studies
Graduate School of Oceanography
University of Rhode Island
Narragansett Bay Campus, South Ferry Rd
Narragansett, RI 02882-1197

The University of Rhode Island, Narragansett, RI 02882-1197
Center for Atmospheric Chemistry Studies
Graduate School of Oceanography

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The University of Rhode Island
Graduate School of Oceanography
Center for Atmospheric Chemistry Studies
Narragansett, Rhode Island 02882

J. Douglas Cullen
Co-Principal Investigator

Richard Arimoto
Co-Principal Investigator

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Work Plan for Trace Level Inorganic Analysis of Marine and Estuarine Samples

Contract Number: N66001-92-D0092
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I. Investigations

There are two primary objectives of this study. One objective is to develop a practical methodology for the collection, preparation, and analysis of estuarine water samples for selected trace metals. The other objective is to develop methods for instrumental neutron activation analysis to be used for the determination of trace metal concentrations in marine tissue and sediment samples. A summary of the samples that will be analyzed and the number of samples in each category is given in Table1.

Table 1. Sample categories and number of samples within each category that will be analyzed for selected trace metals.

Task/Category	Seawater	Elutriate	Tissue	Sediment
Model Calibration	20			10
Model Validation	20			10
Loading Rate Study	20	10	10	
Geochemical Assimilation		10	10	
Monitoring and Verification	20			
Subtotal No. of Samples	80	20	20	20
Number of Batches	4	1	1	1
QA Samples (@3/Batch)	12	3	3	3
Total Number of Samples	92	23	23	23

The technical approach to each objective is similar in that a performance based Quality Control and Quality Assurance program will be developed to evaluate the samples analyzed. Our performance-based program will be built on commercially available Standard Reference Materials and specially prepared Laboratory Control Samples. A Method Detection Limit (MDL) study also will be included in our effort to assess the performance of our analytical protocols. Specific tasks appropriate to each objective are given in greater detail below. Ultimately, field and laboratory operating procedures will be detailed in written reports.

A major part of our involvement in this project is field sampling to be conducted during three cruises in the late summer and fall of 1993. The purpose of these cruises is to evaluate the distribution of trace metals within the estuarine environment of the Great Bay and Piscataqua River in New Hampshire and Maine. We will also collect water column samples from selected stations within the Piscataqua River so that modelers can evaluate certain predictive capabilities of their hydrodynamic models. Seep samples also will be collected from the vicinity of the landfill on Seavey Island on each of the scheduled cruises.

Progress reports will be submitted monthly, and a final report will be submitted as (1) a hard copy with appropriate flags and (2) with files in ASCII format.

II. Tasks Related to the Measurement of Trace Metals in Seawater.

An overview of the specific tasks for the collection, preparation, and analysis of trace metals in seawater are given in Table 2, along with the projected task completion dates. Following the overview, an expanded version of these specific tasks is detailed in narrative form.

A. Cleaning Procedures and Documentation

Our efforts will be directed towards quantifying nanomolar levels of selected trace metals in the estuarine waters of Great Bay and Piscataqua River. As a first step, all materials that will come in contact with the samples must be cleaned using at a minimum analytical grade acids. All sample collection devices, collection bottles, filtration apparati, filters, and reagents must be specially prepared before use. We will document the cleaning steps involved in the preparation of each material and detail these steps in an operating procedure.

Table 2. Overview of tasks related to the measurement of dissolved and particulate metal in seawater, elutriate, and/or seep samples.

Operation	Expected Completion Date
1. Cleaning procedures	October, 1993
2. Method Detection Limit (MDL) Studies	
a. Dissolved metal MDL	August, 1993
b. Particulate metal MDL	September, 1993
c. Hg MDL (option metal)	October, 1993
3. Field Sampling (3 cruises)	November, 1993
4. Documentation of preconcentration and other analytical procedures for seawater	December, 1993

We will also document the shipboard operating procedures that we use to collect and process seawater samples. Again, our efforts will focus on providing a trace metal clean sampling environment for all shipboard processing.

B. Method Detection Limit (MDL) Study for Dissolved and Particulate Metals in Seawater.

As part of our study, we will establish the MDL for dissolved and particulate trace metals. For dissolved metals we will use NASS-4 Open Ocean Seawater Reference Material for Trace Metals, available from the National Research Council of Canada. For our particulate MDL, we will prepare a Laboratory Control Sample (LCS) which will involve the measurement of an acid-recoverable fraction of an Estuarine Sediment Standard Reference Material (SRM 1646), available from the National Institute of Standards and Technology.

1. Dissolved Metal MDL

For dissolved Cd, Cu, Ni, and Pb the MDL will be established for the ammonium pyrollidine dithiocarbamate (APDC)-cobalt chloride coprecipitation technique. NASS-4 will be preconcentrated prior to the analysis of each metal by graphite furnace atomic absorption spectrophotometry (GFAAS). The MDL for dissolved Fe, Mn, and Cr will be established using direct injection GFAAS.

2. Particulate Metal MDL

The acid-recoverable fraction of SRM 1646 will be used as the material to establish the particulate MDL for Cd, Cu, Ni, Pb, Fe, Mn and Al. Particulate metals will be analyzed by GFAAS, although it may be necessary to analyze Al by INAA.

3. MDL for Acid-Recoverable Hg

As specified in our proposal, Hg is an optional metal for this project in the event that Cr is not measured in the dissolved fraction of seawater. Our laboratory is currently modifying existing analytical equipment to develop the capability of quantifying this element in estuarine water. In concert with our efforts to measure other metals, we plan to perform a MDL using the acid-recoverable fraction of SRM 1646 as described earlier. If the results of our efforts to quantify Hg are not successful, then we will report instead Cr concentrations for all of the dissolved samples collected.

C. Field Sampling (Estuarine Cruises)

We have planned three, two-day cruises for the Piscataqua River and Great Bay Estuary to collect water column and seep samples for trace metal analysis. The Research Vessel *Gulf Challenger*, available through the University of New Hampshire, will be used to conduct the estuarine surveys. On each cruise, we plan to use conductivity-temperature-depth (CTD) measurements to characterize the physical nature of the water column and to identify areas within the water column for trace metal sampling. Teflon®-lined Niskin samplers (General Oceanics, Model No. 101101.7T), will be used to collect seawater samples, and once on board, water samples will be filtered through pre-tared, acid-cleaned, 47 mm diameter, 0.4μm pore size Nuclepore® membranes, using Nalgene® polysulfone filtration units. The operationally defined dissolved component of the water sample (filtrate), will be transferred to specially

cleaned polyethylene bottles and acidified to pH 2 with Baker Ultrex® HCl. The pre-tared membranes will be rinsed with Milli-Q® water and transferred to pre-cleaned petri dishes. All filtration operations will be carried out onboard, in a laminar flow clean bench. A more detailed account of onboard operating procedures will be documented after completing all scheduled cruises.

Seep samples will be collected from Seavey Island in the vicinity of the landfill. We anticipate the collection of between three and five seep samples during each cruise. These samples will be filtered immediately after collection to exclude particulate materials from the sample. After filtration, seep samples will be acidified and ultimately analyzed for dissolved metal. Documentation of the procedures used for the collection and preparation of seep samples will be detailed in written reports.

D. Documentation of Preconcentration and Analytical Procedures.

We will use the APDC-cobalt chloride coprecipitation technique as our preconcentration method. We will document (1) the cleaning steps used to prepare reagents for trace metal analysis, (2) the cleaning steps for all materials to come into contact with the sample, and (3) the steps involved in the preconcentration procedure. The QA/QC plan and validation plan for metals analyzed by the preconcentration technique will be developed subsequent to the MDL study. At a minimum, the QA/QC plan will define our data in terms of accuracy, precision, completeness, detection limit, representativeness, and comparability.

The same performance based QA/QC elements that we will use to validate our preconcentration procedure will be extended to the direct injection GFAAS techniques for dissolved Fe, Mn, and Cr, as well as our particulate metal efforts. In this regard, we will define the accuracy, precision, and detection limits for all our analytical methods.

III. Tasks Related to the Measurement of Trace Elements in Tissues and Sediments.

We will use at least two instrumental neutron activation analysis (INAA) protocols to quantify trace elements associated with marine tissue and sediment samples. The "short" and "long" activation protocols that we use simply refer to the length of time a sample is exposed to a neutron flux, and these activation irradiation schemes are summarized in Table 3, along with the trace elements that we expect to quantify. Although irradiation

and counting times for specific elements are more-or-less fixed by nuclear physics, the methods for irradiating the samples in the nuclear reactor must be developed. As a first step in the analytical process we will experimentally evaluate the geometry for irradiating and counting the marine tissue and sediment samples.

Table 3. Instrumental neutron activation analysis regimes and the expected trace elements resulting from the analysis of marine tissues and sediments using short and long irradiation protocols.

Neutron Activation Protocol	Irradiation Time	Decay Time	Count Time	Trace Elements
Short	1-2 min	2-10 min.	500 s	Al, Cu, Mn, V
Long	1-3 d	3 wk	40,000 s	Ag, Ba, Co, Cr, Cs, Fe, Sb, Sc, Se, Th, Zn

We will use the short neutron activation program to address the issue of irradiation geometry, and for the reference materials we will use SRM 1566a (Oyster Tissue), and SRM 1646 (Estuarine Sediment), available from the U.S. National Institute of Standards and Technology. Once the irradiation geometry is established, a MDL study will be initiated for each matrix using the "short" and "long" neutron activation procedures.

Subsequent to the MDL studies for tissues and sediments, we will develop our QA/QC plan. Finally, we will document all of our operating procedures involved in preparing, irradiating, and counting the tissue and sediment samples. Our goal in this portion of the project is to provide a methodology that can be used as an independent analytical technique to verify and augment other methodologies, and to provide unique information on some of the elements that are not be measured by other more commonly used techniques for marine tissue and sediment analysis.

IV. Data Deliverables and Technical Support

We will provide the data deliverable required for reporting the results of routine chemical analysis conducted according to the QA/QC specifications. At a minimum, the data deliverable will include a hardcopy of the data and a data diskette that is formatted according to specifications with respect to headers, data types and record length, variables, and appropriate data flags.

Technical support will be provided to the Navy at information exchange meetings by appropriate scientists working on the project. This will include attendance at meetings, and at public hearings to present and/or discuss the results of the water column, tissue, and sediment studies.

V. Milestones

Our overall schedule of tasks and the anticipated completion dates for each task are presented in Table 4.

Table 4. Milestone schedule for trace level inorganic analysis of marine and estuarine samples.

Task	1992					1993					
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Cleaning Procedures	x	x	x	x							
Cruise Preparation	x	x	x								
Scheduled Cruises		x	x	x							
Analytical Efforts											
Seawater MDL Studies		x	x								
Seawater Samples			x	x	x						
Elutriate Samples				x	x						
Marine Tissue					x	x	x				
Marine Sediment						x	x	x			
Reports											
Progress Reports	x	x	x	x	x	x	x	x	x	x	x
Draft Project Report									x		
Final Project Report											x

Meetings

Meetings will be held as necessary over the course of the project, and we will participate in a public meeting to discuss the results of our findings.

STANDARD OPERATING PROCEDURES

POINT OF CONTACT:

Larry G. Ward
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Road
Durham, NH 03824

I. OBJECTIVE

Collect and sample sediment cores for textural, radionuclide, bacteriological or toxicological analyses.

II.NECESSARY MATERIALS AND EQUIPMENT

- Research vessel with adequate winches for handling gravity corer (at least 150 kg capacity for subtidal samples).
- Benthos Gravity Corer or equivalent with (for subtidal samples) transparent liners, end caps, stainless steel core catchers, PVC check valve and manganese bronze nose cone.
- Hand corer: handle with clamp to fit 1 to 3 meter sections of core pipe (~7.6 cm diameter aluminum irrigation pipe)
- If necessary, appropriate clothing and protection for sampling in contaminated sites (rubber boots, raingear - jacket and pants, rubber gloves, glasses)
- Cooler or plastic garbage barrel with ice
- Duct tape
- Core extruder - rubber stopper attached to wood pole
- Core tray
- Aluminum weighing dishes
- Teflon or plastic knives
- Knife, piano wire or scalpel
- Holder to place core while cutting barrel
- Vibra-corer system

III.METHODS

A. Gravity Coring for subtidal Samples

1. If necessary, prior to use, wash each component of the coring system which will come in contact with any samples and thoroughly rinse with tap water. In addition, rinse the equip-ment with ambient water between uses in the field. Cut the core liner into a 1.6 m and 1.0 section prior to coring and unite with duct tape. Place the 1.0 m section on top.

2. When on station, first take a grab sample to determine the suitability of the bottom for gravity coring. Rocky bottoms will damage the coring apparatus while sandy bottoms are very hard to penetrate. Soft, muddy bottoms are most suitable.

3. Assemble the core liner, core catcher and nose cone in the gravity corer. Lift the corer to the desired height and allow to either free fall or lower to the bottom with resistance on the winch system (depending on desired penetration into the sediment). The more rapid fall velocities allows more penetration; however, more disturbance of the sediment may occur. Make every effort to minimize the disturbance of the surface of the sediment during the coring procedure. This is accomplished by controlling the fall velocity of the corer.

4. Retrieve core from bottom using the winch system, and raise out of the water.

5. Leave the gravity corer in the vertical position while removing the core liner (with sediment) from the gravity corer barrel. Remove the nose cone from the core barrel and place a plastic cap on the bottom of the core liner (tape in place). Remove the upper 1 m section of the core liner by cutting the duct tape and allowing the water to drain. Siphon off the remaining water above the sediment. The sediment will normally be below the junction of the two lengths of core liner. If it is not, then the core liner must be left united. Alternatively, do not cut the 2.6 m core liner (see A.1). Normally, the core catcher is left in the barrel until the core is brought back to the laboratory. If the core catcher needs to be removed in the field, siphon off the water above the sediment, hold a plug at the base of the core to stop the sediment from sliding downward, remove the core catcher and recap the core liner.

6. After recovery of the sediment core (still in the core liner), maintain the core in a vertical position. Cap the top of the 1.6 m section of the core containing the sediment, place the base of the core in a large cooler with ice and cover the entire core with an opaque shielding. Maintain the core in a vertical position until analyzed. Measure the length of the sediment core in order to determine if settling occurs. The sediment core should be remeasured prior to extrusion.

7. Store the sediment core at ~4°C in the dark in a vertical position until sampled in the laboratory. The cores should be extruded and subsampled as soon as possible. Samples for textural analyses can be stored for relatively long periods as long as the sediments do not dry out. However, samples for toxicological analysis should be processed relatively quickly. A maximum holding time of 2 weeks was recommended for sediments for toxicological analyses (Puget Sound Estuary Program 1991).

B. Hand Coring for Marsh Samples

1. If necessary, wash each component of the coring system which will come in contact with any samples and thoroughly rinse with tap water.

2. Assemble the corer handle to a 1 to 3 meter section of core pipe. The lower end of the core pipe should be sharpened with a file to facilitate cutting of root material. Place the corer in a vertical position and force into marsh with a twisting motion and downward force. Be extremely careful not to compress the sediments. This can be determined by comparing the surface of the sediment in the core barrel with the marsh surface. The difference should be less than a few percent (5% used here). If a suitable core cannot be taken with the hand corer, attach a vibra-corer (Lansky et al. 1979, Smith 1987) and proceed with coring using the same restrictions.

3. Retrieve the core by capping the barrel with a pipe expansion plug. Either pull or dig the pipe out of the marsh being careful not to disturb the sediments inside the barrel. After extrusion, fill the core hole and restore to original setting (as close as possible).

4. Be careful while transporting not to allow the core to slump or become disturbed. Store the core as described in A7.

C. Core Extrusion

This sequence assumes that the core has had the water siphoned off and that the core has been chilled to ~4oC. However, the chilling is not mandatory. It serves to maximize the cohesiveness of the sediment for ease in handling.

1. Gently, place the core gently in a horizontal position and remove the caps. Be sure that the surface does not slump.

2. Extrude the core by using a plunger to push the core out of the tube (from the bottom) onto a core tray being careful not to disturb the sediment. The core may also be extruded by tilting the core liner gently and allowing the core to slide out the bottom of the liner onto the core tray. It may be necessary to cut the aluminum core barrels to retrieve the sediment core.

3. The core is now ready for description and subsampling. Be sure to remove the outside of the sediment sample where contact was made with the core liner.

4. Remove the surface layer of the core with a scalpel, sharp knife or piano wire. Subsamples can be removed with the same apparatus. However, if toxicological analyses are to be done(i.e., trace metals, etc.), use a teflon or plastic knife.

D. Core Subsampling

1. Determine desired sampling depths. At each depth remove 2 to 3 cc of sediment for moisture and combustible content as described in the SOP on Sediment Textural Analysis. Subsequently, remove the appropriate size samples for textural, radionuclide, microbiological or toxicological analyses and place in sterile, appropriately cleaned plastic bags. The samples are then frozen, stored in a refrigerator, or dried until analyzed.

2. An alternative method is to carefully section the entire core at 2 cm intervals and archiving (freezing or storing at 5oC) all the sections. This saves the entire core for future analyses.

IV. TROUBLE SHOOTING/HINTS

A. Coring

The main problem that may arise is the substrate is too hard to allow penetration by the gravity corer due to coarse sediments or bedrock. It is not advisable to attempt coring if the substrate is very coarse as the nose cone may be damaged. In the event that a gravity core can not be taken, it may be possible to take suitable cores in shallow depths with a vibra-coring system or by forcing a core barrel directly into the sediment.

B. Extrusion

Care must be taken to be certain that the surface or bottom of the core does not slump during extrusion. This can be accomplished by making a temporary cap for the top or bottom with a stopper with the appropriate diameter. After the core has been extruded and is in the core tray, examine the sediment carefully looking for any indications of disturbance to the core and note them. Things to look for include smearing, bioturbation, slumping, or materials being dragged down the side of the barrel (e.g. eelgrass). This is only a partial list. If the core has been disturbed, it may not be suitable for analysis.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

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Puget Sound Estuary Program. 1991. Recommended Guidelines for Conducting Laboratory Bioassays on Puget Sound Sediments (Interim Final). Prepared for U.S. Environmental Protection Agency, Region 10, Office of Puget sound, Seattle, WA.

Smith, D.G. 1987. A mini-vibra-coring system. Journal Sedimentary Petrology 57:757-794.

POINT OF CONTACT:

Larry G. Ward
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Road
Durham, NH 03824

I. OBJECTIVE

Characterization of estuarine sediments: determination of grain size statistics, moisture content, combustible content (as a measure of particulate organics) and particulate carbon and nitrogen of a sediment sample.

II. NECESSARY MATERIALS AND EQUIPMENT:

- Standard set of sieves, sieve shaker and wet sieves (63μ and 2mm)
- Graduated cylinders (1 L), 20 ml pipette, 50 ml beakers, 1 L beakers, stirring rod
- Dispersant (sodium hexametaphosphate - Calgon)
- 30% Hydrogen peroxide (H_2O_2) or equivalent oxidizer
- Laboratory drying oven
- Muffle furnace
- Balance accurate to 0.0001 g
- Deionized water
- Aluminum weighing dishes
- Squeeze bottles
- Funnel (greater than 20 cm diameter)
- Stirring rod for a 1 L graduated cylinder
- Dessicator
- CN Analyzer

III. METHODS

A. Moisture And Combustible Content

1. Place 2 to 3 cc of sample in a preweighed aluminum dish and weigh to the nearest 0.0001 g to determine the wet weight. Subsequently, dry in an oven at 50-75 °C for 24 hrs or until completely dry. Remove from oven and cool in a desiccator to room temperature. Place sample in weighing room for about 1 hr or until the sample stops gaining weight due to ambient air moisture. Reweigh to determine the moisture content - the % weight loss ((wet weight - dry weight) / wet weight). Next, heat sample in muffle furnace at 450°C for 4 hrs to determine combustible content - the % weight loss after heating [(dry weight - combusted weight) / dry weight] (Ball 1964). Again, after removing the sample from the oven, place in a dessicator until cooled to room temperature, then place in ambient air until weight is stable.

B. Particulate Carbon and Particulate Nitrogen Analysis

1. Place ~1cc of sample in an aluminum weighing dish and dry in an oven at 75° for 24 hr or until dry. Remove and grind in a mortar and pestle until the sample is homogeneous.

2. Carefully weigh (to 0.001mg) approximately 1.5 to 1.75 mg of sediment into a tared tin sample cup (< 1 cm container).

3. Carefully remove the cup and seal it by crushing the top edge together and then crushing with needle nose pliers.

4. The sample is then oxidized by flash combustion and analyzed by chromatography in a Carlo Erba Nitrogen Analyzer or equivalent instrument.

C. Size Analysis: Wet Sieving

1. Place the sample in a large beaker (1 L) and add approximately 20-30 ml of 30% H_2O_2. Add a small amount of H_2O_2 at a time to be sure the sample does not foam over the top of the beaker (~10-15 ml initially, then 10-15 ml after 24 hours, etc.). The amount of sediment sample to use depends on the analyses to be run. Approximately 10 - 15 g dry weight is needed for pipette analysis and 30 - 70 g for sieve analysis. Stir to break up the sample.

2. After as much of the organics as possible have been removed as indicated by lack of bubbling upon adding more H_2O_2 and the remaining liquid has been decanted, remove the sea salts which may be in the sample. This is done by adding deionized water, stirring and decanting. Place the sample in a bottle and fill with dispersant (~0.2 g/L of Calgon in deionized water). Stir vigorously to be certain that the sediment particles are separate. If the sample appears to have gravel particles (greater than 2 mm), wet sieve the sample through a 2 mm stainless steel sieve to remove the gravel. Rinse the gravel with deionized water into a preweighed beaker, dry and set aside (to determine % gravel).

3. Wet sieve the sample through a 63 μ stainless steel sieve. This is done by placing the wet sieve in a plastic funnel of slightly larger diameter which drains into a 1 L graduated cylinder. Place the sample on the wet sieve and gently rinse all the sediment finer than 63μ through the sieve with dispersant in a squeeze bottle. Be careful not to touch the sieve with your hand or anything that will damage the mesh.

4. Wash the material on the wet sieve into a preweighed beaker using deionized water and dry. This is the sand fraction (63μ to 2mm). The material in the graduated cylinder is the mud fraction (silt and clay).

D. Sieve Analysis of the Sand Fraction

1. Weigh the dry and disaggregated sand fraction to the nearest 0.01 g.

2. Select a set of sieves that cover the range of sediment sizes to be analyzed and place the screens in order with the coarsest at the top and the pan on the bottom. Use a maximum of 1/2 phi intervals. Pour the sand sample on the top sieve and place in the sieve shaker for 15 minutes.

3. Carefully remove the sediment from each sieve and weigh to 0.01 g.

4. Total the weight of the sand in each size interval (sieve). If the weight is not within 2% of the original sediment weight, then rerun the analysis.

5. If the analysis is okay, then proceed with determining the size frequency distribution and graphic grain size statistics (mean size, sorting, skewness and kurtosis) as outlined in Folk (1980).

6. Add any sediment that passes through the 63 μ sieve during the shaking processes to the fine fraction to be used for the pipette analysis.

F. Pipette Analysis of the Silt and Clay (Mud) Fraction

1. Add dispersant to the mud fraction until the graduated cylinder has exactly 1 L in it. Thoroughly mix with stirring rod for approximately 2 to 5 minutes being very careful not to splatter any of the sample. Accurately determine the amount of dispersant added to the sample as this has to be subtracted out during the statistical analysis. This can be done by mixing a large quantity of dispersant, removing 20 ml samples with a pipette, drying and determining dispersant weights.

2. Allow the sample to stand overnight to see if any flocculation occurs. If the sample flocculates, the sample preparation has to be redone or additional dispersant added.

3. If the sample is suitable for analysis, restir for 2 to 5 minutes with a stirring rod or with a mechanical stirrer. Be sure to stir the length of the column.

4. The moment the stirring is stopped is time = 0 and the analysis has begun. At the prescribed time intervals and depths outlined in Folk (1980), carefully pipette out 20 ml samples from the graduated cylinder and place in preweighed (to 0.0001 g) 50 ml beakers or equivalent. Take the appropriate samples to determine the amount of sediment in each size interval from 4 to 14 phi as described in Folk (1980). Normally, the 10 - 14 phi interval is determined by computation.

5. Dry the sediment in each beaker 50-75°C for a minimum of 24 hours or until completely dry. After drying, place the beakers initially in a desiccator until the sample is at room temperature, then place in the weighing room until the samples are at equilibrium with ambient humidity and reweigh.

IV. TROUBLE SHOOTING

Included in Methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Determine the size frequency distribution and graphic statistics (mean, sorting, skewness and kurtosis) using the methods outlined in Folk (1980). If both sieve and pipette analyses were done for a sample, then combine the data sets to determine a single set of statistics (Folk 1980).

VI. REFERENCES

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Folk, R. L. 1980. Petrology of Sedimentary Rocks. Hemphill Publishing Company, Austin, Texas. 185 pp.

POINT OF CONTACT:

Larry G. Ward
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Road
Durham, NH 03824

I. OBJECTIVE

Water sample collection from the field and subsequent laboratory determinations of total suspended sediment concentrations and organic content using combustion or loss-on-ignition methodology.

II. NECESSARY MATERIALS AND EQUIPMENT

- Submersible pump
- 1 L plastic storage bottles
- Vacuum pump
- Filter funnel and flask
- Filters (~0.3 μ nominal pore diameter, e.g. Gelman type AE glass fiber filters)
- Balance accurate to ± 0.1 mg
- Deionized water
- Forceps
- Petri dishes
- Drying oven (~50 to 75°C)
- Muffle furnace (450°C)
- Aluminum dishes for combusting filters

III. METHODS

A. Collection of Water Samples

1. Lower submersible pump attached to plastic or rubber (garden) hose or other type of water sampler on a calibrated rope to desired sampling depth.

2. Allow pump to rinse and purge at depth.

3. Rinse the clean plastic water bottles three times in ambient water, vigorously shaking each time. Fill the water bottle with the water sample and store on ice in the dark (or @ 4 - 5°C) until filtered. If filtering will not occur with 24 hrs, freeze the sample. Take as much water sample as needed to accurately determine the TSS concentration.

B. Determination of Total Suspended Sediment (TSS)

1. Prewash glass fiber filters in deionized water to remove any loose fibers and place on filter funnel to vacuum out water. Transfer to an aluminum dish or tray and place in muffle furnace at 450°C for ~1 hour to remove any organic matter. Do not leave filter in oven longer than 1 hour or hotter than 450°C as they may become somewhat brittle. Adjust as necessary.

2. Preweigh each filter to the ± 0.1 mg and place in a petri dish.

3. Place preweighed filter on filtering apparatus using forceps. Vigorously shake water sample (to resuspended all particles) and pour into graduate cylinder (normally 1 L cylinder). Record volume to nearest ml (estimating if necessary). Under low vacuum (less than 5 to 10 inches of mercury) filter the sample. Use as large as possible of water sample to maximize weight of TSS in comparison to filter weight. Limiting factor is filtering time (after filter clogs with sediment).

4. After filtering has been completed, rinse filter three times with deionized water to remove any salts. Remove filter funnel and rinse edge of filter. About 10 ml per rinse is usually adequate.

5. Remove filter while under vacuum (to remove as much water as possible) and place in petri dish. A small aluminum foil tab may be placed under the filter to prevent sticking as the filter dries.

6. Place the filter in an oven at ~50°C until completely dry. Slightly warmer temperature (<75°C) may be used in the oven if necessary.

7. After drying, place the filters in desiccator until cool or until ready to reweigh filter. Remove from desiccator and allow to equilibrate to ambient humidity. It is important to control or at least keep humidity as low as possible (e.g. air conditioning) to minimize weight gain due to moisture. Fluctuation in humidity can be a major source of error. After weights have stabilized, reweigh to nearest ± 0.1 mg.

8. TSS weight in mg/l is dried sediment and filter weight minus filter weight divided by the volume filtered.

C. Estimating Organic Content Via Combustion Method

1. Place the weighed filters with the sediment in an aluminum dish and place in a preheated muffle furnace (~450°C) for one hour. Remove filter and place in desiccator until cool.

2. Remove filter from desiccator and allow it to equilibrate with ambient humidity. Reweigh combusted filter. Again, control humidity either by keeping it constant or minimized through all steps of these procedures.

3. The % combustible or loss-on-ignition content is the dry weight of the filter with sediment minus the combusted weight divided by the dry weight. The % combustible or loss-on-ignition is an approximation of the organic content. The remaining materials on the filter is the ashed weight and approximates the inorganic content.

III. TROUBLE SHOOTING

The major sources of error found in these procedures result from the following: 1) problems with ambient humidity, and 2) low weights of suspended material in comparison to the weights of the filters. To minimize these analytical errors, do the following:

1. Control humidity by air conditioning, etc. Try to keep constant through all weighings.

2. Maximize the volume of sample being processed to maximize the weight of the TSS.

3. Run numerous controls (e.g. every 10 filters) to determine analytical errors due to handling and weighings. Include rinsing some of the filters as in step A4.

IV. STATISTICS

Not applicable.

V. REFERENCES

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Strickland, J.D.H., and J.R. Parsons. 1968. A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada Bulletin 167, Ottawa. 311 pp.

POINT OF CONTACT:

David M. Burdick or Frederick T. Short
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Road
Durham, NH 03824

I. OBJECTIVE

To locate and collect ground water flowing from uplands to marine waters through the intertidal zone (intertidal seeps) for chemical analyses.

II.NECESSARY MATERIALS AND EQUIPMENT

- Pore water sippers with teflon body and tubing (after Short et al. 1985)
- Temperature corrected optical refractometer
- Acid-washed bottles, 125 mls, labeled
- Hand powered vacuum pump
- Teflon trowel
- Flags with PVC stakes
- Boots, gloves and other protectiver clothing, as needed
- Field book for recording data

III. METHODS

A. Location of sampling locations

1. Examine the shoreline of the area to be studied at low tide during the time of spring tides. Mark all visible flows of water or areas with evidence of water flow with the flags and mark the location of the areas on a map. Evidence includes hearing water flow under rocks and channels in the intertidal sediments made by water.

2. Measure and record the salinity of the waters at each site. Dig a pool with the trowel to collect the water if the flow is largely subsurface.

3. Since sampling ground water is the primary objective, choose sampling locations based on the maximum number of samples that can be analyzed, the salinity of the water, the relative flow rate, the location of the seep, and any other factors specific to the area or to the goals of the study.

B. Collection of Water Samples

1. Insert an acid-washed teflon sipper into the seep area. Connect the hand pump to the sipper with a sample bottle (one that will serve as a rinse bottle) in between. Draw a vacuum within the rinse bottle and sipper using the hand pump until water begins to flow into the rinse bottle. After about 30 mls are collected, break the vacuum, remove the rinse bottle and discard its contents.

2. Attach a clean, labeled sample bottle to the pump and sipper and draw a vacuum with the pump until the sample bottle has about 80 mls in it. Repeat with a second bottle if replicate samples are desired.

3. At each new sampling location, insert a clean sipper and repeat steps #1 and 2 until all sites are sampled.

III. TROUBLE SHOOTING

1. If insertion of the sipper is difficult, use the trowel to make a hole for the sipper.

2. If a vacuum cannot be drawn, check all sipper fittings for tightness and be sure the entire frit area is submerged.

3. If only a little water is drawn and the flow is too slow or stops (even though there is ample vacuum), the sediments may be too fine grained, thus clogging the frit. In this case, dig a hole with the trowel to below water level, let the fine sediments settle, and replace the sipper so that the frit is mainly in the water column above the sediment. The sipper may have to be backflushed with the seep water to clear the frit, or replaced.

IV. STATISTICS

Since this procedure is performed to identify seeps and assess their potential for carrying contaminants from uplands to marine waters, no advanced statistical procedures are envisioned. Means of replicates could be calculated as well as the means for entire shorelines, or portions thereof, if appropriate.

V. REFERENCES

Short, F. T. M. W. Davis, R. A. Gibson and C. F. Zimmerman. 1985. Evidence for phosphorus limitation in carbonate sediments of the seagrass *Syringodium filiforme*. Estuarine, Coastal and Shelf Science 20:419-430.

AUTHORS:

Diane Nacci
Stephanie Cayula
Jonathan Serbst
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

Hal Walker
US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

This method was devised to measure the general and reproductive health of the sea urchin, *Arbacia punctulata*. This SOP describes procedures to measure survival, growth and fecundity of adult animals (test diameter, wet weight, gamete count) following exposures to potentially toxic materials via food, media (water or suspended or bedded sediment) or direct injection. This SOP incorporates adaptations of toxicity test procedures (*i.e.*, sperm and larval tests) to measure the capacity of gametes succeed to fertilize and develop to swimming and feeding lifestages in uncontaminated or contaminated media. Procedures are included to store tissues for future chemical, biochemical (*e.g.*, stress protein content) and genotoxicity (*i.e.*, single cell gel) analyses.

II. NECESSARY MATERIALS AND EQUIPMENT

- Facilities for holding and exposing test organisms.
- Calipers for measuring test diameter.
- Environmental chamber or equivalent facility with temperature control ($20 \pm 1^{o}C$) for controlling temperature during exposure.
- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.
- Balance -- Analytical, capable of accurately weighing to 0.0001 g.
- Reference weights, Class S -- for checking performance of balance.
- Air pump -- for supplying air.
- Air lines, and air stones -- for aerating water containing adults.
- Vacuum suction device -- for washing eggs.
- pH and DO meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of these two parameters, portable, field-grade instruments are acceptable.
- Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.
- Fume hood -- to protect the analyst from formaldehyde fumes.
- Dissecting microscope -- for counting diluted egg stock.
- Compound microscope -- for examining and counting sperm cells and fertilized eggs.
- Sedgwick-Rafter counting chamber -- for counting egg stock.
- Hemacytometer, Neubauer -- for counting sperm.
- Count register, 2-place -- for recording sperm and egg counts.
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Ice bucket, covered -- for maintaining live sperm.
- Centrifuge tubes, conical, 15 mL -- for washing eggs.
- Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension.
- Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL

for making test solutions.
- Glass dishes, flat bottomed, 20-cm diameter -- to hold adults during gamete collection.
- Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.
- Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) for collecting sperm and eggs.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipets, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET^R, or equivalent.
- Tape, colored -- for labelling tubes.
- Markers, water-proof -- for marking containers, etc.
- Sea Urchins (approximately 4 of each sex).
- Scintillation vials, 20 mL, disposable -- to prepare test concentrations.
- Parafilm -- to cover tubes and vessels containing test materials.
- Gloves, lab coat, disposable -- for personal protection from contamination.
- Safety glasses
- KCl solution, 0.5 M.
- Acetic acid (HAC), 5%, reagent grade, in sea water -- for preparing killed sperm dilutions.
- Formalin, 10% in seawater -- for preserving eggs.
- Formalin, 10% in seawater with Rose Bengal -- for preserving and staining larvae.
- pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check.
- Membranes and filling solutions for dissolved oxygen probe or reagents for modified Winkler analysis.
- Low melting agar (LMA) dissolved in TRIS-acetate buffer, heating block (30 C), microscope slides coated with LMA, coplin jars filled with lysis buffer: for details refer to ERLN SOP for Conducting Single Cell Gel Electrophoresis Assay.
- Laboratory quality assurance samples and standards for the above methods.
- Reagent water -- defined as distilled or deionized water that does not contain

substances which are toxic to the test organisms.
- Saline test and dilution water -- The salinity of the test water must be 30 ^{o}/oo. The salinity should vary by no more than $\pm$ 2 ^{o}/oo among the replicates.

III. METHODS

A. URCHIN COLLECTION AND MAINTENANCE CONDITIONS

1. Adults are purchased from biological supply houses or collected by divers. Species should be verified by biologists. Adults are transported from collection sites moist (but not submerged) with sea water at moderate ambient temperatures. Upon receipt, animals should be vigorous (spines waving), without obvious signs of stress (*e.g.*, gamete shedding). Animals should be transferred to and maintained in flowing or recirculating sea water aquaria aerated vigorously and held at 15 C, and fed kelp (*Laminaria sp*), *ad libitum*. Healthy animals will attach to tank sides or kelp immediately. Unhealthy animals should be discarded; spawning animals should be segregated.

2. Animals are sexed using electrical stimulation and held separately. Temperature and animal conditions (survival) should be monitored daily. Obviously sick or dead animals and decaying food matter should be removed daily. Tanks should be cleaned and kelp replaced weekly. Before exposures begin, animals of each sex are sized (test diameter) using calipers and distributed randomly into treatments.

B. COLLECTION AND MEASUREMENT OF GAMETES TO ASSESS REPRODUCTIVE CONDITION: FEMALE

1. To characterize a single replicate treatment following exposure, remove all urchins from test tank. Select four urchins female urchins and process concurrently for toxicity testing. Each of these animals is measured (test diameter) and weighed, after blotting with paper towel. A similar process will be repeated with male animals, after measurement and egg collection is completed. For remaining urchins, only qualititative data on fecundity will be taken after the test procedure is set-up. Data is recorded on appropriate data sheets.

2. Place each of the four selected urchins oral side up on an appropriately-sized container, *e.g.*, 100 ml beaker, specimen cup or weigh boat. Containers are labelled 1 - 4, to maintain identity of egg stocks. Simlarly labelled conical centrifuge tubes will be used to measure total egg volumes for each female.

3. Fill a 1 ml syringe with 0.5 M KCl and inject each sea urchin with 0.25 ml through several injections around the peristomal membrane (oral side). Note time of injection. Spawning is sometimes induced by holding the injected urchin in the palm of the hand and swirling it gently with a circular, horizontal motion for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl. Following injection, invert over container with oral side up (anal/gonadopore side down).

4. Fifteen minutes later, inject with a second volume of 0.5 ml. If animals do not produce sufficient gametes following injection of 1.0 ml KCl, they should not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins. Collect gametes from each individual 5 minutes after second injection. Use about 10 ml sea water to wash urchin test and rinse gametes into 15-ml centrifuge tube.

5. After gametes are collected, dissect urchin and confirm that spawning was complete. Remove all soft body tissues from each female (from a single treatment) and transfer to a single scintillation vial. Label and store on ice until frozen.

6. Decant eggs into a labelled conical centrifuge tube and allow to settle (about 5 minutes). Decant sea water from tube and replace with 10 ml clean sea water.

7. Cap the individual egg suspension tubes, mix by several inversions and divide egg stock for testing and storage.

a. A small sample of the eggs from each female should be examined for the presence of significant quantities of irregularly shaped or vacuolated eggs. Results should be noted on the appropriate data sheet.

b. Transfer 1 ml egg suspension from each female to a single tube (labelled as pooled sample), to be washed, counted and used in test procedures.

c. Transfer 1 ml egg suspension from each female to a scint labelled by female number (1 - 4) and letter B, to be used to count total number of eggs for each female.

d. Decant remainder of egg suspension (8ml) into a 50 ml conical vial for storage: decant excess sea water and set aside for freezer storage (archive for chemical and biochemical analyses).

8. Count pooled egg sample for use in testing as described in section C. Count individual egg samples (using procedure described in section C.6) after tests have been set up. Results should be noted on the appropriate data sheet.

9. For remaining surviving urchins (not used in testing), inject with KCl following procedures described previously. Observe microscopically, a small subsample of gametes shed, if any. Note on appropriate data sheet, number of surviving urchins, number producing gametes, and whether thsoe gametes were grossly normal or abnormal.

C. COUNTING AND PREPARING GAMETES: EGGS

1. Wash the pooled egg sample three times using control water with gentle centrifugation (500xg or lowest possible setting) for 3 min using a tabletop centrifuge. If the wash water becomes red, the eggs have lysed and may not fertilize acceptably. Comments on egg quality should be added to data sheets.

2. Count the eggs:
   a. Remove the final wash water and transfer (by filling the centrifuge tube with control water and repeatedly inverting to resuspend the eggs) the washed eggs to a beaker containing a small volume (about 50 mL) of control water. Add more control water to bring the eggs to a volume of 200 mL ("egg stock").
   b. Mix the egg stock using gentle aeration. Cut the point from a pipet tip, then transfer 1 mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted 1:10 from egg stock).
   c. Mix the contents of the vial using inversion. Transfer 1 mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope ("egg count").
   d. Calculate the concentration of eggs in the stock. Eggs/mL = 10x (egg count).

3. Dilute the egg stock to 2000 eggs mL by the formula below.
   a. If the egg count is equal to or greater than 200:

      (egg count) - 200 = volume (mL) of control water to add to egg stock

   b. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as above.

4. The test procedure can be continued at this point. The egg stock may be held at room temperature for several hours before use in testing.

5. After the egg test stock has been set up, heat 1 tube of low melting agar (LMA) and allow to cool to 30 C in heater block. Remove 2, 12 ml samples (about 50,000 eggs) from egg stock and transfer into 2, 15 ml conical centrifuge tubes. Centrifuge gently

(lowest setting) for 2 minutes and decant overlying sea water. Transfer egg pellets to microfuge tube and add 250 ul melted LMA to tube. Resuspend eggs by pipetting. Transfer 50 ul aliquots to four microscope slides, coated with LMA. Cover with coverslip and allow gel to set. Remove cover slip and transfer slides to coplin jar containing cold lysis buffer. Enter slide data into appropriate data sheet.

6. Count and calculate egg number per female at a convenient time.
   a. Prepare for each female 2 vials containing 9 ml sw, labelled with female number and the letters C and D.
   b. Add 9 ml sw to the 1 ml sample of egg suspension saved for each female ("B"). Mix suspension by inverting capped vial and transfer 1 ml egg suspension from "B" to "C". Mix suspension by inverting capped vial and transfer 1 ml egg suspension from "C" to "D".
   c. Mix "C" by inversion and transfer 1 ml sample to a Sedgwick-Rafter counting chamber. Observe under dissecting microscope, if too many eggs too count accurately (>500), then discard sample and count a subsample from "D". Record total egg number, which dilution counted and any comments on egg quality on appropriate data sheet.

D. COLLECTION AND MEASUREMENT OF GAMETES TO ASSESS REPRODUCTIVE CONDITION: MALE

NOTE: Handle sexes separately; this minimizes the chance of accidental egg fertilization. Throughout the test process, it is best if a different worker, different pipets, etc, are used for males (semen) and females (eggs). Frequent washing of hands is always a good practice.

1. Select four male urchins from a single treatment condition. Each animal is measured (test diameter) and weighed, after blotting with paper towel. Data are recorded on appropriate data sheets.

2. Place urchin, oral side up on an appropriately-sized container, *e.g.*, 100 ml specimen cup, labelled 1-4. Fill a 3 - 5 ml syringe with 0.5 M KCl and inject each sea urchin with 0.5 ml through peristomal membrane. Note time of injection. Spawning is sometimes induced by holding the injected urchin in the palm of the hand and swirling it gently with a circular, horizontal motion for several seconds. This may provide an additional physical stimulus, or may aid in distributing the injected KCl. Inject with a second volume of 0.5 ml injection after 15 minutes.

4. If animals do not produce sufficient gametes following injection of 1.0 ml KCl, they should probably not be reinjected as this seldom results in acquisition of good quality gametes and may result in mortality of adult urchins. Collect gametes from each individual after each injection, using a total volume of 10 ml sea water for each male. Store tubes on ice.

5. After spawning ceases, dissect urchin and confirm that spawning was complete. Transfer all soft body tissues from males from a single treatment to a single scintillation vial. Label and store on ice until frozen.

6. Transfer portions of collected sperm for toxicity testing, counting or storage:
   a. Transfer 1 ml sperm suspension from each male to a scint vial labelled by individual number and "B": count individual sperm samples after tests have been set up.
   b. Microsopically observe 1 drop of live sperm suspension from each male under high power. Note whether sperm are moving.

c. If no motile sperm are observed, do not use sample from this individual for pooled sample. If motile sperm are observed, then procede to transfer individual sample to pooled sample. Transfer 1 ml sperm suspension form each male (with motile sperm) to vial labelled pooled sample.

d. Decant remainder of sperm suspension (8ml) into a scintillation vial for storage and set aside for freezer storage (archive for chemical and biochemical analyses).

6. Count pooled sperm sample for use in testing as described in section E. Count individual sperm samples (using procedure described in section E.3) after tests have been set up. Results should be noted on the appropriate data sheet.

7. For remaining surviving urchins (not used in testing), inject with KCl following procedures described previously. Observe microscopically, a small subsample of gametes shed, if any. Note on appropriate data sheet, number of surviving urchins, number producing gametes, and whether thsoe gametes were grossly normal or abnormal.

E. COUNTING AND PREPARING GAMETES: SPERM

1. Using control water, make a sample of sperm test stock at a concentration of about $5 * 10^7$ sperm/mL. Estimate the sperm concentration as described below.
   a. Mix by inversion pooled stock of sperm collected in sea water ("A-pooled"). The maximum volume should equal 4 ml (1 per male). Transfer 1 ml to a vial labelled "B-pooled", containing 9 ml sea water.
   b. Mix "B" by inversion and transfer 1 ml to vial labelled "C-pooled". Mix "C" by inversion and transfer 1 ml to vial labelled "D-pooled".
   c. Transfer 10 ul samples from "D" to each of two sides of a hemacytometer. Let sample settle for 15 minutes before counting.
   d. Using a compound microscope (250-400x), count the number of sperm in the central 400 squares on each side of the hemacytometer and average (X). If the average of both sides is less than 50, clean hemacytometer, sample from solution "C" and repeat counting process. If the average is greater than 500, make a solution "E" by transferring 1 ml from "D" into 9 ml sea water, sample from "E" and repeat the counting process.

2. Use the count average to calculate the concentration of sperm in the sampled solution, the concentration of sperm in stock "A", and the dilution of "A" required to make an appropriate sperm test test.

   For counts made on "D":
   Sperm concentration in "D" = $X * 10^4$ sperm/ml
   Sperm concentration in "A" = "D" $* 10^3 = X*10^7$ sperm/ml
   Sperm number necessary for sperm stock
   $= 5 * 10^8$ sperm/ml * 10 ml
   $= 5 * 10^9$ sperm
   Volume of "A" containing $5 * 10^9$ sperm
   $= (5 * 10^8 \text{ sperm})/(X * 10^7 \text{ sperm/ml})$
   $= 50/X$ ml
   Volume of "A" to transfer into sperm stock = 50/X ml

For counts made on "C":
Sperm concentration in "C"= $X*10^4$ sperm/ml
Sperm concentration in "A"= $X*10^6$ sperm/ml
Volume of "A" to transfer into sperm stock = 500/X ml

For counts made on "E":
Sperm concentration in "E"= $X*10^4$ sperm/ml
Sperm concentration in "A"= $X*10^8$ sperm/ml
Volume of "A" to transfer into sperm stock = 5/X ml

To make sperm test stock (10 ml total volume):
Volume of sea water to add= 10 - (volume of "A" transferred into stock)

3. Maintain sperm test stock on ice until test begins.

4. After the sperm test stock has been set up, heat 1 tube of low melting agar (LMA) and allow to cool to 30 C in heater block. Add 250 ul melted LMA to a microfuge tube. Make a 1:10 dilution of sperm test stock: transfer 100 ul sperm test stock into a vial containing 0.9 ml sea water. Transfer 10 ul diluted sperm stock (about 2.5 x 10^4 sperm) into microfuge tube and resuspend sperm by pipetting. Transfer 50 ul aliquots to four microscope slides, coated with LMA. Cover with coverslip and allow gel to set. Remove cover slip and transfer slides to coplin jar containing cold lysis buffer. Enter slide data into appropriate data sheet.

5. Count and calculate sperm number per male at a convenient time.
   a. Prepare for each male 2 vials containing 8 ml sw and 1 ml HAC, labelled with male id number and the letters C and D.
   b. Add 9 ml sw to the 1 ml sample of sperm suspension saved for each male ("B"). Mix suspension by inverting capped vial and transfer 1 ml sperm suspension from "B" to "C". Mix suspension by inverting capped vial and transfer 1 ml sperm suspension from "C" to "D".
   c. Mix "C" by inversion and transfer subsample to a hemacytometer. Mix "D" by inversion and transfer subsample to a hemacytometer. Allow sperm to settle (10 minutes) and observe under coumpound microscope. If too many sperm to count accurately (>500), then discard sample and count the subsample from "D".

Record total sperm number and which dilution counted on appropriate data sheet.

F. TOXICITY TESTING: SPERM AND LARVAL DEVELOPMENT ASSAYS

1. Six replicates are prepared for each test concentration: 3 replicates will be used for the sperm test and 3 replicates for the larval test. For the sperm test, use 5 mL of solution in disposable liquid scintillation vials. For the larval test, use 18 mL of solution in disposable scint vials. A total volume of 69 mls is required per treatment.

2. Reproductive success will be assessed routinely by testing gametes using clean sw and treatment exposure water ("tank water"). Particle-free tank water will be prepared for testing by centrifuging samples for 10 minutes at 10,000 rpm.

3. All test samples should be equilibrated to 20 C before sperm addition.

4. Two hours after sperm collection and immediately after the final dilution is made, add 100 uL of appropriately diluted sperm to each test vial. Record the time of sperm addition.

5. Incubate all test vials at 20 $\pm$ 1°C for 1 h.

6. Mix the diluted egg suspension (2000 eggs/mL), using gentle bubbling. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate at 20 $\pm$ 1°C.

7. Twenty minutes following egg addition, terminate sperm test and measure fertilization success.

Ba. At 20 minutes after egg addition, terminate the vials for the sperm test. Terminate the test and preserve the samples by adding 1 mL of 10% formalin in seawater to each vial. Then add 13 mL sea water to each vial so that total volume is 20 mL (5 ml solution + 1 ml eggs + 1 ml formalin + 13 ml sw).

b. Vials may be evaluated immediately or capped and stored for as long as two days before being evaluated. As holding time amy affect egg appearance, all test vials should be read at one sitting.

c. To determine fertilization, mix vials by inversion and transfer a 1 mL sample of eggs from a test vial to a Sedgwick-Rafter counting chamber. Observe the eggs using a compound microscope (100 X). Count all eggs in the sample. Record the number counted and the number unfertilized. Fertilization is indicated by the presence of a fertilization membrane surrounding the egg. Adjustment of the microscope to obtain proper contrast may be required to observe the fertilization membrane.

8. At 48 hours after egg addition, terminate the vials for the larval test and measure larval survival and development.

a. Terminate the test and preserve the samples by adding 1 mL of 10% formalin in seawater containing Rose Bengal to each vial. The total volume of each vial should total 20 ml (18 ml solution + 1 ml eggs + 1 ml formalin).

b. Count and record the number of normally developed and total number of larvae in 2, 1 mL samples of each vial. Mix vial by inversion and transfer 1 mL eggs from the test vial to a Sedgwick-Rafter counting chamber. Observe the larvae using a dissecting microscope (100 X). If time permits, larval size may determined using image analysis.

NOTE: Because samples are fixed in formalin, a ventilation hood is set-up surrounding the microscope to protect the analyst from prolonged exposure to formaldehyde fumes.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data. Calculate the percent of unfertilized eggs for each replicate.

2. The endpoints of toxicity tests using the sea urchin are based on the reduction in percent of eggs fertilized. Dunnett's Procedure (Dunnett, 1955) is used to estimate whether treatments differ from controls.

3. Use spreadsheet format (see sample accompanying) to calculate and summarize numbers of gametes per treatment group.

VI. REFERENCES

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POINT OF CONTACT:

Diane Nacci
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

Eugene Jackim
US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

Detection of DNA strand breaks is based on an established quantitative relationship between an increased rate of DNA strand separation under alkaline conditions, referred to as DNA alkaline unwinding, and increased DNA strand breaks (Ahnström & Erixon, 1973; Rydberg, 1975).

In the Single Cell Gel (SCG) electrophoresis assay, relative numbers of DNA strand breaks are estimated in individual cells (Singh et al 1988). This is accomplished by embedding crude tissue homogenates or cell suspensions in agar, dissolving cell membranes and electrophoresing the preparations under alkaline conditions. DNA unwinding and migration during alkaline electrophoresis, visualized using the fluorescent dye, ethidium bromide, is demonstrated as nuclear "tail" or "comet". The length of this tail may be quantified and correlated with the relative number of DNA strand breaks.

II. NECESSARY MATERIALS AND EQUIPMENT

- Deionized water (diH20)
- Ice bucket and ice or cold packs
- Dissecting equipment
- Glass homogenizers (3 ml capacity) with glass pestles, disposable
- Table top microfuge
- Microfuge tubes, 1.5 and 0.5 ml, disposable
- Refrigerator (4° C)
- Water bath or heater block, 30-35°C
- Balance -- Analytical, capable of accurately weighing to

0.0001 g
- Weighing boats or glascene weighing papers
- Reference weights, Class S -- for checking performance of balance
- pH meter
- Syringes, 1 ml, with small gauge needles, disposable (e.g., 26 ga)
- Borosilicate tubes, disposable, 5 ml
- Glass screw-cap vials, disposable, 20 ml (e.g., liquid scintillation vials)
- Filter sterilizing apparatus, disposable, tissue culture grade, 500 ml
- Thermometers, glass or electronic, laboratory grade
- Wash bottles -- for deionized water, for rinsing small glassware and instrument, electrodes and probes
- Volumetric flasks, graduated cylinders and bottles -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making and storing test solutions
- Pipets, automatic -- adjustable, 10-1000uL, and appropriate tips
- Pipets, serological -- 1-10 mL, graduated
- Pipets, pasteur, glass disosable
- Pipet bulbs and fillers -- PROPIPET^R, or equivalent
- Tape, colored -- for labelling tubes and bottles
- Markers, water-proof -- for marking containers, etc.
- Gloves, lab coat, disposable -- for personal protection and to avoid contamination
- Safety glasses
- Parafilm^R
- Fully Frosted Microscope Slides
- No.1 Coverslips
- Glass coplin-type jars with screw caps
- Teflon-coated forceps for handling slides
- Horizontal Electrophoresis System (Submarine Type)
- Electrophoresis Power Supply
- Staining Tray

Chemicals

- NaCl, reagent grade
- TRIZMA HCl (Tris[hydrodroxymethyl]aminomethane), reagent grade
- TRIZMA acetate (Tris[hydrodroxymethyl]aminomethane acetate), reagent grade
- EDTA, reagent grade
- Sodium hydroxide (NaOH), reagent grade
- Hydrochloric acid(HCl), reagent grade
- Sodium monophosphate (NaH_2PO_4), reagent grade

- Sodium diphosphate (Na_2HPO_4, reagent grade
- Potassium monophophate (KH_2PO_4), reagent grade
- Potassium Chloride (KCl), reagent grade
- Glucose, biological grade
- HEPES (N-[2-Hydroxymethyl]piperazine-N'-[2-ethanesulfonic acid], sodium salt), reagent grade
- Triton X-100
- Dimethyl sulfoxide (DMSO), reagent grade
- Ethidium Bromide (EB)
- Ethidium Homodimer (EH, supplied by Molecular Probes, Inc, Eugene, OR)
- Sodium Lauryl Sarcosinate (SLS)
- Low Melting Point Agarose (LMA), Molecular biology grade
- Normal Melting Agarose (NMA), Molecular biology grade

III. METHODS

Solutions

HEPES Solution, 1.0 M
Weigh out 47.7 g HEPES (m.w. 238.3). Add 150 ml diH_2O, adjust pH to 7.4 if necessary. Q.s. 200 ml.

Hank's (HBSS) or Earle's (EBSS) Buffered Saline Solution

Chemical	HBSS,g	EBSS,g
NaCl	0.800	0.990
KCl	0.040	0.040
KH_2PO_4	0.006	0.006
Na_2HPO_4	0.009	0.009
$NaHCO_3$	0.085	0.220
Glucose	0.100	0.100
HEPES solution	2.5 ml	2.5 ml

Q.s. to 100 ml with diH20. Filter sterilize.

Kenny's Salt Solution

Chemical	g/L
NaCl	23.5
KCl	0.7
K_2HPO_4	0.1
$NaHCO_3$	0.2

Weigh out chemicals. Dissolve with 900 ml diH_2O. Adjust pH to 7.5. Q.s. 1L. Filter sterilize and store refrigerated.

Agarose Solutions: 0.5% in TRIS-Acetate EDTA (TAE)
Stock solutions
0.2 M Tris acetate(TA) solution, pH 7.5
0.2 M EDTA

Add 200 ml TA solution and 5 ml EDTA solution to about 900 ml diH20. Adjust pH to 7.5, if necessary. Q.s. to 1L (final concentrations 40 mM TA and 1 mM EDTA). Filter sterilize and store refrigerated.

Agarose solutions (LMA and NMA) are made with 0.5 g agarose per 100 ml TAE (final concentration, 0.5% agarose). Boil solutions to dissolve. Dispense about 5 ml per liquid scintillation vial. Cool and cap. Store at room temperature.

Lysing Solution:

Chemical	wt, g	Final Concentration, M
NaCl	146.4	2.5
EDTA	37.2	0.1
TRIZMA-HCl	1.2	0.01

Combine chemicals in 500 ml ddH20. Set pH to 10 with 12 gm solid NaOH. Add 10 g sodium sarcosinate (final concentration, 1% w/v). Bring volume to 890 ml with ddH_2O. Filter sterilize and store at room temperature.

For final lysis buffer made on day on use. To 44.5 ml lysis solution, add 0.5 ml Triton X-100 (final concentration, 1% v/v) and 5 ml DMSO (final concentration, 10% v/v).

Electrophoresis Buffer :
Stock Solutions

NaOH Solution, 10 N
Add 40 g NaOH to 100 ml ddH20. Mix carefully. Store at room temperature. Replace every 10 days.

EDTA Solution, 0.2 M
Add 37.2 g to 500 ml ddH20. Filter sterilize and store refrigerated.

For 1X Buffer (made fresh before each run) : mix 30 ml NaOH Solution (final concentration, 300 mM) and 5.0 ml EDTA Solution (final concentraton, 0.001 M), q.s. to 1L, mix well.

Neutralization Buffer : 0.4 M Tris Solution
Add 63.04 g TRIZMA-HCl to about 900 ml diH20. Adjust pH to 7.5 using 10N NaOH. Q.s. to 1L. Filter sterilize and store at room temperature.

Ethidium Bromide Staining Solution :
Use extreme caution while handling this potent mutagen. All weighing should be done in a hood while personnel are protected by appropriate gear (gloves, coat). All contaminated materials should be disposed of as carcinogenic waste.
Add about 2-4 mg EB to pre-tared liquid scintillation vial. Calculate volume required for final concentration 1 mg/5 ml (20 ug/ml). Add this amount of diH20. Wrap vial in foil. For EB working solution, mix 1 ml with 9 ml diH_2O (2 ug/ml).
Store light protected at room temperature.

Ethidium Homodimer Staining Solution :
Add 2 ml of sterile EBSS (without calcium or magnesium) to a vial containing 25ul of 12 mM EH in DMSO (as available from supplier). Vortex to ensure thorough mixing (stock concentration is 150 uM).
Transfer 250 ul EH stock to 10 ml PBS to make EH working solution (concentration is 4 uM).
Store solutions frozen, protected from light in a sealed plastic bag containing dessicant. Do not open bag until contents have reached room temperature.

Positive Control Solution: Hydrogen Peroxide
Immediately before exposure, make a 1:1000 dilution of 30% H2O2 by adding 10 ul to 10 ml ddH20. For a final concentration of 200 mM H2O2, add 23 ul of this 1:1000 dilution per ml exposure media.

Single Cell Gel Electrophoresis : Slide Preparation

Preparation

Base-coating slides: Melt one or more vials of NMA in microwave. Add 100 ul melted NMA to each pre-numbered slide and immediately add a coverslip (being careful to minimize bubbles). Place the slides on a tray over ice for 3 minutes to solidify. Slides may be base-coated in advance amd stored for several days in moist slide boxes.

Preparing LMA: Melt one or more scintillation vials of LMA in microwave. Dispense into small glass vials or

plastic centrifuge tubes (about 2 ml volume, to fit heater block holes) and allow to come to 30 C in heater block before use.

Tissue Preparation

NOTE: Procedures that include live tissues should be performed under dim yellow lights to prevent additional DNA damage.

Exposures may occur prior to sampling. Positive controls may be generated in vitro by exposing cells to 0.2 M H_2O_2 for 10-30 minutes on ice.

Whole Blood : Withdraw blood using syringe rinsed in 0.2 M EDTA. Immediately, mix 5 ul whole blood with 50 ul LMA, layer onto base-coated slide.

Tissue Isolation: Remove a small piece of the organ, place in 1 ml of cold buffer (EBSS, HBSS, Kenny's or PBS containing 0.02 M EDTA) on ice. Mince into fine pieces, let settle, remove 5 ul cell suspension, add to 50 ul LMA, layer onto base-coated slide. For easily disaggregated tissues (eg. spleen and testes) add two to three ml more buffer and remove 5 ul for slides.

Culture Isolation : Monolayer cultures - scrape off cells into the culture media in the cell dish using a teflon scrapper (do not trypsinize cells!) to yield approximately 1 x 10^6 cells/ml. Add 5 ul cell suspension to 50 ul LMA, layer onto base-coated slide.

General Slide Making

Gently slide off the coverslip and add 50 ul of warm LMA (30 C) mixed with approximately 10,000 cells in 5 ul volume or less. Replace coverslip and return slide to the tray on ice for another 5 minutes.

Gently slide off coverslip and add a third layer of 50 ul LMA to the slide. Replace coverslip and return to the tray on ice for 5 minutes.

Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution. Protect from light and place in 4°C refrigeration for a minimum of 1 hour. Slides should be stored for at least one hour before proceeding but may be stored for extended periods of time

(weeks to months) in cold Lysing Solution.

Single Cell Gel Electrophoresis

NOTE: Perform electrophoresis under dimmed yellow light.

After at least 1 hour at 4 C, gently remove slides from the Lysing solution. Place slides on the horizontal gel box near the anode (+) end, sliding them as close together as possible.

Fill the buffer reservoir with freshly made electrophoresis buffer until liquid level completely covers the slides. Let slides sit in buffer for 15-60 minutes (with power OFF) to allow unwinding of the DNA.

Turn on the power supply to 25 volts and adjust current to 300 mAmps by slowly raising or lowering the buffer level. Allow slides to run for 5-40 minutes.

Turn off the power. Gently lift the slides from the buffer and place in the staining tray. Using a pasteur pipet, drip neutralization buffer over slides to coat. Let sit for 5 minutes. Repeat twice.

Drain slides, add 50 ul of stain (EB or EH) working solution and cover with coverslip. Coat edges of cover slip with nail polish, store slides in moist chamber. View immediately or refrigerate to store.

Observation

Within 3 days after making slides, observe microscopically. Remove slides from refrigerator. Blot away condesation before use. For slides stained with EB, observe using fluorometric scope (green light, 365nm excitation - 420nm emission) at 400x set up with a micrometer. For slides stained with EH, observe using standard flourescein filter set (blue light, EH excitation at 485-500 nm and emission at 645 nm). Focus on individual cells, estimate tail lengths. Record lengths for 25 cells per slide.

E. Acceptability of Test Results

Criteria for acceptability have not yet been established.

F. Hazards and safe usage

Ethidium bromide is a DNA-interactive dye and should be considered mutagenic. Users should take protective measures and discard solutions appropriately.

IV. TROUBLE SHOOTING

Substances which interfere positively or negatively with fluorescence may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.

V. STATISTICAL ANALYSIS AND DATA USAGE

Statistical differences between groups are determined by analyzing data using ANOVA followed by Dunnet's test (SAS).

VI. REFERENCES

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Single Cell Gel Procedure

Oyster Hemolymph Cells

Cell Density: Effect on tail length of exposed and non-exposed cells

1. Base coat 25 slides using 100ul/slide regular agar base (0.5%). Label slides 105-1--25. Make lysis buffer (150 ml), store on ice.

2. Sample oyster hemolymph from 6-8 notched (non-exposed) oysters:

   Rinse syringes with 200 mM EDTA (optional). Withdraw about 0.2-0.3 ml from each oyster (to obtain final volume of about 1600 ul, total). Pool hemolypmh from all oysters in microfuge tube. Sample and perform cell count using hemocytometer. Divide hemolymph into two microfuge tubes containing 750ul fluid each.

3. Centrifuge cell suspensions at 2000rpm x 10 seconds. Reconstitute with Kenny's salt solution containing EDTA to 1 ml. Add 23ul DI to tube-1 (CTL), 23ul H2O2 solution (see SOP) to tube-2 (EXP). Mix by inversion and allow to stand at RT for 15 minutes.

4. Mix cell suspensions using pipettor. Distribute cell suspensions from each exposure group into 3 tubes as follows: 400ul(HI), 200ul(MED) and 100ul(LO). Centrifuge cell suspensions (6 tubes) at 2000rpm x 10 seconds. Discard supernatant.

5. Add 200ul melted LMA to each tube. Make 4 slides from each cell suspension. Continue slide making.

   Make slides using 50ul agar-cell suspension. Allow slides to gel. Cover with 50 ul LMA and drop into lysis buffer, ASAP. Store slides in lysis buffer for at least 1 hour.

6. Electrophorese one slide from each group using brief unwinding and electrophesis times (15 minutes unwinding and 5 minutes electrophoresis). Electrophorese one slide from each group using relatively long unwinding and brief electrophoresis times (60 minutes unwinding and 5 minutes electrophoresis). Neutralize, stain and measure as per SOP.

Slide Number	Treatment	Density	Comment
105-1	CTL	LO	
105-2			
105-3			
105-4			
105-5	CTL	MED	
105-6			
105-7			
105-8			
105-9	CTL	HI	
105-10			
105-11			
105-12			
105-13	EXP	LO	
105-14			
105-15			
105-16			
105-17	EXP	MED	
105-18			
105-19			
105-20			
105-21	EXP	HI	
105-22			
105-23			
105-24			
105-25	EXTRA		

Tissue Preparation for Eventual Western Blot Analysis and Archiving

Point of Contact:
Scott A. Steinert, Computer Sciences Corporation
c/o NCCOSC RDTE DIV 521
53475 Strothe Road Rm 276D
San Diego, CA 92152-6325

I. OBJECTIVE

The following procedure can be used to preserve tissue samples for archiving purposes and for eventual use for Western blot analysis. The procedure has been used by this lab to preserve tissue samples for stress protein analysis with no observed loss or gain of stress protein concentration over a six month storage period.

II. MATERIALS & EQUIPMENT

-Dissecting instruments; scissors, forceps, scalpel, and for bivalve mollusks a shucking knife.
-1.5 ml microfuge tubes, with snap or screw top.
-Styrofoam ice chest
-Dry ice
-Gloves
-Storage freezer, -70°C

III. METHODS

Test organisms are placed in clean plastic bags and held on ice until tissues can be dissected. It is recommended that tissue preparation not be delayed for more than a few hours. For mussels, *Mytilus edulis*, sever the posterior adductor muscle with a shucking knife and split apart valve halves. Remove enough tissue, preferably gill and mantle, to fill a quarter of the volume of a 1.5 ml tube, blot excess fluid with an absorbent towel, place in labelled 1.5 ml tube, cap, place on dry ice, eventually transfer to -70-80°C freezer. When possible prepare replicate samples. If samples are to be transported do so on dry ice.
*If there is access to liquid nitrogen storage facilities store tissues in liquid nitrogen in liquid nitrogen compatible storage tubes.

Standard Operating Procedures For
The Preparation of Marine Tissues for Organic and Inorganic Analysis

October 28, 1992

Author: Bryan Taplin
Science Applications International Corp.
Environmental Research Laboratory
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

The following document defines the standard operating procedures for the preparation of marine tissues, specifically for the American lobster and the Summer/Winter flounder, for organic and inorganic analysis.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Dissecting knife: stainless steel with replaceable blade

B. Stainless steel filleting knife

C. Teflon pad

D. Teflon coated stainless steel spatula

E. Stainless steel scissors

F. Acid stripped/muffled screw capped jars (no aluminum foil) with teflon lined closures - sizes: 4 and 16 oz.

G. Lobster claw crusher

H. Teflon coated stainless steel forceps

III. COLLECTION AND PREPARATION OF TISSUE

A. All dissection and removal of tissue from marine samples should be conducted using good laboratory practices. All dissecting utensils and cutting surfaces should be free of contaminants prior to dissection.

B. Dissection and removal of tissue should be done on a teflon or polyethylene cutting surface to minimize metal contamination. Dissecting utensils should be stainless steel or teflon coated stainless steel. The cutting board and dissecting impliments should be cleaned between treatments and composite reps with alconox soap, rinsed with tap water 3x, rinsed with distilled water 3x, before being solvent rinsed with methanol.

C. Prior to dissection, all animals should be rinsed with seawater, making sure all particulate material and sediment has been removed. Lobsters are dissected live, using a stainless steel knife by making an incision ventrally and equidistant from each side (see attached diagram for detailing anatomy of the lobster) . The incision runs from the tip of the rostrum to the end of the tail. Once this cut has been made, the animal can be opened latterally and the organs exposed for removal. The hepatopancreas organ should be removed first to prevent rupturing of the membrane. This organ is located on either side of the ventral mid-line in the posterior region of the carapace. This organ can be removed using stainless steel tweezers. After the hepatopancreas has been removed, the muscle tissue can be excised from the tail using tweezers and spatula. The two claws of the lobster, ripper and crusher claw, can be broken open using a claw crusher and the tissue removed using the same instruments.

Flounder are dissected by making a ventral incision on either side of the head, extending diagonally to the sides of the animal. The outer skin is removed from the flounder by breaking the head off and pulling the head and attached outer skin away from the body. Once the outer skin has been removed, the muscle tissue can be removed using a stainless steel fillet knife. Edible flesh is removed by cutting along the vertebrae and cutting away at a 45 degree angle towards the sides of the animal. The liver of the flounder is located in the visceral area on the ventral, unpigmented side of the animal. This organ is removed by making three incisions along the outer borders of the visceral pouch with a pair of stainless steel scissors. Care should be taken to minimize any damage to the underlying organs when the incisions are made. Once the the contents of the visceral pouch been exposed, the liver is the largest organ in the visceral area and usually is reddish in color. The liver can be separated from the other organs using a pair of forceps and scissors.

D. All tissue is placed into acid stripped/muffled jars with teflon lined caps with the appropriate sample information. Lobster hepatopancreas and flounder liver can be placed into 4 oz jars while lobster muscle tissue and and flounder flesh can be placed into 16 oz. jars. Samples should be placed into sealed zip lock bags, labeled appropriately, and placed in ice for transport back to ERL-N. Chain of custody procedures should be implemented once the samples have arrrived.

E. Sample size for each tissue type should be at least 20 - 25g on a wet weight basis. Some tissues, such as the lobster hepatopancreas and flounder liver, will be less. A smaller sample size (15g) may be sufficient for these tissues. This amount of sample should provide sufficient material for both organic and inorganic analyses.

IV. TROUBLE SHOOTING

A. Biomass considerations are important in determing the number of animals needed to collect. Sample size for lobsters will be contingent on the age class of the animal. Juvenile lobsters having carapace length between 4.0 - 4.5 cm, on average, contain 12.67 g (wet weight) of muscle tissue and 2.32 g (wet weight) hepatopancreas tissue per animal. Larger size classes of lobster will contain proportionally more tissue on a wet weight basis.

B. Dissection of flounder and lobster should be done to minimize cross contamination. Best available knowledge should be used in assessing the contamination level at a particular site. If prior chemistry data exist from a known collection site, this information should be used to determine the order of processing of samples. Generally one should proceed from clean to dirty in processing samples.

C. Removal of outer skin from flounder should be done correctly. Incisions should be made on the ventral side, flanking the head, and extending out towards the sides of the animal. The incisions should not penetrate the pigmented side of the animal. Once the incisions have been made, the vertebrae can be broken, and the outer skin removed. This is done by turning the animal over to the dorsal side (pigmented side), and grasping the head and pulling it towards the tail. If done correctly, the outer skin should peel off.

D. Removal of the liver from the flounder should also be done correctly. The visceral pouch can be identified by first turning the animal onto the ventral side (unpigmented side) and feeling for the soft cavity located just posterior to the head (usually located on the left side). Using a pair of scissors, the first cut should be made along the outside border of the fish, extending the length of the cavity. The last two incisions should be made just along the borders of the cavity extending inwards. It is very important not to damage the organs in the visceral pouch, as this would make identification of organs difficult. When dissecting, one should hold scissors just above the surface of the skin and keep upward tension during cutting.

V. STATISTICAL ANALYSIS AND DATA USAGE

Samples collected and prepared for organic and inorganic analysis are part of the Portsmouth Harbor Phase II field sampling operation. Data collected will be used to address any ecological effects associated with Portsmouth Harbor shipyard.

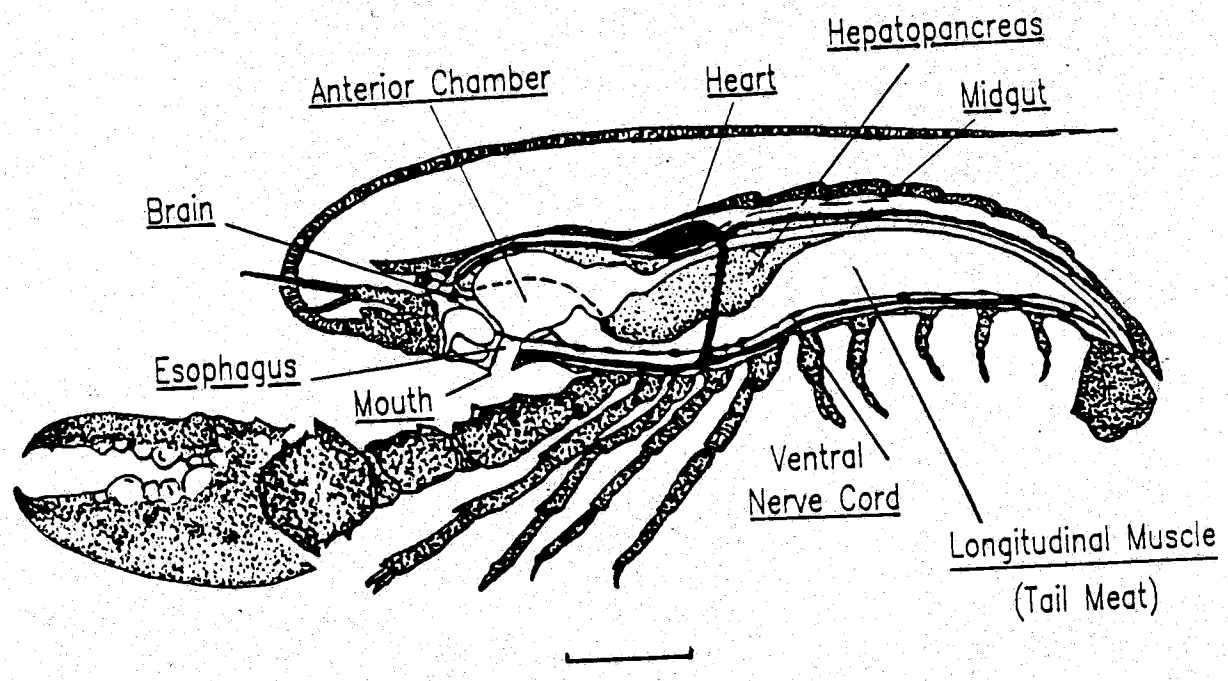
VI. REFERENCES

Cobb, S.J., 1976. The American Lobster: the biology of Homarus americanus. University of Rhode Island, kingston Rhode Island.

AMERICAN LOBSTER

Internal Anatomy

(Sagital view, showing major organs)



POINT OF CONTACT:

Cornelia Mueller
Science Applications International Corporation
c/o Environmental Research Laboratory
27 Tarzwell Drive
Narragansett, RI 02882

I. OBJECTIVE

This document describes the procedures required to excise the spleen from flounder for histopathological preparation and examination.

II. NECESSARY MATERIALS AND EQUIPMENT

- Small stainless steel dissecting scissors
- Prelabelled scintillation vials containing 15-20 ml fixative[a] prepared by ERLN
  [a]Caution - Dietrich's fixative contains 95% ethyl alcohol, 40% formaldehyde, and glacial acetic acid
- Stainless steel forceps
- Cutting board (optional)

III. METHODS

1. Label the scintillation vial with a sample number[b].
   [b]Note - ERLN will not accept responsibility for samples received without a Chain-of-Custody form.

2. Place the flounder specimen pigmented side down with the anterior end pointing to the left and the posterior end to the right on the cutting board. In this position the body cavity is found just posterior to the gill slit close to the investigator.

3. Insert a small pair of dissection scissors in the anal vent.

4. Make an incision toward the vertebral column, anteriorly to the gill slit, away from the vertebral column and finally back toward the anal vent.

5. Remove the abdominal wall over the body cavity.

6. The liver, a large reddish brown organ, is centrally located in the exposed cavity. Lift this organ and separate from the underlying intestinal mesenteries.

7. The spleen, a discrete bright red organ, is located in these mesenteries. The spleen is generally about one twentieth the size of the liver. Lift the spleen with the forceps and carefully cut away from attached mesenteries.

8. Place the spleen in the prelabelled scintillation vial containing fixative and recap tightly for transport to ERLN[b].
[b]Note - ERLN will not accept responsibility for samples received without a Chain-of-Custody form.

IV. TROUBLE SHOOTING

Not applicable.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VII. REFERENCES

Not applicable.