NATIONAL CENTERS FOR COASTAL OCEAN SCIENCE

MUSSEL WATCH PROGRAM

Expanded Long-term Great Lakes Contaminant Monitoring Project Plan
Version III

Mussel Watch Program: Expanded Long-term Great Lakes Monitoring Project Plan

W. E. Johnson, K. L. Kimbrough, A. P. Jacob, and G. G. Lauenstein
NOAA/NOS/NCCOS/CCMA
1305 East-West Highway
Silver Spring, Maryland 20910

Abstract

NOAA's Mussel Watch Program is a key part of the Nation's coastal monitoring infrastructure. At the regional level, the Mussel Watch Program works with state and local stakeholders to provide relevant information to support decision making. The program is primarily recognized for its status and trends assessment of chemcial contaminants measured in bivalve tissue (mussels and oysters) nationwide (Dreissena spp. in the Great Lakes).

This document serves as the Quality Assurance Project Plan (QAPP) for NOAA's expanded Mussel Watch Program (MWP) monitoring effort in the Great Lakes. Mussel Watch is leveraging its long-term monitoring mission in the Great Lakes and adding enhancments (additional sites and indicators) that address specific issues of the action plan of Great Lakes Restoration Initiative (http://GLRI.US).

The GLRI action plan covers the period 2010 through 2014 and identifies specific urent needs including cleaning up Areas of Concern. The U.S.-Canada Great Lakes Water Quality Agreement (Annex 2 of the 1987 Protocol) defines AOCs as "geographic areas that fail to meet the general or specific objectives of the agreement where such failure has caused or is likely to cause impairment of beneficial use of the area's ability to support aquatic life." Through its historic and ongoing efforts the Mussel Watch Program engages federal, state, tribal and community stakeholders to leverage knowledge and resources to assess and remove Beneficial Use Impairments (BUIs), and monitor the recovery of AOCs.

Included in the document are descriptions of sampling, analyses, reporting, and associated quality assurance and quality control methods. Discussions of the Mussel Watch Program background, project scope and objectives are presented.

Mussel Watch Program: Expanded Long-term Great Lakes Project

Quality Assurance Project Plan Approval Page

June 2012

_____ _____

Dr. Marie Colton Date
GLRI Program Manager
NOAA/OAR/GLERL
Tel: 734-741-2244
marie.colton@noaa.gov

_____ _____

Mr. Juan Ramirez Date
Chemistry Laboratory Manager
TDI-Brooks International, Inc.
Tel: 979-693-3446
juanramirez@tdi-bi.com

_____ _____

Dr. Mark Monaco Date
Center Director CCMA
NOAA/NCCOS/CCMA
Tel: 301-713-3028
mark.monaco@noaa.gov

_____ _____

Mr. Thomas Nalepa Date
Taxonomy Assessment
NOAA/OAR/GLERL
Tel: 979-693-3446
thomas.nalepa@noaa.gov

_____ _____

Dr. Greg Piniak Date
Branch Chief COAST
NOAA/NCCOS/CCMA/COAST
Tel: 301-713-3028
greg.piniak@noaa.gov

_____ _____

Dr. Michael H. Fulton Date
Toxicity Laboratory Manager
NOAA/NCCOS/CCEHBR
Tel: 843-762-8576
mike.fulton@noaa.gov

_____ _____

Dr. Kimani Kimbrough Date
Project Manager
NOAA/NCCOS/CCMA/COAST
Tel: 301-713-3028
kimani.kimbrough@noaa.gov

_____ _____

Ms Rebecca Held Date
NOAA GLRI Quality Manager
NOAA/OAR/GLERL
Tel: 734-741-2244
rebecca.held@noaa.gov

_____ _____

Dr. W. Edward Johnson Date
Quality Assurance Lead
NOAA/NCCOS/CCMA/COAST
Tel: 301-713-3028
ed.johnson@noaa.gov

Table of Contents

Table 1. QAPP distribution list

Name	Organization
Russell Callender	NOAA/National Centers for Coastal Ocean Science (NCCOS)
Mark Monaco	NOAA/NCCOS
Greg Piniak	NOAA/NCCOS
Michael Fulton	NOAA/NCCOS
W. Edward Johnson	NOAA/NCCOS/Mussel Watch Program
Kimani Kimbrough	NOAA/NCCOS/Mussel Watch Program
Rebecca Wynne	NOAA/NCCOS/Mussel Watch Program
Annie Jacob	NOAA/NCCOS/Mussel Watch Program
Mary Baker	NOAA/Office of Response and Restoration (ORR)
Marie Colton	NOAA/Great Lakes Environmental Research Laboratory (GLERL)
Rebecca Held	NOAA/GLERL
Thomas Nalepa	NOAA/GLERL (retired)
Glenn Warren	EPA/Great Lakes National Program Office (GLNPO)
Juan Ramirez	TDI-Brooks, International, Inc.

Table 2. Project task organization

Name	Affiliation	Responsibility
NOAA - National Centers for Coastal Ocean Science		
Russell Callender	National Centers for Coastal Ocean Science	Acting Director
Mark Monaco	Center for Coastal Monitoring and Assessment	Director
Greg Piniak	Coastal Oceanographic Status and Trends	Branch Chief
Michael Fulton	Center for Coastal Environmental Health and Biomolecular Research	Estuaries and Land Use Branch Chief/Toxicity
NOAA - National Status and Trends Program		
W. Edward Johnson	Mussel Watch Program	Quality Assurance Manager
Kimani Kimbrough	Mussel Watch Program	Project Manager
W. Edward Johnson	Mussel Watch Program	Project Field Coordinator
Rebecca Wynne	Mussel Watch Program	Project Communications Manager
Annie Jacob	Mussel Watch Program	Project Data Manager
NOAA - Office of Response and Restoration		
Mary Baker	Office of Response and Restoration	NOAA Lead
NOAA - Oceanic and Atmospheric Research		
Rebecca Held	Great Lakes Environmental Research Laboratory	GLRI Coordinator
Thomas Nalepa	Great Lakes Environmental Research Laboratory (retired)	Taxonomy Assessment
Marie Colton	Great Lakes Environmental Research Laboratory	GLERL Director and NOAA's GLRI Director
U.S. EPA		
Glenn Warren	Great Lakes National Program Office	EPA, NOAA Interagency Agreement Project Lead
Contractors		
Juan Ramirez	TDI-Brooks International, Inc.	Chemistry

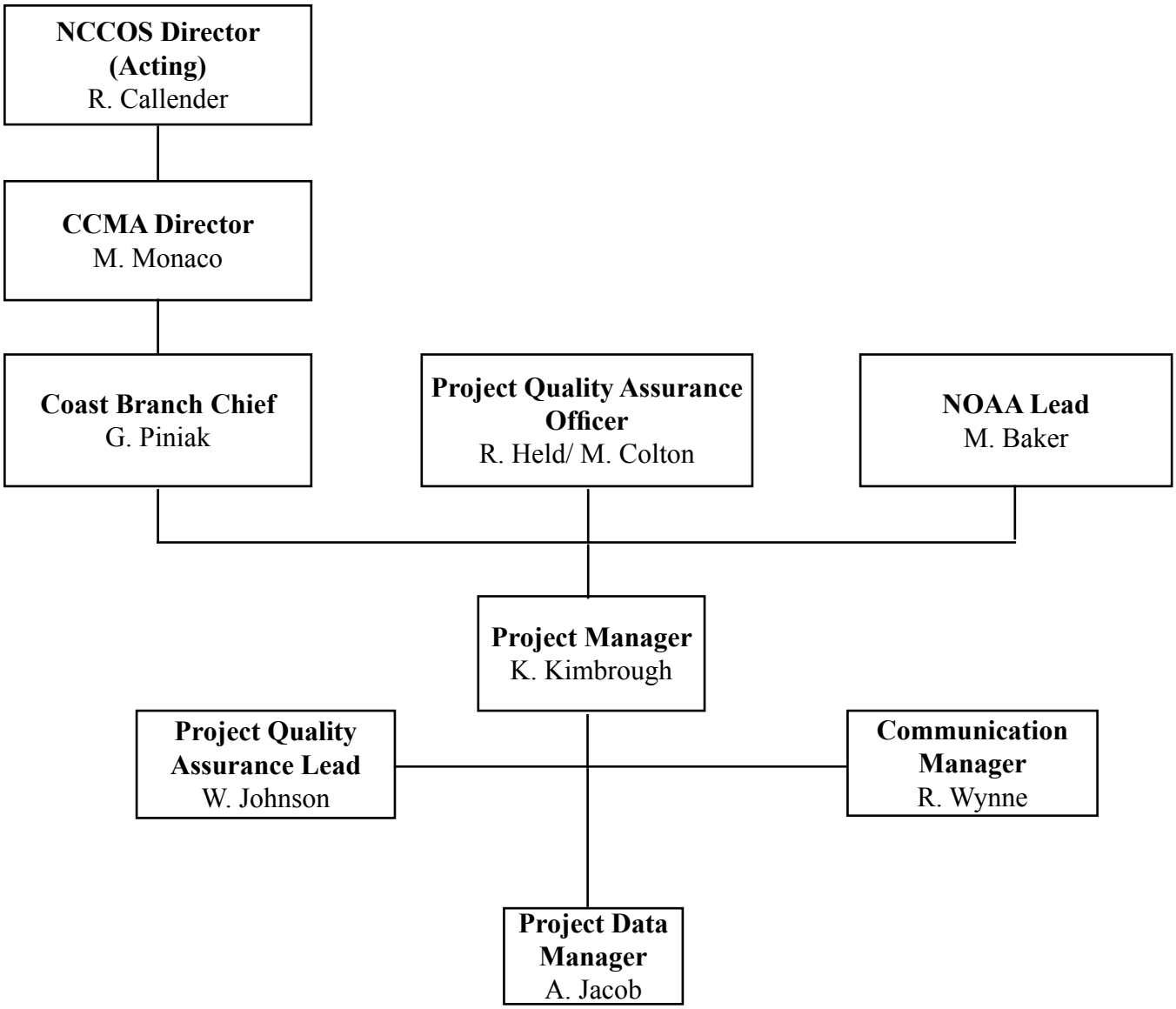


Figure 1. Organizational chart that shows lines of authority and reporting responsibilities.

Section 2: Project Description and Background

National

NOAA's Mussel Watch Program (MWP) monitors the status and trends of chemical contamination of U.S. coastal waters. The Program began in 1986 and is one of the longest running, continuous coastal monitoring programs that is national in scope. The Program is based on annual collection and analysis of oysters and mussels. These bivalves are sessile organisms that filter particles from water; thus, measuring contaminant levels in their tissue is a good indicator of local contamination. Mussel Watch data are useful for characterizing the environmental impact of new and emerging contaminants, extreme events (hurricanes and oil spills), and for assessing the effectiveness of legislation, management decisions, and remediation of coastal contamination levels. As a result of monitoring all major estuaries for chemical contamination, Mussel Watch results can be used to identify geographic areas of concern and potential human exposures to elevated levels in seafood.

Program Goal

To support ecosystem-based management through an integrated nationwide program of environmental monitoring, assessment, and research to describe the status and trends of our Nation's estuaries and coasts.

NOAA established Mussel Watch in response to a legislative mandate under Section 202 of Title II of the Marine Protection, Research and Sanctuaries Act (MPRSA) (33 USC 1442), which called on the

Figure 2. National Mussel Watch Program sites.

Secretary of Commerce to, among other activities, initiate a continuous monitoring program "to assess the health of the marine environment, including monitoring of contaminant levels in biota, sediment and the water column." As part of the NOAA Authorization Act of 1992, the overall approach and activities of NOAA's National Status and Trends Program (NS&T), including Mussel Watch, were codified under provisions of the National Coastal Monitoring Act (Title V of the MPRSA).

In 1986, the inaugural year of the Mussel Watch Program, 145 sites were sampled. Today, Mussel Watch is comprised of nearly 300 monitoring sites, where more than 140 chemical contaminants, chosen through consultation with experts and scientists from academia and government, are measured. Many of these contaminants are listed as Environmental Protection Agency (EPA) Priority Pollutants (Keith and Teillard, 1979). Legislation has been passed to regulate most of the organic contaminants analyzed by the Mussel Watch Program (http://NSandT.noaa.gov). The majority are toxic to aquatic organisms, and some are taken up and stored in animal tissues with the potential to be transferred through food chains to humans.

Great Lakes MWP Objectives

This project will expand under Great Lakes Restoration Initiative (GLRI) to include Areas of Concern (AOCs). The program will be enhanced with additional sites and indicators including mussel tissue, sediment chemistry, sediment toxicity and benthic infauna in support of:

1. Assessment and removal of Beneficial Use Impairments (BUIs);
2. Assessment of AOC remediation effectiveness;
3. Monitoring AOC recovery and delisting;
4. Leveraging MWP national data to provide a national perspecitve to Great Lakes contamination;
5. Policy, remediation, regulatory and legislative decisions

Great Lakes

MWP has documented the continuing presence of legacy contaminants such as PCBs, DDTs and other chlorinated contaminants, many of which were produced by industries along the shores of the Great Lakes. In addition, MWP evaluates trends in metal contamination, including cadmium, mercury, and lead concentrations, among others. These contaminants have the potential to cause harm to humans, aquatic organisms, and wildlife.

The Mussel Watch Program began collecting mussels from sites in the Great Lakes in 1992 as part of its national contaminant monitoring program. Today there are 25 long-term Mussel Watch sites based on historical presence from Duluth, MN on Lake Superior to Cape Vincent, NY at the St. Lawrence River (Figure 3). Zebra and quagga mussels of the genus *Dreissena*, which are invasive species and native to western Europe, are collected in the Great Lakes. Furthermore, *Dreissena* spp. are a vector for contaminant trophic transfer and bio-magnification since they are preyed upon by fish (gobies) which in turn may be fed upon by larger fish which are likely taken for human consumption (Kwon, et al., 2006).

MWP is an important tool that can be used to determine the success of efforts to improve the environmental quality of the Great Lakes. With the measurement of dozens of legacy contaminants, trace elements, contaminants of emerging concern and, a large suite of compounds related to the use of fossil fuels (including oil); NOAA's MWP can act as a barometer of environmental improvement for the Great Lakes.

Data and documents of the Mussel Watch Program including this work are available online (http://NSandT.noaa.gov) as static data files and through a data query tool that allows custom data searches.

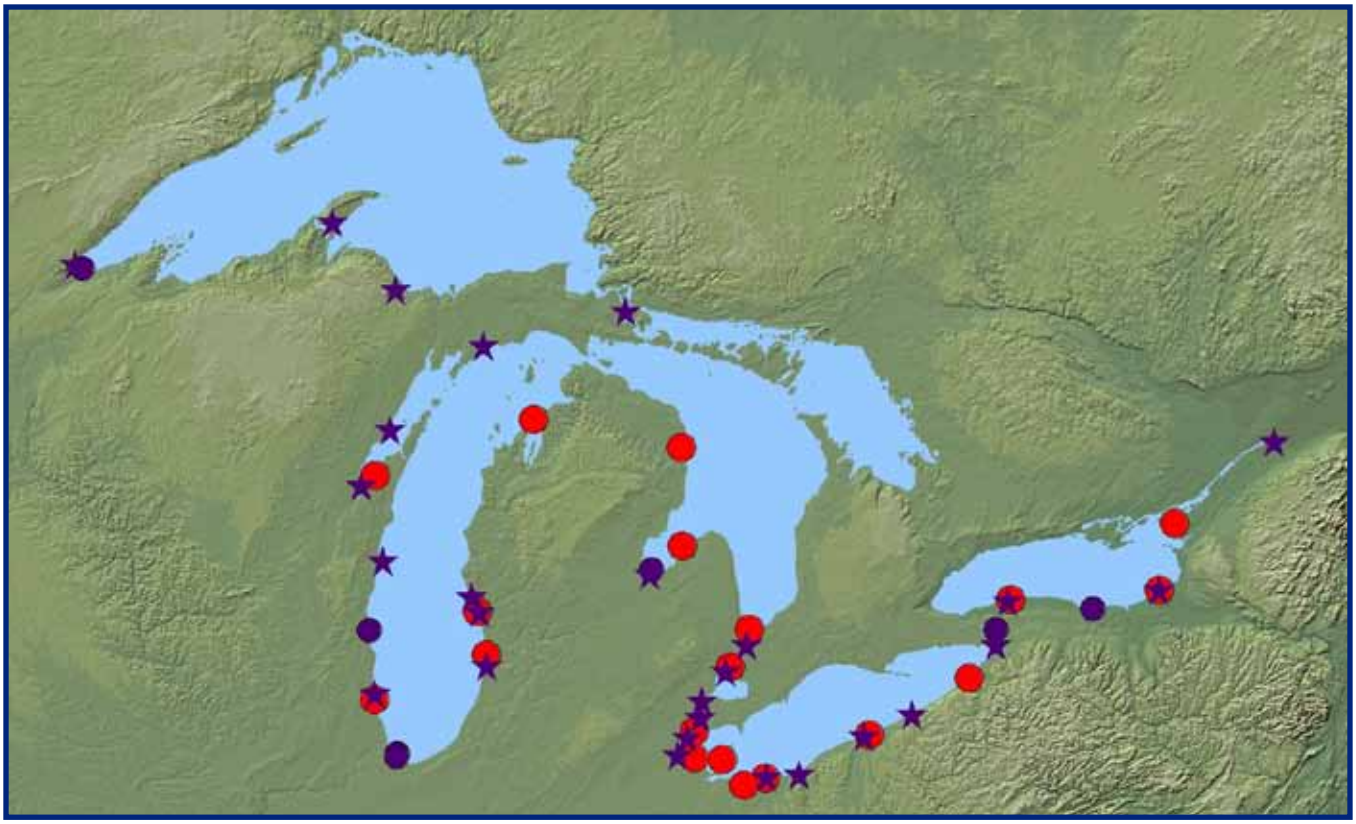


Figure 3. Great Lakes Mussel Watch Program sites. (●Mussel Watch sites, ●Mussel Watch/AOC sites, ★AOC sites).

Reports, methods documents and other publications related to this project are available online at http://www2.coastalscience.noaa.gov/publications/ccma/. Field logs, chain-of-custody logs, are retained on file for a minimum of ten years at NCCOS headquarters in Silver Spring, MD location and available on request. Data acquisition system files are stored electronically by the laboratories until the project is complete and final reporting is accepted and approved.

MWP contaminant data can be used to support a variety of remedial and restoration decisions. The data can be used in investigation and sample plan design, ecological risk assessment, cleanup level derivation, development and evaluation of remedial alternatives, development of mitigation strategies, sediment remediation design--including time-critical removal actions, natural resource damage assessment (pathway evaluation, injury determination, restoration project development, and scaling damages), and long-term effectiveness monitoring programs and other management activities needed to cleanup and restore the Great Lakes ecosystem. The project work schedule is shown in Figure 4.

Results will lead to a better understanding of the sources, releases, fate, transport, persistence, bioaccumulation, and toxicity of persistent toxic substances.

This project is complementary to NOAA's Great Lakes Sediment Contamination Database and as such, will help expedite cleanup and restoration. As BUIs are removed and AOCs are delisted, MWP will provide the long-term monitoring information for verifying the improvement in environmental conditions.

Table 3. Samples collected as part of the Expanded Great Lakes MWP. For more details see methods document found in the appendies.

Sample Type/Matrix	*Classification*
Mussel chemistry	Critical
Sediment chemistry	Critical
Benthic infauna characterization (Sediment)	Information only
Sediment grain size	Critical
Sediment total organic carbon	Critical
Clostridium perfringens (Sediment)	Information only
Mussel histopathology	Critical
Sediment toxicity Microtox®	Information only

Table 4. Trace elements analyzed as part of the Expanded Great Lakes MWP. For more details see methods document found in appendix 1.

Symbol	Element	Symbol	Element
Al	Aluminum	Hg	Mercury
Sb	Antimony	Ni	Nickel
As	Arsenic	Se	Selenium
Cd	Cadmium	Si	Silicon
Cr	Chromium	Ag	Silver
Cu	Copper	Tl	Thallium
Fe	Iron	Sn	Tin
Pb	Lead	Zn	Zinc
Mn	Manganese		

Table 5. Organic contaminants analyzed as part of the Expanded Great Lakes MWP.

Compound class	Organic compound
PCB (39 compounds) Polychlorinated biphenyls	PCB8/5, PCB18, PCB28, PCB29, PCB31, PCB44, PCB45, PCB49, PCB52, PCB56/60, PCB66, PCB70, PCB74/61, PCB87/115, PCB95, PCB99, PCB101/90, PCB105, PCB110/77, PCB118, PCB128, PCB138/160, PCB146, PCB149/123, PCB151, PCB153/132, PCB156/171/202, PCB158, PCB170/190, PCB174, PCB180, PCB183, PCB187, PCB194, PCB195/208, PCB199, PCB201/173/157, PCB206, PCB209
PAH Polycyclic aromatic hydrocarbons (Sum of 19 parent PAH compounds plus 19 groups of alkylated PAHs)	naphthalene, biphenyl, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, dibenz[*a,h*]anthracene, indeno[*1,2,3-cd*]pyrene, benzo[*ghi*]perylene C1-Chrysenes, C1-Dibenzothiophenes, C1-Fluoranthenes/Pyrenes, C1-Fluorenes, C1-Naphthalenes, C1-Phenanthrenes/Anthracenes, C2-Chrysenes, C2-Dibenzothiophenes, C2-Fluorenes, C2-Naphthalenes, C2-Phenanthrenes/Anthracenes, C3-Chrysenes, C3-Dibenzothiophenes, C3-Fluorenes, C3-Naphthalenes, C3-Phenanthrenes/Anthracenes, C4-Chrysenes, C4-Naphthalenes, C4-Phenanthrenes/Anthracenes
Pesticides & their transformation products:	
DDT (Sum of 6 compounds)	2,4'-DDD; 2,4'-DDE; 2,4'-DDT; 4,4'-DDD; 4,4'-DDE; 4,4'-DDT;
Dieldrin (Sum of 2 compounds)	Aldrin, Dieldrin;
Chlordane (Sum of 4 compounds)	Alpha-Chlordane, Heptachlor, Heptachlor-Epoxide, Trans-Nonachlor;
Endosulfans (Sum of 3 compounds)	Endosulfan I, Endosulfan II, Endosulfan Sulfate;
Butyltin (Sum of 3 compounds)	Monobutyltin, Dibutyltin, Tributyltin;
Chlorpyrifos	Chlorpyrifos;
Mirex	Mirex
PBDE Flame Retardants Polybrominated Diphenyl Ethers (Sum of 34 PBDEs)	BDE 1, BDE 10, BDE 100, BDE 11, BDE 116, BDE 118, BDE 119, BDE 12, BDE 126, BDE 13, BDE 138, BDE 15, BDE 153, BDE 154, BDE 155, BDE 166, BDE 17, BDE 181, BDE 183, BDE 190, BDE 194, BDE 195, BDE 196, BDE 197, BDE 198_199_203_200, BDE 2, BDE 201, BDE 202, BDE 204, BDE 205, BDE 206, BDE 207, BDE 208, BDE 209
Perflouronated compounds (Sum of 2 compounds)	Perfluorooctane sulfonate (PFOS, Perfluorooctanoic acid (PFOA)

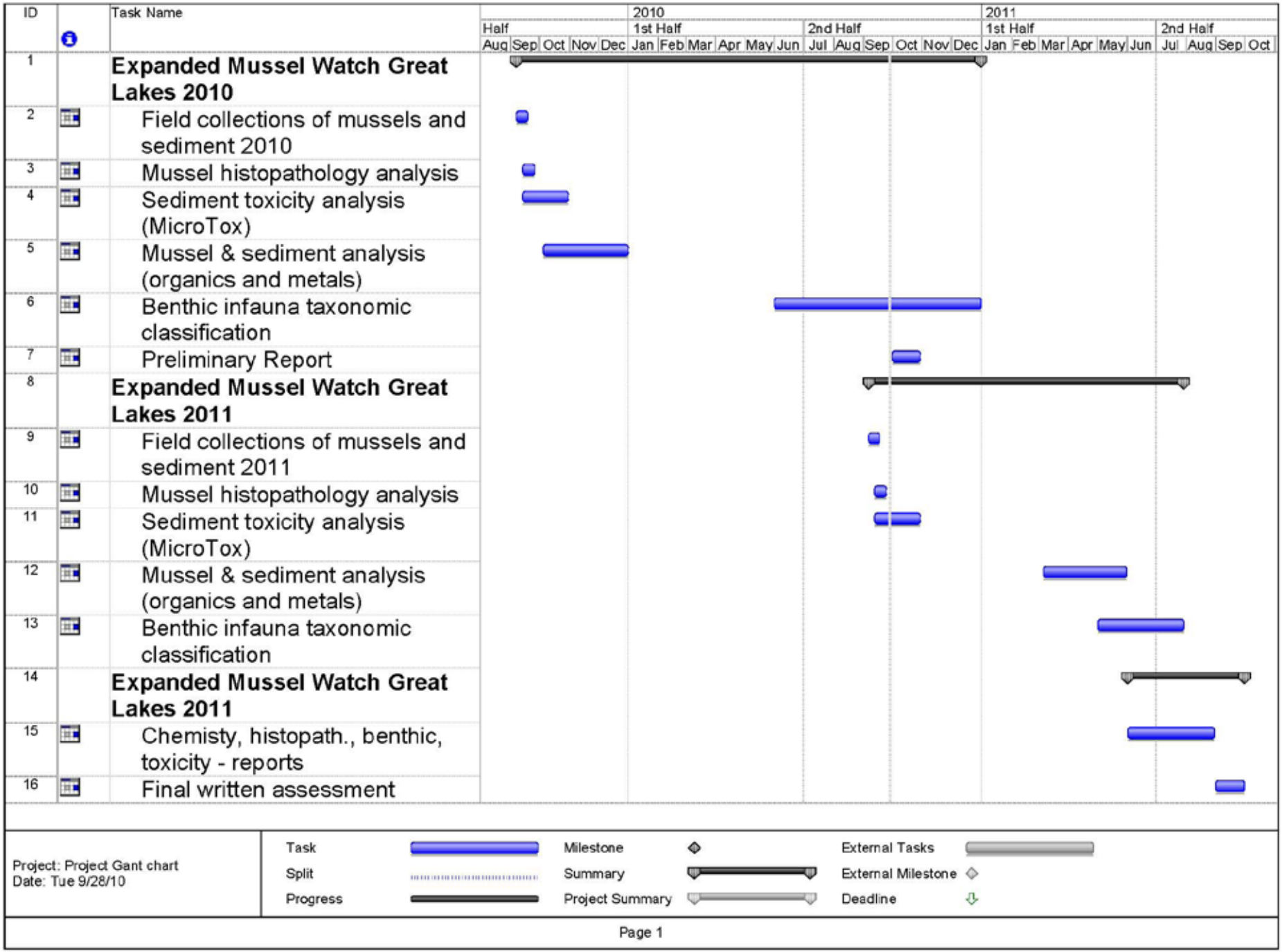


Figure 4. Expanded Mussel Watch Great Lakes Project work schedule.

Data Quality Objectives

Results help achieve the following GLRI goals:

- To significantly reduce exposure to persistent toxic chemicals from historically contaminated sources through source reduction and other exposure reduction methods.
- To protect the health and integrity of wildlife populations and habitat from adverse chemical and biological effects associated with the release of persistent toxic substances.

Project Objectives

- Revisit long-term Mussel Watch sites in the Great Lakes basin;
- Establish new Mussel Watch sites within AOC boundaries that are not currently sampled as part of the existing Mussel Watch Program;
- Assess chemical and microbial contamination in both tissue and sediment matrices. Collect mussels, and sediment from each site and conduct analyses for chemical and microbial contamination; sediment toxicity, and benthic infaunal characterization;
- Assess the results of sample analyses and compare and contrast the observations within AOCs to those observed outside of the AOC boundaries. Reconcile these results in the context of local Remedial Action Plans (RAPs).

Bivalve sample attainment is dependant on the presence of abundant resident population. At sites where environmental conditions that support bivalves do not exist a sediment sample will be collected. In few instances, fine grained sediments cannot be found in the vicinity of bivalve sites. In such cases, sediment sample will not be collected and another sample matrix will not be substituted.

Measurements will support the weight of evidence approach to assessing remedy effectiveness and providing pre- and post remediation measurements. Action levels and criteria do not exists for bivalve tissue measurements. As a results, measurements will be compared to historic MWP data to gain perspective on concentration.

If corrective action is needed the responsibility is that of the Project QA Lead. Other relevant project participants will support all corrective actions dictated by the Project QA Lead.

Special Training Requirements/Certifications

Project participants have a range of educational preparation in their respective fields ranging from MS to Ph.D. level training. Other than their respective academic preparation no special training requirements or certifications are needed. Licensed boat personnel and equipment support is provided through a collaboration between MWP and NOAA's Great Lakes Environmental Research Laboratory.

Section 3: Site Selection and Sample Collection

Site Selection

A targeted sampling design is used for this study. Long-term Mussel Watch sites will be collected and new Mussel Watch sites will be established in Areas of Concern. The sample collection sites are provided in Figure 3 and Tables 6 and 7. Locations of new sites are approximate pending site visits. The precise location of new sites within AOC boundaries is a function of the availability of

mussels that can be collected by diving or using a small epibethic dredge.

Criteria for bivalve site selection

- Sites should integrate contaminant accumulation from nearby or surrounding areas and should be outside effluent discharge zone unless designated to monitor incoming contaminants.
- Substrates are limited to rock or concrete (including rip-rap and jetties), and sand or mud. Structures such as wooden pilings and metallic navigation aids are avoided in order to eliminate potential contamination.
- Indigenous populations of mussels are selected, transplanted mussels are not used;
- Bivalve size ranges is 2 - 3 cm for *Dreissena* spp.
- Sites must have sufficient bivalves to obtain approximately one-half gallon of mussels.
- Sites must be suitable for follow-up sampling (e.g., not anticipated to be physically disrupted by development activities or dredging).
- Sites are collected in August/September. Once established new sites must be documented with Global Positioning Satellite System (GPS).
- In the case where a sediment collection site exceeds 2 km from the nearest bivalve collection site then it is defined as a different site and given a unique site name and code.
- The site must integrate contaminants from multiple sources in the surrounding area but should not reflect inputs from an individual point source of contamination unless the site was chosen specifically to document a hot-spot.

Mussels

Mussel samples are collected for the analysis of organic, inorganic and microbial contaminants. In addition, these are analyzed for gondal index and histopatholgy. Mussels may be collected by hand or dredge. All samples are accompanied by chain of custody forms which included the date and time of sample collection and the site name.

Sediment

Samples for sediment chemistry, toxicity, and the benthic community analyses are collected concurrently with a Young-modified, van Veen grab sampler (0.04 m^2) deployed from a boat (Figure 5.)

Briefly, the grab sampler and sampling utensils are acid washed with 10% HCl and then rinsed with distilled water at the beginning of each study, benthos samples are attempted first. Three replicate samples for benthic community analyses are collected. The entire contents of an acceptable grab (at least 5-cm deep at the center of the grab) are retained and sieved in the field with a 0.5 mm sieve. Materials retained on the sieve are carefully transferred to plastic jars and immediately preserved in 10% buffered formalin solution containing rose bengal stain.

The sediment grab is thoroughly rinsed with site water followed by acetone and then distilled water immediately prior to collection of sediment for chemistry or toxicity samples.

Usually, 3 or 4 deployments of the sampler (minimum of 3) are required to provide a sufficient volume of surficial sediment (0.6 to 1.0 L) for the toxicity tests and chemical analyses. The upper 2-3 cm of the sediment are sampled to ensure the collection of recently deposited materials. Sediments are removed from the grab with a acetone rinsed stainless steel spoon and composited in a high-density, polyethylene (HDPE) bucket that has been acetone rinsed. Between each deployment of the sampler the bucket is

Table 6. Long-term Mussel Watch sites.

Site	Site Name	State	Latitude	Longitude	Location
GBBS	Green Bay Bayshore Park	Wisconsin	44.63700	-87.808167	Bayshore Park
LESP	Lake Erie Stony Point	Michigan	41.95521	-83.23416	Stony Point
LEDK	Lake Erie Dunkirk	New York	42.52922	-79.27704	Dunkirk
LERB	Lake Erie Reno Beach	Ohio	41.66845	-83.23415	Reno Beach
SBPP	Lake Erie Peach Orchard Pt.	Ohio	41.65948	-82.82415	Peach Orchard Pt.
LEOW	Lake Erie Old Woman Creek	Ohio	41.38500	-82.5187	Old Woman Creek
LELR	Lake Erie Lorain	Ohio	41.46117	-82.207	Lorain
LEAB	Lake Erie Ashtabula	Ohio	41.922683	-80.71802	Ashtabula
LHTB	Lake Huron Thunder Bay	Michigan	44.922167	-83.4135	Thunder Bay
LHBR	Lake Huron Black River Canal	Michigan	43.044333	-82.438667	Black River Canal
LMNC	Lake Michigan North Chicago	Illinois	42.304667	-87.827333	North Chicago
LMHM	Grand Calumet River (AOC)	Indiana	41.69865	-87.50825	Hammond Marina
LMHB	Lake Michigan Holland Breakwater	Michigan	42.773167	-86.215	Holland Breakwater
LMCB	Lake Michigan Calumet Breakwater	Indiana	41.72717	-87.495	Calumet Breakwater
LMMU	Lake Michigan Muskegon	Michigan	43.225833	-86.347	Muskegon
LMMB	Milwaukee Estuary (AOC)	Wisconsin	43.032167	-87.895167	Milwaukee Bay
LOOC	Lake Ontario Olcott	New York	43.35472	-78.68867	Olcott
LORC	Rochester Embayment (AOC)	New York	43.2651	-77.49577	Rochester
LOOS	Lake Ontario Oswego	New York	43.4528	-76.5508	Oswego
LOCV	Lake Ontario Cape Vincent	New York	44.14489	-76.32452	Cape Vincent
LSAB	Lake St. Clair Anchor Bay	Michigan	42.649167	-82.711	Anchor Bay
LSMP	St Louis River and Bay (AOC)	Minnesota/ Wisconsin	46.71094	-92.02236	Minnesota Point
NRNF	Niagara River (AOC)	New York	43.05093	-78.89618	Niagara Falls
SBSR	Saginaw River and Bay (AOC)	Michigan	43.6735	-83.836667	Saginaw River
SBSP	Saginaw Bay Sandpoint	Michigan	43.909833	-83.400167	Sandpoint
TBLL	Traverse Bay Leelanau State Park	Michigan	45.205667	-85.536833	Leelanau State Park

Table 7. New Mussel Watch sites established in AOCs as part of this expanded project.

Site	Site Name	State	Latitude	Longitude
DRSE	Detroit River South End (AOC)	Michigan	42.10342	-83.1357
LERR	Lake Erie River Raisin (AOC)	Michigan	41.89329	-83.3248
LEBU	Lake Erie Buffalo River (AOC)	New York	42.88003	-78.89157
LEMR	Lake Erie Maumee River (AOC)	Ohio	41.70142	-83.45871
LEBR	Lake Erie Black River (AOC)	Ohio	41.47436	-82.18159
LECR	Lake Erie Cuyahogo (AOC)	Ohio	41.499417	-81.71878
LEAR	Lake Erie Ashtabula River (AOC)	Ohio	41.911233	-80.78768
LEPB	Lake Erie Presque Isle Bay (AOC)	Pennsylvania	42.137767	-80.09525
LOEC	Lake Ontario Eighteenmile Creek (AOC)	New York	43.338733	-78.71878
LOOR	Lake Ontario Oswego River (AOC)	New York	43.46834	-76.50973
LOSL	Lake Ontario St Lawrence River (AOC)	New York	44.97987	-74.89162
LMWH	Lake Michigan Waukegan Harbor (AOC)	Illinois	42.361891	-87.822156
LSTL	Lake Superior Torch Lake (AOC)	Michigan	47.157767	-88.4169
LSSM	Lake Superior St Marys River (AOC)	Michigan	46.2865	-84.211333
LMWL	Lake Michigan White Lake (AOC)	Michigan	43.40697	-86.35371
LMML	Lake Michigan Muskegon Lake (AOC)	Michigan	43.22020	-86.30467
LMIK	Lake Michigan Kalamazoo River (AOC)	Michigan	42.67461	-86.20730
LHSR	Lake Huron Saginaw River (AOC)	Michigan	43.62178	-83.84213
LCSC	St Clair River (AOC)	Michigan	42.819496	-82.48411
LCCR	Lake St Clair Clinton River (AOC)	Michigan	42.59217	-82.80069
LMMR	Rouge River Detriot River (AOC)	Michigan	42.28032	-83.11807
LMGF	Lake Michigan Green Bay Fox River (AOC)	Wisconsin	44.54452	-88.00118
LMMQ	Lake Michigan Manistique River (AOC)	Michigan	45.94578	-86.2497
LMMM	Lake Michigan Menominee River (AOC)	Michigan/ Wisconsin	45.0908	-87.58925
LMSR	Lake Michigan Sheboygan River (AOC)	Wisconsin	43.7516	-87.69733
LSCR	Lake Superior Carp River (AOC)	Michigan	46.501	-87.51
LSLR	Lake Superior St Louis River (AOC)	Minnesota / Wisconsin	46.74645	-92.12383

covered with a HDPE lid to minimize sample oxidation, photolysis and atmospheric contamination. The material is carefully homogenized in the field with the acetone rinsed, stainless steel scoop before being dispensed into sample containers redundantly labeled on the lid and the side of the container with a unique station ID number/code, sample date and project code. Samples are immediately placed in a cooler with water ice.

Sample handling

At each station a log sheet is filled in at the time of sampling that records local conditions, precise location, actual sample time and a list of all samples collected. All sample container lids are sealed with tape to minimize contamination while in storage and shipment. Sediment samples are immediately placed in coolers on water ice and shipped within 3 days except as noted below.

Samples which are not harmed by freezing (e.g., sediment chemistry) and once frozen can be maintained frozen for the duration of the mission may be held and shipped at the end of the mission. Similarly, benthic community samples that have been sieved and preserved with 10% buffered formalin containing rose bengal stain may be stored at room temperature, out of direct sunlight and shipped at the end of the mission. All other samples should be shipped within three days of collection.

Samples which should never be frozen include sediment for grain size analysis, sediment used for whole-sediment toxicity assays, and bivalve samples for gonadal index and histopathology.

At the time of shipment, all samples are organized chronologically by station and sample type (e.g. bioassay, chemical analysis etc.). Any discrepancies between sample containers and log sheets that can not be resolved by the collection team shall be duly noted by the team leader on the field data sheet and the Chain of Custody form.

Table 8. Samples collected as part of the Expanded Great Lakes MWP. For more detail see methods documents found in appendices.

Sample Type	*Field Holding Conditions :*	*Lab Holding Conditions :*	*Shipping*
Benthos	10% buffered formalin/R. Bengal	10% buffered formalin/R.Bengal	end of mission
Sediment Grain Size	cooler filled with ice chips	water ice/refrigerate	Within 3 days with water ice
Sediment Metals/Organics/TOC	cooler filled with ice chips	freeze	Within 3 days with water ice
Mussels Metals/Organics	cooler filled with ice chips	water ice/refrigerate	Within 3 days with water ice
Mussels Histopathology	cooler filled with ice chips	water ice/refrigerate	Within 3 days with water ice
Sediment Toxicity	cooler filled with ice chips	water ice/refrigerate	Within 3 days with water ice

Sediment samples are packed into coolers with bubble wrap or other suitable packing material and sufficient bagged or bottle frozen water ice or gel packs. Benthos samples are placed inside two heavy duty plastic bags used to line a cardboard box or other suitable shipping container. After loading the jars the bag is filled with absorbent material (vermiculite) and the plastic bags securely tied.

Before sealing each shipping container a duplicate chain of custody sheets are compiled for each container enumerating each sample by unique station ID, number of containers per station, and collection date and time. One sheet is retained and one is sent by overnight delivery to the receiving laboratory with the samples. Receiving laboratories are notified of pending sample shipments and provided tracking numbers when samples are shipped. Chain of custody forms are included with each sample as described above (Figure 6).

Section 4: Analytical Methods and Quality Control Requirements

Analytical methods for trace elements and organic compounds are detailed in Kimbrough and Lauenstein (2006) and Kimbrough et al. (2006). The methods include details about instrument calibration, maintenance, and control procedures to ensure data quality. The quality of the chemistry data generated by the National Status and Trends Program is overseen by a performance based quality assurance program (Cantillo and Lauenstein, 1993; Cantillo and Lauenstein, 1995). All NS&T cooperating laboratories are required to participate. Brief and general descriptions of the procedures are out lined below. The methods are available online at http://ccma.nos.noaa.gov/about/coast/nsandt/musselmethods.aspx and/or http://www2.coastalscience.noaa.gov/publications/ccma/all.aspx.

Along with partner laboratories, sampling and analytical methods for monitoring chemicals in oysters, mussels and sediment have been developed. The Mussel Watch Program uses a performance based quality assurance (QA) process to ensure data quality. This effort has been in operation since 1985 and is designed to document sampling protocols, analytical procedures and laboratory performance. Analytical laboratories used by the Mussel Watch Program are required to participate in exercises with assistance from the National Institute of Standards and Technology (NIST) and the National Research Council of Canada (NRC) to ensure data are comparable in accuracy and precision (Willie, 2000; Schantz et al.,

Figure 5. Sediment samples taken for Expanded MWP Great Lakes Project

CHAIN OF CUSTODY RECORD

Client:_____

Project ID:_____

B&B Contact:_____

Sampler Signature:_____

Sample ID	Sample Date	Sample Time	Sample Matrix	Preservative	Containers		Analyses							Other Instructions / Comments
					Type	No.								
					Total # of Containers									

Relinquished By	Company Name	Date	Time	Received By	Company Name	Date	Time
Printed Name:				Printed Name:			
Signature:				Signature:			
Printed Name:				Printed Name:			
Signature:				Signature:			

Matrix:

T=Tissue G=Gas
S=Soil/Sediment Ws=Waste
R=Rinseate HW=Hazardous Waste
P=Product W=Water

Sample Container: Vol/material

G=Glass C=Core
P=Plastic B=Bag

Figure 6. Chain of Custody Form

2000).

Chemical Standards

Stock standard solutions are prepared from either high purity neat materials, from the U.S. Environmental Protection Agency's Pesticide and Industrial Chemicals Repository, or as certified neats and solutions from commercial vendors (Accu Standard, New Haven, CT and Chem Service, West Chester, PA). Stock and working standards are prepared in class "A" volumetric flasks with measurements of neat materials made with certified analytical balances. Fortification and surrogate standard solutions are prepared similarly. Each stock solution is given a tracking code and this code is recorded in permanent records of the preparation procedures of each standard (calibration, internal, fortification and surrogate), and equipment maintenance, repair and calibration are maintained in laboratory notebooks. Dilutions of stock standard solutions are prepared in class "A" volumetric flasks with aliquots taken by gas tight analytical syringes. These solutions are then used to prepare the matrix spike samples and instrument calibration standards. A tracking code is assigned to connect each dilution with its stock solution and all pertinent information is recorded in laboratory notebooks. Matrix spike samples are processed and analyzed in the exact manner as all other field samples.

A method blank is run with every 20 samples, or with every sample set, whichever is more frequent. If blank levels for any component exceeded three times the MDL, samples analyzed in that sample batch are re-extracted and reanalyzed. If insufficient sample was available for extraction, the data was reported and appropriately qualified. Matrix spike/matrix spike duplicate samples were run with every 20 samples, or with every sample batch, whichever was more frequent. Surrogate standards were spiked into every sample and quality control sample.

Method Accuracy and Precision

The recovery of surrogate standards will be used to monitor method performance. Analytical instruments are calibrated daily (prior to each analysis sequence, in the middle of the sequence, and at the end of the sequence) with a minimum of 5 calibration standards prepared in extract matrix solution. Normal sequence size is 40 samples. A standard is reanalyzed every 10^{th} sample within a sample batch to monitor system performance. Calibration curves are created electronically and checked for consistency throughout the sequence. An acceptable calibration curve will have a linear slope with a linear correlation factor (r^2) of ≥ 0.985. Further more, the slope value should not vary more than +/- 5% over the course of the sequence. A print out of all calibration curves for all compounds of interest is kept on file with the chromatograms produced from each sequence.

Analytical instruments are maintained at the highest possible performance condition through routine maintenance and necessary repair. In the event of critical repair needs, authorized manufacturer service technicians are employed. Detailed logs of the daily use, number and type of sample extracts, routine maintenance, repairs, tunes, and calibrations are kept and reviewed daily.

Trace Elements

Metals occur naturally in the environment, but human use of metals, particularly since the industrial age, has resulted in excessive releases. Anthropogenic sources of metals include fossil fuel and waste burning, mining and ore processing, chemical production, and agriculture. These sources are largely

responsible for the elevated environmental concentrations observed in coastal waters. Transport of metals to coastal and estuarine water occurs primarily from runoff and atmospheric deposition. The relative contribution from each mechanism varies by metal, proximity to sources, and chemical phase (dissolved or particulate-bound). Metals can exist in the environment in several forms of varying toxicity. The analytical methods used by the Mussel Watch Program do not distinguish between these various forms, but instead report values as total metal (aggregation of all species of a metal).

Quality control samples were processed in a manner identical to actual samples. A method blank was run with every 20 samples, or with every sample batch, whichever was more frequent. If corrected blank concentrations for any component exceeded three times the MDL, then whole sample set is re-extracted and reanalyzed. If there is insufficient sample available for re-extraction, then the data are reported and appropriately qualified. Matrix spike/matrix spike duplicate (MS/MSD) samples are run with every 20 samples, or with every sample set, whichever was more frequent. The appropriate spiking level is ten times the MDL. Reference materials were extracted with each set of sediment samples and were analyzed when available. The method detection limit was determined following the procedures outlined in CFR 40, Appendix B, Part 136 (1999).

Organic Chemicals

Organic chemicals reported here are mostly manufactured and released to the environment either intentionally (e.g., pesticides) or through manufacturing or disposal processes, such as PCBs. Others, such as PAHs, occur both naturally and as a result of human activities. Some of the chemicals presented here are industrial by products and represent major components of other manufactured chemicals. An example of this is the pesticide dieldrin, which itself is a pesticide but also a degradation product of aldrin.

All samples and quality control samples were spiked with DBOFB, PCB 103 and PCB 198. The surrogate standard solution was spiked into the samples prior to extraction in an attempt to minimize individual sample matrix effects associated with sample preparation and analysis. A matrix spike and a duplicate were analyzed with each sample set or every 20 field samples, whichever was more frequent. The acceptable matrix spike recovery criteria were 50 - 125% recovery for at least 80% of the analytes. Criterion for duplicates was ≤30% relative percent difference (RPD). The method detection limit was determined following the procedures outlined in CFR 40, Appendix B, Part 136 (1999). Most target compounds, surrogates and internal standard were resolved from one another and from interfering compounds. When they were not, coelutions were documented. A standard reference material sample was analyzed per batch of sediment samples or every 20 samples whichever was more frequent.

Toxicity testing

NOAA requires its contractors and research collaborators to engage in substantial, explicit and documented quality control and quality assurance protocols. This is to ensure that data produced by different laboratories for studies in different estuaries and coastal bays are consistent and comparable. In most instances, the sediment toxicity testing procedures are standardized with specific experimental controls and data reporting procedures. Details of the proposed toxicity testing procedures can be found in the following documents.

- Cytochrome P450 Test: ASTM E 1853-96

The following narrative summarizes the QA/QC requirements of NOAA using an example of the amphipod mortality test.

Each sediment sample is logged on a standardized form (not prescribed by NOAA but approved by NOAA) and assigned a sample tracking number at the time of arrival. The sample number is used to track the sample from arrival, through testing, and for disposal. Proper state and federal regulations are followed to insure the safe disposal of all samples. The original form is maintained in a permanent file. The information on the login sheet serves as documentation of proper handling within the laboratory, as well as how the sample was held. Arrival and collection dates are recorded. Samples must be grouped according to their time of collection since testing of each sample must begin within 10 days of collection.

Benthic Taxonomy and Sorting

See Appendix 3 for method and procedures.

Section 5: Data Management

Data are first reviewed by the laboratory and subsequently by NOAA Mussel Watch staff. Electronic raw data files are retreived by NOAA via a secure ftp site. The data base manager imports the raw data files (usually in Excel format) and transforms the data into a realtional data strucuture. The relational data if reviewed and comparison to historic data. Both raw data files and relational data base files are stored on NOAA servers. All data stored on NOAA servers are backed up daily, and weekly backups are stored off site for added security. Data and metadata are available for download at http://NSandT.noaa.gov.

Section 6: References

Cantillo, A.Y. and G.G. Lauenstein. 1995. Use of reference materials in coastal monitoring quality assurance. Fresenius' Journal of Analytical Chemistry 352:152-156.

Cantillo, A.Y. and G.G Lauenstein. 1993. Performance based quality assurance of the NOAA National Status and Trends Program, In: The Proceedings of the Fifth International Symposium on the Harmonization of Internal Quality Assurance Schemes for Analytical Laboratories held in Washington, DC, USA, 22-23 July 1993.

Federal Registry (1999) Vol. 40, http://www.gpo.gov/fdsys/pkg/CFR-2011-title40-vol23/pdf/CFR-2011-title40-vol23-part136-appB.pdf

Keith, L.H., and W. A. Teillard. 1979. Priority pollutants I: a perspective view. Environmental Science and Technology 13:416-423.

Kimbrough K.L., G.G. Lauenstein, and W.E. Johnson (eds.). 2007. Organic Contaminant Analytical Methods of the National Status and Trends Program: 2000-2006. U.S. Dept. Comm. NOAA Technical Memorandum 30, NOS NCCOS, Silver Spring, MD.

Kimbrough, K.L. and G.G. Lauenstein (eds.). 2006. Trace Metal Analytical Methods of the National

Status and Trends Program: 2000-2006. US Dept. Comm., NOAA Technical Memorandum 29, NOS NCCOS, Silver Spring, MD.

Kwon, T.D., S.W. Fisher, G.W. Kim, H. Hwang, and J.E. Kim. 2006. Trophic transfer and biotransformation of polychlorinated biphenyls in zebra mussel, round goby, and smallmouth bass in Lake Erie, USA. Environmental Toxicology and Chemistry 25(4)1068-1078.

Schantz, M.M., R.M. Parris, S.A. Wise. 2000. NIST/NOAA NS&T intercomparison exercise program for organic contaminants in the marine environment: description and results of 1999 organic intercomparison exercises. NOAA Technical Memorandum NOS NCCOS CCMA, 146.

Willie, S., 2000. NOAA national status and trends program thirteenth round intercomparison exercise results for trace metals in marine sediments and biological tissues. NOAA Technical Memorandum NOS NCCOS CCMA, 142.

Appendix 1: Trace Element Methods

This document contains analytical methods that detail the procedures for determining major and trace element concentrations in bivalve tissue and sediment samples collected as part of the National Status and Trends Program (NS&T) for the years 2000-2006. Previously published NOAA Technical Memoranda NOS ORCA 71 and 130 (Lauenstein and Cantillo, 1993; Lauenstein and Cantillo, 1998) detail trace element analyses for the years 1984-1992 and 1993-1996, respectively, and include ancillary, histopathology, and contaminant (organic and trace element) analytical methods.
The methods presented in this document for trace element analysis were utilized by the NS&T Mussel Watch and Bioeffects Projects. The Mussel Watch Project has been monitoring contaminants in bivalves and sediment for 25 years, and is the longest active contaminant monitoring program operating in U.S. coastal waters. Approximately 300 Mussel Watch sites are monitored on biennial and decadal timescales using bivalve tissue and sediment, respectively. The Bioeffects Project applies the sediment quality approach, which uses sediment contamination measurements, toxicity tests and benthic macroinfauna quantification to characterize pollution in selected estuaries and coastal embayments. Contaminant assessment is a core function of both projects.
Although only one contract laboratory was used by the NS&T Program during the specified time period, several analytical methods and instruments were employed. The specific analytical method, including instrumentation and detection limit, is noted for each measurement taken and can be found at http://NSandT.noaa.gov. The major and trace elements measured by the NS&T Program include: Al, Si, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Sn, Sb, Ag, Cd, Hg, Tl and Pb.

REFERENCES

Lauenstein, G. G. and A. Y. Cantillo (eds.) (1998) Sampling and analytical methods of the National Status and Trends Program Mussel Watch Project 1993-1996 Update: TERL Trace Element Quantification Techniques, Volume III. NOAA Technical Memorandum NOS ORCA 71, Silver Spring, MD. 219 pp.

Lauenstein, G. G. and A. Y. Cantillo (eds.) (1993) Sampling and analytical methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992: Comprehensive descriptions of elemental analytical methods, Volume III. NOAA Technical Memorandum NOS ORCA 71, Silver Spring, MD. 219 pp.

TRACE ELEMENT QUANTIFICATION TECHNIQUES

R. J. Taylor and B. L. Brattin
Department of Veterinary Integrative Biosciences
Texas A&M University
College Station, TX

ABSTRACT
Sample preparation and analysis methods have been developed and refined that allow the accurate and precise determination of major and trace elements in sediment and biological tissue samples. Sample preparation emphasizes homogenization and total digestion steps that minimize contamination. Analyses utilize atomic spectroscopy techniques, including a full suite of quality assurance and quality control samples, with an emphasis on certified reference materials, in order to produce reliable data. These methods allow measurement of both background and elevated concentrations in samples collected as part of NOAA's National Status and Trends Program.

1.0 INTRODUCTION

This method documents the analytical procedures used for major and minor element analysis of marine sediments and tissue samples collected by NOAA's National Status and Trends Program. These procedures were used by the Trace Element Research Laboratory (TERL), Department of Veterinary Integrative Biosciences, Texas A&M University to analyze samples collected from 2000 to 2006.

2.0 EQUIPMENT AND SUPPLIES

2.1 INSTRUMENTATION

Perkin-Elmer model DRC-2. Inductively coupled plasma mass spectrometry (ICP-MS) Perkin-Elmer, Norwalk, CT. Autosampler, Perkin-Elmer AS 93 Plus
Spectro A.I. CIROS inductively coupled plasma optical emission spectrometry (ICP-OES). Spectro A.I., Fitchburg, MA. Autosampler, Spectro A.I. model AS400
Perkin-Elmer SIMAA 6000 graphite furnace atomic absorption spectrometry (GFAAS) Perkin-Elmer, Norwalk, CT. Autosampler, Perkin-Elmer model AS 72
Perkin-Elmer Analyst 100 flame atomic absorption spectrometry (FAAS). Perkin-Elmer, Norwalk, CT
PSA Millennium Excalibur atomic fluorescence spectrometer. PS Analytical, Orpington, Kent, UK. Autosampler, PS Analytical model AS300
Cetac M7500 cold vapor atomic absorption Hg analyzer. Cetac Technologies, Omaha, NE. Autosampler, Cetac model ASX510
OI Analytical MDS 7295 microwave digestion system. OI Analytical, College Station, TX
CPI ModBlock graphite block digestion system. CPI, Santa Rosa, CA

2.2 SUPPLIES

Argon, liquid	Nitrogen, liquid
Acetylene, welder's grade	Oxygen, compressed
Nitrous oxide	Hollow cathode lamps. Perkin-Elmer, Norwalk, CT
Electrodeless discharge lamps (EDL). Perkin-Elmer, Norwalk, CT	Autosampler cups, 1.1 mL, polystyrene, Perkin-Elmer N1012010
Boosted discharge hollow cathode lamps (BDHCL). Photron, Victoria, Australia	Autosampler vials, 5 mL polypropylene, 60818-281. VWR Scientific Products, West Chester, PA
Graphite tubes, THGA, Perkin-Elmer B0504033	

2.3 LABWARE

Balance, 0.01 g, Fisher 200 Ainsworth toploader. Fisher Scientific, Pittsburgh, PA	Balance, analytical, 0.0001 g, Mettler H10
Balance, 0.01 g, Mettler PC2000	Balls, 1 cm diameter, Teflon
Balls, 3.5 cm diameter, Teflon	Pipette, Finnpette, adjustable, 1000 - 5000 mL 9402020. Curtin-Matheson Scientific, Houston, TX
Bench, clean, with HEPA filter. Liberty Industries, East Berlin, CT	Pipette transfer, polyethylene
Bottles, screw-cap bottles, polyethylene, wide-mouth, 1 oz., Nalgene 2104-0001	Pipettes, Eppendorf, fixed volume: 10 mL, 22350102; 25 mL, 22350307; 50 mL, 22350404; 100 mL, 22350501; 200 mL, 22350609; 500 mL, 22350706; 1000 mL, 22350803
Drying oven, 60 °C, NAPCO 332. Curtin-Matheson Scientific, Houston, TX	Vials, snap-cap, polystyrene; 5, 15, and 40 dram. Baxter Scientific Products, McGaw Park, IL
Drying oven, 130 °C, Thelco	Vials, snap-cap, polyethylene, 70 mL. CPI, Santa Rosa, CA2.4
Freeze dryer system, Labconco Freezone 12L. Labconco, Kansas City, MO	Pipette tips, for Finnpette, Finntip 62. Labsystems
Large jars, Teflon lined caps	Pipette tips, polypropylene for Eppendorf pipettes, 10 - 100 mL Model 22 34190-1 and 200-1000 mL, 22 35 090-1

2.4 REAGENTS

Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) [7722-76-1], Spectropure Grade, P30. Spex, Edison, NJ
Ascorbic acid ($C_6H_8O_6$) [50-81-7], A-7506. Sigma, St. Louis, MO
Boric acid (H_3BO_3) [10043-35-3], 10659, Grade 1. Johnson Matthey, West Chester, PA
Citric acid ($C_6H_8O_7$) [77-92-9], 0110. J. T. Baker, Phillipsburg, NJ
Hydrochloric acid (HCl) [7647-01-0], concentrated (37%), Ultrex 6900-05. J. T. Baker, Phillipsburg, NJ
Hydrofluoric acid (HF) [7664-39-3], concentrated (48%), 9560-06. J. T. Baker, Phillipsburg, NJ
Magnesium nitrate [$Mg(NO_3)_2 \cdot 6H_20$] [13446-18-9], MG60-50. Spex, Edison, NJ
Nickel oxide (Ni0) [1313-99-1], powder. Spex, Edison, NJ
Nitric acid (HNO_3) [7697-37-2], concentrated (70%), 2704-7x6. Mallinckrodt, Paris, KY

Nitric acid (HNO_3) [7697-37-2], concentrated (70%), Ultrex 6901-05. J. T. Baker, Phillipsburg, NJ
Palladium metal [7440-05-3], Specpure, 560001. Johnson Matthey, West Chester, PA
Stannous chloride, ($SnCl_2 \cdot 2H_2O$) [10025-69-1], 8176. Mallinckrodt, Paris, KY
Water, redistilled in quartz sub-boiling still

2.5. MATRIX MODIFIERS

Ammonium phosphate: 0.04 g/mL in quartz-distilled water
Ascorbic acid: 2% w/v made with quartz-distilled water
Citric acid: 2% w/v made with quartz-distilled water
Hydroxylamine hydrochloride
Magnesium nitrate: 0.02 g/mL in quartz-distilled water
Palladium nitrate: 1000 mg Pd/mL made by dissolving 0.05 g Pd metal in 2 mL Concentrated Ultrex HNO_3 and diluted to 50 mL with quartz-distilled water

2.6. STANDARDS

INORGANIC VENTURES, LAKEWOOD, NJ

Table 1. Baxter Ricca standards, 1000 ppm. Ricca Chemical Co., Arlington, TX

Individual elements standards, 1,000	
Element	**Stock number**
Hg	CGHG1-1
Individual element standards, 10,000 ppm.	
Ag	CGAG10-1
Al	CGAL10-1
As	CGAS10-1
Cd	CGCD10-1
Cr	CGCR10-1
Cu	CGCU10-1
Fe	CGFE10-1
Mn	CGMN10-1
Ni	CGNI10-1
Pb	CGPB10-1
Sb	CGSB10-1
Se	CGSE10-1
Sn	CGSN10-1
Tl	CGTL10-1
Zn	CGZN10-1

QC-1 mixed standard, 100 ppm: Al, Fe, Zn; 10 ppm: Ba, Be, B, Cd, Ag, Sr
QC-2 mixed standard, 100 ppm: Sb, As, Cr, Co, Cu, Pb, Mn, Mo, Ni, Se, Tl, Sn, Ti, V
ICP-MS custom mixed standard, 10 ppm: Ag, Al, As, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Th, Tl, U, V, Zn

CPI, SANTA ROSA, CA

ICP-MS internal standard, 10 ppm:

Li-6, Rh, Bi, Ho, Sc, Tb, In

3.0 SAMPLE TREATMENT
3.1 OYSTER AND MUSSEL TISSUE

BIVALVE SHUCKING
Whole oysters and mussels were rinsed with distilled water to remove extraneous material and shucked with a stainless steel knife (using care not to touch the tissue). Whole soft tissue was removed with plastic forceps and rinsed with distilled, deionized water to remove sediment particles from gills and exterior tissue surfaces. Soft parts were transferred to a tared Ziploc polyethylene bag, and the number of individuals shucked and placed in the bag was recorded. When soft tissue from all individuals from a site had been collected, they were weighed on a top loading balance to measure the total sample wet weight. The pooled samples were stored in a freezer.

BULK HOMOGENIZING
Ziploc bags containing pooled tissue were removed from the freezer and allowed to thaw. The entire pooled sample was transferred to an acid-washed Teflon jar and 3 large Teflon balls were added. The Teflon lids were securely tightened and the jars placed in Ziploc bags and shaken in an industrial paint shaker for 20 min. After the bulk sample was homogenized, an aliquot of the sample was transferred to a clean 40 dram snap vial and frozen.

FREEZE DRYING
The frozen aliquot from the bulk homogenization step was placed in a freeze drier and allowed to dry for several days, depending upon the total mass of tissue being dried at one time. In some cases it was necessary to remove the samples from the freeze drier and drain accumulated water from the trap before continuing with the drying step.

HOMOGENIZATION OF DRY ALIQUOT
When samples were thoroughly dried, three small Teflon balls were inserted into each snap cap vial, the lids were affixed, and the samples placed in a Spex shaker mill for 1 min. The Teflon balls were then removed, and the samples stored in closed vials until weighing.

DIGESTION
Approximately 0.2 g samples of dried tissue were weighed to the nearest 0.0001 g and transferred to tared, acid-washed Teflon bombs. A 3 mL aliquot of HNO_3 was added and the bombs sealed in a digestion system and cooked according to the appropriate method (Section 3.2.4). The samples were allowed to cool and 1 mL of H_2O_2 was added to each sample, then heated to promote the reaction. After the samples were allowed to cool, 1 mL of HCl was added to each sample, and then heated gently. The samples were cooled and 15 mL of deionized distilled water was added. The bombs were closed, mixed by shaking, and weighed to 0.01 g to determine the total solution weight. The digest solution was transferred to labeled 1 oz polyethylene bottles. Solution density was determined by weighing known volumes with calibrated Eppendorf pipettes in order to determine solution volume.

For analysis of Hg, tissue samples were digested using a modified version of the Environmental Protection Agency (EPA) method 245.6. Approximately 0.15 to 0.3 g (dry weight) of sample was weighed into a 70 mL snap cap vial. Concentrated H_2SO_4 (2.5 mL) and 1.5 mL of concentrated HNO_3 were added and the samples heated in a digestion block at 90 - 95 °C for 30 min. After cooling, 10 mL of distilled water, 10 mL of 5% (w/w) $KMnO_4$, and 5 mL of 5% (w/w) of $K_2S_2O_8$ were added to each tube, and the samples left overnight without heating. Before analysis, 5 mL of 10% (w/w) $NH_2OH \cdot HCl$ were added to reduce excess $KMnO_4$ and the volume brought to 40 mL with distilled water.

3.2 BOTTOM SEDIMENT

Bottom sediment samples were prepared for analysis by freeze drying and wet digestion.

HOMOGENIZATION

Wet bulk sediment was stored frozen until sample processing began. Sediment was thawed and homogenized with a clean plastic spatula. A homogeneous aliquot of the bulk sample was transferred to a labeled 40 dram snap cap vial and frozen. The remainder of the sample was archived in the freezer.

FREEZE DRYING

The snap cap vial containing the sediment sub-sample was placed in a freeze drier for the period of time required for complete drying. Depending upon the amount of water in the freeze drier, this ranged from 12 - 76 hr.

HOMOGENIZATION OF DRY ALIQUOT

In some cases, homogenization of freeze dried sediment was accomplished by simply placing the snap cap vials in a Spex shaker. When this was not sufficient, the samples were individually ground in alumina mortar and pestles and the powdered samples returned to the vials in which they were freeze dried.

DIGESTION

Approximately 0.2 g of homogenized, dried sediment was weighed to the nearest 0.0001 g and transferred to tared 70 mL snap cap vials. A 3 mL aliquot of HNO_3 was added and the vials were placed in a 95 °C digestion block for a total of approximately 6 hr. During this time, the vials were periodically removed from the block and swirled to mix. After this period, the vials were removed from the block and allowed to cool before 2 mL of concentrated HF was added. The vials were then returned to the block for approximately 2 hr. After cooling, 15 mL of 4% boric acid were added and the vials returned to the block for another 1 to 2 hr. After the samples were allowed to cool, the content of the vials were mixed by shaking, and the vials weighed to 0.01 g to determine the total solution weight. Digest solutions were then transferred to labeled 1 oz polyethylene bottles. Solution density was determined by weighing known volumes with calibrated Eppendorf pipettes in order to determine solution volume.

For analysis of Hg, sediment samples were digested using a modified version of EPA method 245.5. Approximately 0.1 to 1.0 g (dry weight) of sample was weighed into a 70 mL snap cap vial. Concentrated H_2SO_4 (2.5 mL) and 1.5 mL of concentrated HNO_3 were added and the samples heated in a digestion block at 90 - 95 °C for 30 min. After cooling, 10 mL of distilled water, 10 mL of 5% (w/w) $KMnO_4$, and 5 mL of 5% (w/w) of $K_2S_2O_8$ were added to each tube, and the samples again heated in a digestion block at 90 - 95 °C for 30 min. Before analysis, 5 mL of 10% (w/w) $NH_2OH \cdot HCl$ were added to reduce excess $KMnO_4$ and the volume brought to 40 mL with distilled water.

4.0 CALIBRATION AND ANALYSIS

Calibration standards were prepared by serial dilution of commercially available standards using calibrated micropipettes, a top loading balance, deionized distilled water and acids to match the matrix of the samples and methods. Concentrations of working standards were verified by comparison with independent standards traceable to the National Institute of Standards and Technology (NIST) Standard Reference Materials.

In all cases, final working standards were prepared in an acid matrix that matched that of the samples being analyzed. For some elements, it was necessary to further attempt to match the major ion composition of the samples. This was most apparent in graphite furnace AAS when the peak shape of the samples was significantly different from that of the standards. For example, the standards may have a relatively broad, Gaussian-shaped peak while the sediment samples may have an extremely sharp peak, indicative of rapid volatilization of the metal. In this case, the standards were prepared in a solution that had Si, Al, Fe, Ca, and Mg added at final concentrations of 3000, 400, 200, 100, and 100 ppb, respectively.

5.0 CALCULATIONS

Trace metal concentrations were calculated by comparing analytical signals of unknowns with those of calibration standards, and then multiplying the observed concentration by the instrumental and digestion dilution factors.

The least-squares fit of the data was calculated, treating Abs (or Abs-sec., emission intensity, mass/charge ratio, etc.) as the dependent variable (y), and concentration as the independent variable (x). If the concentration range extended into the non-linear range, a second order fit was used. The intercept, the first and second order coefficients (if appropriate), and R, the correlation coefficient, were calculated.

$Abs = a + b\ (conc_{obs})$

$conc_{obs} = Abs - ab$

where $conc_{obs}$ was the calculated observed concentration, Abs was the instrumental signal (e.g., Absorbance for atomic absorption instruments, emission for ICP-OES, or counts per second for ICP-MS), a was the intercept, and b was the slope of the regression line.

5.1 DILUTION FACTOR

The dilution factor, DF, resulting from sample digestion was calculated using the equation

DF = [bomb tot. - bomb tare]spl. wt. x soln. dens.

where bomb tare was the tare weight of the digestion vessel (g); bomb tot. was the total weight of the digestion vessel plus digest solution (g); spl. wt. was the weight of the dry sample (g); and soln. dens. was the density of the digest solution (g/cm^3).

5.2 CONCENTRATION

The concentration in the original sample was calculated according to the relationship:

If $conc_{obs} < DL$, final concentration $\leq (DL)\ (DF_{instr})\ (DF_{dign})$

If $conc_{obs} \geq DL$, final concentration $= (conc_{obs})\ (DF_{instr})\ (DF_{dign})$

where $conc_{obs}$ was the concentration observed in the aqueous sample; DL was the detection limit of the analytical technique; DF_{instr} was the dilution factor of the analytical technique, if necessary; and DF_{dign} was the dilution factor of the sample digestion.

6.0 INSTRUMENTAL ANALYSIS
6.1 INDUCTIVELY COUPLED PLASMA - MASS SPECTROMETRY

Silver, aluminum, chromium, cadmium, nickel, lead, antimony, and tin in sediments or tissues

METHOD:

Inductively coupled plasma - mass spectrometry

DIGEST MATRIX:

1 to 10 dilution of digestate with reagent water

INSTRUMENT SETTINGS:

Instrument settings change on a daily basis as sensitivity is optimized. This is especially true for autolens settings and dual detector calibration. The following software settings give typical values for parameters that remain relatively constant in day-to-day operation:

Power: 1400 watts	PROCEDURE:
Plasma gas flow: 15 L/min.	Scan mode: peak hopping
Auxiliary flow: 1.2 L/min.	Dwell time per AMU: 50 msec.
Nebulizer flow: 1.0 L/min.	Sweeps/reading: 20
Analog stage voltage: -2200 V	Integration time: 1000 msec.
Pulse stage voltage: 1200 V	Replicates: 3
Quadrupole rod offset: -3 V	Autolens: On
Cell rod offset: -9 V	Blank subtraction: After internal standard
RPQ: 0.25	Measurement unit: cps
Cell path voltage: -24 V	Mode: Dual (pulse/analog)
Sample uptake: 1 mL/min.	Internal standards: Ga, Rh, In, Bi
Internal standard uptake: 0.1 mL/min.	

STANDARDS:

Calibration standards (representative of sample concentration) are prepared from dilutions of NIST-traceable multi-element standards. The low concentration standard is based on instrument sensitivity (e.g., 0.05 ppb for Pb, 0.5 ppb for Al). Mid and high standards are at 20 and 200 ppb, respectively. Other reference materials (NIST 1640 Trace Elements in Water) are used as check standards. Calibration verification is performed periodically with a blank and mid-range standard (20 ppb).

TYPICAL SENSITIVITY:

Sensitivity is approximately 5 x 10^7 counts per sec. per ppm for In - 115 .

CALIBRATION:

Weighted linear, least-squares regression.

7.2 INDUCTIVELY COUPLED PLASMA - OPTICAL EMISSION SPECTROMETRY

Aluminum, arsenic, chromium, copper, iron, manganese, nickel, silver, and zinc in sediments or tissues.

METHOD:

Inductively coupled plasma - optical emission spectrometry

DIGEST MATRIX:

Direct analysis of digestate

INSTRUMENT SETTINGS:

The following software settings give typical values for parameters that remain relatively constant in day-to-day operation:

Power: 1400 watts
Plasma gas flow: 12 L/min.
Auxiliary flow: 1.0 L/min.
Nebulizer flow: 0.7 L/min.
PROCEDURE:
Integration time: 45 sec.
Integration option: “Best SNR”
Internal standards: Au, In, Yb

STANDARDS:

Calibration standards are prepared from dilutions of NIST-traceable single element standards. Calibration verification standards are prepared from dilutions of NIST-traceable multi-element standards. All standards are prepared to match sample matrix.

CALIBRATION:

Weighted linear, least-squares regression.

7.3 ATOMIC FLUORESCENCE SPECTROMETRY

Arsenic and selenium in sediments or tissues

METHOD:

Hydride generation - atomic fluorescence spectrometry

DIGEST MATRIX:

1 to 10 dilution of digestate, final 3N HCl matrix

INSTRUMENT SETTINGS: The following software settings give typical values for parameters that remain relatively constant in day-to-day operation:

ANALYTE:	As	Se
Primary lamp current:	27.5 mA	20 mA
Boost lamp current:	35 mA	25 mA
Gain:	100	10
Pump 1 speed:	100	100
Pump 2 speed:	50	50
Range (ppb):	0 – 50	0 - 10
Mode:	Pk height	Pk area
Typical sensitivity (1 ppb):	20	500

PROCEDURE:

Delay time: 30 sec.
Analysis time: 20 sec.
Memory time: 50 sec.

STANDARDS:

Calibration standards are prepared from dilutions of NIST-traceable single element standards. Calibration verification standards are prepared from dilutions of NIST-traceable multi-element standards. All standards are prepared to match sample matrix.

CALIBRATION: Weighted linear, least-squares regression.

7.4 ATOMIC ABSORPTION SPECTROMETRY

Hg in sediments or tissues

METHOD:

Cold vapor - atomic absorption spectrometry

DIGEST MATRIX:

Direct analysis of digestate

INSTRUMENT SETTINGS:

The following software settings give typical values for parameters that remain relatively constant in day-to-day operation:

Argon flow rate: 150 mL/min.
Pump rate: 50%
Range: 0 – 50 ppb
PROCEDURE:
Sampling time: 23 sec.
Delay time: 53 sec.
Read time: 5 sec.
Replicates: 5
Background read time: 10 – 14 sec.
Rinse time: 70 sec.

STANDARDS:

Calibration standards are prepared from dilutions of NIST-traceable single element standard. Calibration verification standards are prepared from dilutions of a different NIST-traceable single element standard. All standards are prepared in 7% v/v HCl matrix.

CALIBRATION:

Weighted linear, least-squares regression.

7.5 MICROWAVE DIGESTION PROGRAM

Trace metals, including Hg in sediments or tissues

INSTRUMENT SETTINGS:

The following software settings give typical values for parameters that remain relatively constant in day-to-day operation:

PROCEDURE:

Pressurized steps (nitric acid, hydrofluoric acid):

STAGE	POWER (%)	SETTING (psi)	DWELL (min.)	MAX (min.)
1	50	20	2	5
2	75	40	5	6
3	75	60	2	3
4	75	80	2	3
5	75	100	2	3
6	75	120	2	3
7	75	140	15	16

Non-pressurized steps (hydrogen peroxide, hydrochloric acid, boric acid):

POWER (%)	SETTING (°C)	DWELL (min.)	MAX (min.)
25	85	5	10

7.6 DETECTION LIMITS

The analytical detection limits were determined by following procedures outlined in the Federal Register (1984). Method detection limits for specific measurements can be found online at http://NSandT.noaa.gov.

Table 2. Mussel Watch Project tissue major and trace elements, method limits of detection (µg/g dry weight) for 2000 – 2003*.

	Tissue MDL	**Sediment MDL**
Sample size	**200 mg**	**300 mg**
Element (method)	**n=9**	**n=2**
Ag (ICP-MS)	0.05	0.06
Al (ICP)	6.32	24.6
As (ICP)	2.53	1.97
Cd (ICP)	0.25	0.07
Cr (ICP)	0.63	0.20
Cu (ICP)	0.63	0.20
Fe (ICP)	1.27	9.83
Hg (CVAAS)	0.03	0.02
Mn (ICP)	0.25	0.10
Ni (ICP)	0.63	0.49
Pb (ICP-MS)	0.06	0.07
Sb (ICP-MS)	N/A	0.10
Se (AFS)	0.03	0.03
Si (ICP)	N/A	246
Sn (ICP-MS)	0.13	0.20
Zn (ICP)	1.18	0.20

Note: Tissue MDLs were calculated by averaging the concentration for each analyte for all blanks analyzed with the 2003 samples (not including Great Lakes samples which were analyzed with 2004 samples). Sediment MDLs were calculated by averaging the concentration for each analyte for all blanks analyzed with the 2003 sediments.

* All samples from 2000-2003 were analyzed in 2003.

Table 3. Mussel Watch Project tissue major and trace elements, method limits of detection (µg/g dry weight) for 2004.

	Tissue MDL	Sediment MDL
Sample Size	**200 mg**	**300 mg**
	n=9	**n=2**
Ag (ICP-MS)	0.04	0.06
Al (ICP)	4.88	24.6
As (ICP)	1.95	1.97
Cd (ICP)	0.20	0.07
Cr (ICP)	0.49	0.20
Cu (ICP)	0.49	0.20
Fe (ICP)	1.10	9.83
Hg (CVAAS)	0.01	0.02
Mn (ICP)	0.20	0.10
Ni (ICP)	0.49	0.49
Pb (ICP-MS)	0.043	0.07
Sb (ICP-MS)	N/A	0.10
Se (AFS)	0.05	0.03
Si (ICP)	N/A	246
Sn (ICP-MS)	0.09	0.20
Zn (ICP)	0.50	0.20

8.0 REFERENCES

Federal Register. 1984. vol. 49, No. 209:198-199.

Appendix 2: Organic Methods

This document describes the analytical methods used to quantify core organic chemicals in tissue and sediment collected as part of NOAA's National Status and Trends Program (NS&T) for the years 2000-2006. Organic contaminat analytical methods used during the early years of the program are described in NOAA Technical Memoranda NOS ORCA 71 and 130 (Lauenstein and Cantillo, 1993; Lauenstein and Cantillo, 1998) for the years 1984-1992 and 1993-1996, respectively. These reports are available from our website (http://www.ccma.nos.gov)

The methods detailed in this document were utilized by the Mussel Watch Project and Bioeffects Project, which are both part of the NS&T program. The Mussel Watch Project has been monitoring contaminants in bivalves and sediments since 1986 and is the longest active national contaminant monitoring program operating in U.S. costal waters. Approximately 300 Mussel Watch sites are sampled on a biennial and decadal timescale for bivalve tissue and sediment respectively. Similarly, the Bioeffects Assessment Project began in 1986 to characterize estuaries and near coastal environs. Using the sediment quality triad approach that measures; (1) levels of contaminants in sediments, (2) incidence and severity of toxicity, and (3) benthic macrofaunal conmmunities, the Bioeffects Project describes the spatial extent of sediment toxicity. Contaminant assessment is a core function of both projects. These methods, while discussed here in the context of sediment and bivalve tissue, were also used with other matricies including: fish fillet, fish liver, nepheloid layer, and suspended particulate matter.

The methods described herein are for the core organic contaminants monitored in the NS&T Program and include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), butyltins, and organochlorines that have been analyzed consistently over the past 15-20 years. Organic contaminants such as dioxins, perfluoro compounds and polybrominated biphenyl ethers (PBDEs) were analyzed periodically in special studies of the NS&T Program and will be described in another document.

All of the analytical techniques described in this document were used by B&B Laboratories, Inc, an affiliate of TDI-Brook International, Inc. in College Station, Texas under contract to NOAA. The NS&T Program uses a performance-based system approach to obtain the best possible data quality and comparability, and requires laboratories to demonstrate precision, accuracy, and sensitivity to ensure results-based performance goals and measures.

REFERENCES

Lauenstein, G. G. and A. Y. Cantillo (eds.) (1998) Sampling and analytical methods of the National Status and Trends Program Mussel Watch Project 1993-1996 Update: TERL Trace Element Quantification Techniques, Volume III. NOAA Technical Memorandum NOS ORCA 71, Silver Spring, MD. 219 pp.

Lauenstein, G. G. and A. Y. Cantillo (eds.) (1993) Sampling and analytical methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992: Comprehensive descriptions of elemental analytical methods, Volume III. NOAA Technical Memorandum NOS ORCA 71, Silver Spring, MD. 219 pp.

EXTRACTION OF SEDIMENTS FOR AROMATIC AND CHLORINATED HYDROCARBONS AND POLYBROMINATED FLAME RETARDANTS 2000 – 2006

Juan. A. Ramirez, Bo Wang, Donell S. Frank, Thomas. J. McDonald, Rebecca Price, Susanne J. McDonald and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Determining organic contaminant levels in sediments requires the extraction, isolation, and concentration of analytes from the matrix. Sediment samples are dried, pulverized, and homogenized prior to extraction. Sediments are extracted in dichloromethane using a Dionex® Accelerated Solvent Extractor. The extracts are purified using alumina/silica gel chromatography columns. The volume of the resultant eluent is reduced and analyzed for aromatic and chlorinated hydrocarbons and polybrominated flame retardants by gas chromatography/mass spectrometry and gas chromatography/electron capture detection.

1.0 INTRODUCTION

The procedure described is used to extract, isolate, purify, and concentrate aromatic and chlorinated hydrocarbons and polybrominated flame retardants from sediments. Contaminant concentrations in parts per billion or parts per trillion can be resolved in sediments. Sediment samples are homogenized, dried, and ground using a mortar and pestle. Approximately 15 g of dry sediment are extracted with 100% dichloromethane using a Dionex Accelerated Solvent Extractor (ASE200). The extracts are reduced in volume and then purified using alumina/silica gel column chromatography. Extracts are reduced to 1 mL and analyzed for PAHs, PCBs, pesticides, PBBs, and PBDEs by gas chromatography.

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Dionex, ASE200 Accelerated Solvent Extractor (ASE) with 33 mL extraction cells
Water bath, capable of maintaining a temperature of 55-60 °C
Balance, top loading, tare capacity to 300 g, capable of weighing to 1 mg
Microbalance, capable of weighing to 1 μg
Calibrated weights, certified
Combustion furnace, electric, capable of combusting glassware at 400 °C for at least 4 hours
Oven, capable of maintaining 40 °C temperature maintenance
Conditioning oven, electric, gravity convection, capable of maintaining a stable temperature of up to 200 °C
Tumbler, Lortone rock tumbler or equivalent
Collection vials, 60 mL certified pre-cleaned with open screw caps and Teflon lined septa
Micropipettors, calibrated, 1% accuracy, disposable tips
250 mL flat bottom, boiling flasks
Borosilicate glass chromatography columns, 300 mm x 19 mm, with Teflon stopcock
Kurderna-Danish (K-D tubes), 25 mL, slow dry concentrator tubes
Synder columns, 3-ball
Boiling chips, Teflon
Glass wool

2.2 REAGENTS

Water (CAS 7732-18-5), gas chromatography/HPLC grade or equivalent purity
Acetone (CAS 67-64-1), pesticide grade or equivalent purity
Dichloromethane (CAS 75-09-2), pesticide grade or equivalent purity
Hexane (110-54-3), pesticide grade or equivalent purity
Pentane (109-66-0), pesticide grade or equivalent purity
Copper (CAS 7440-50-8), granular, 20-30 mesh, ACS reagent grade, purified with hydrochloric acid
Hydrochloric acid (7647-01-0), ACS reagent grade
Sodium sulfate (CAS 7757-82-6), anhydrous granular powder, ACS reagent grade, purified by combusting at 400 °C for at least 4 hours and stored at 120°C.
Alumina (CAS 1344-28-1), 80-325 mesh, basic or neutral, purified by combusting at 400°C for at least 4 hours and stored at 120 °C
Silica gel (CAS 1343-98-2), grade 923, 100-200 mesh, purified in an oven at 170 °C for at least 16 hours and stored at 170 °C
Nitrogen (CAS 7727-37-9), 99.8% purity

3.0 PROCEDURE

Sediment samples are frozen upon receipt from the field at –20°C in the contract laboratory. Prior to extraction, sediment samples are thawed and homogenized using a stainless steel spatula. A subsample is removed for percent moisture determination (McDonald *et al.,* 2006). At least 15 grams of sample are dried in an oven at 40 °C and then ground and homogenized using a mortar and pestle. Approximately 15 g of the dried sediment are extracted.

Sediments are extracted with dichloromethane using an ASE200. Dried samples are loaded into 33 mL stainless steel ASE extraction cells. Appropriate surrogate and spikes are added to the top of samples. The ASE extractor tubes are sealed and placed in the ASE cell carousel. The ASE conditions are: 100% dichloromethane as the extraction solvent, 2,000-psi solvent pressure, 100°C cell temperature, and 2 static cycles for 2 minutes each. Extracts are collected in 60 mL collection vials. The extracts are reduced to approximately 10 mL in the 60 mL collection vials in a 55-60 °C water bath. Extracts are then quantitatively transferred to Kurderna-Danish (K-D) tubes and the reduced to 1.0 mL in a 55-60 °C water bath. Quality control samples (e.g., blanks, duplicates, matrix spikes and standard reference materials) are prepared and extracted in the same manner as samples.

Extracts are purified using alumina/silica gel chromatography columns. Combusted and cooled alumina is deactivated by adding 1% (w/w) HPLC water and tumbled for at least 1 hour using a Lortone rock tumbler. Combusted, cooled silica gel is deactivated by adding 5% (w/w) HPLC water and tumbled for at least 1 hour using a Lortone rock tumbler. Borosilicate glass columns (300 mm x 19 mm) are filled with dichloromethane and packed from the bottom with: glass wool, 1-2 g of sodium sulfate, 10 g of deactivated alumina, 20 g of deactivated silica gel, and another 1-2 g of sodium sulfate. The dichloromethane is drained to the top of the column followed by the addition of 50 mL of pentane. The pentane is drained from the top of the upper sodium sulfate layer and discarded. The sample extract (approximately 1 mL) is added to the top of the column and eluted with 200 mL of a 50:50 mixture of pentane and dichloromethane at a flow rate of 1 mL/min. The eluent is collected in a 250 mL flat-bottom flask and reduced to approximately 10 mL in a 55-60 °C water bath. The extract is transferred to a 25 mL K-D container and reduced to 1 mL. The dichloromethane is exchanged with hexane and reduced to a final volume of 1 mL. The concentrate is transferred to a 2 mL amber vial containing pre-cleaned copper granules (copper granules are carefully mixed with concentrated hydrochloric acid followed by thorough rinsing with HPLC-grade water) and stored at 20 °C until analysis. High sulfur-containing samples may require additional copper granules. Additional pre-cleaned copper granules are added if the initial batch of copper granules turns black, indicating the presence of excess sulfur in the extract. Figure 2-1 shows a flow chart of the extraction and purification procedure.

4.0 QUALITY CONTROL (QC)

Solvents are verified to be contaminant-free by lot tests prior to use. All equipment and glassware used to extract samples are thoroughly cleaned by solvent rinsing or combustion at 400 °C. The calibration and accuracy of balances, weights, pipettors and thermometers are checked daily using certified weights and thermometers with calibrations traced to the National Institute of Standards and Technology (NIST). The calibration and accuracy of balances, weight, pipettors, and thermometers are verified yearly by an independent source. A series of quality control samples are processed with each batch of 20 samples or less. The following quality controls are used to ensure the accuracy and precision of sediment data.

- Surrogates. Solutions containing analytes that do not interfere with the analytes of interest are prepared at concentrations approximately 5 to 10 times the method detection limit (MDL). Specified surrogates are added to each sample extracted, including QC samples, at a specified volume (typically 100 μL) immediately prior to extraction.

- Method Blank. Method blanks are extractions of all support material used for extraction of samples, with the exception of sediment. A method blank is analyzed with each extraction batch of 20 or fewer samples. The method blank is extracted and analyzed in a manner identical to samples.

- Matrix Spike. Matrix spikes are extractions of sample matrix fortified with spikes of selected target analytes. Spikes are prepared at concentrations approximately 10 times the MDL. A matrix spike and matrix spike duplicate are analyzed with each extraction batch of 20 or fewer samples. Matrix spikes are extracted and analyzed in a manner identical to samples.

- Laboratory Duplicates. A sample is analyzed in duplicate with each extraction batch of 20 or fewer samples.

- Standard Reference Material (SRM). A sediment standard reference material from NIST (SRM 1941b) is analyzed with each extraction batch of 20 or fewer samples for aromatic and chlorinated hydrocarbons only.

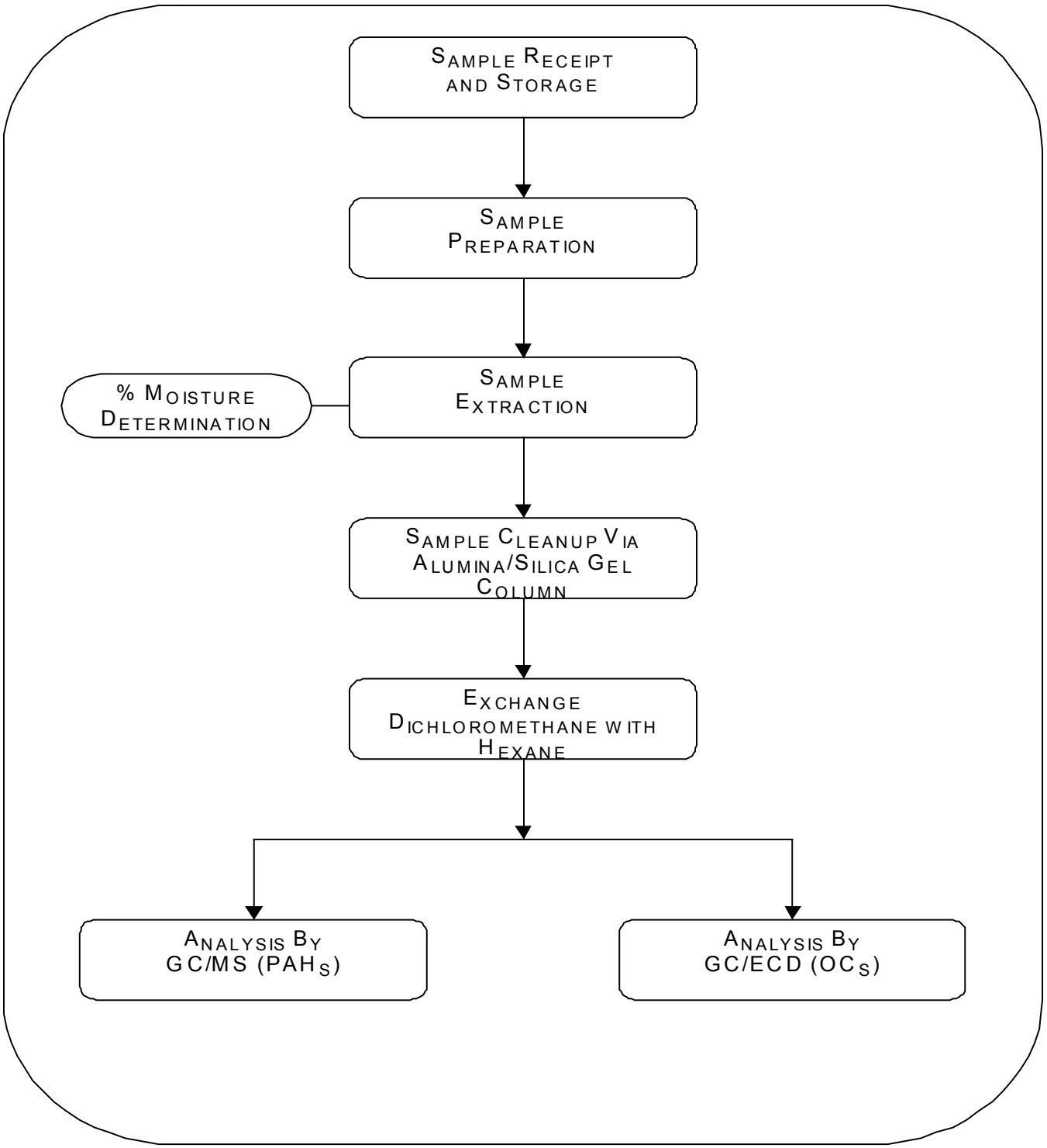


Figure 2-1. Methodology for extraction, isolation and quantification of sediment samples for polycyclic aromatic hydrocarbons (PAHs) and organic contaminants (OC).

5.0 REFERENCES

McDonald, S. J., D. S. Frank, J. A. Ramirez, B. Wang, and J. M. Brooks. 2006. Ancillary Methods of the National Status and Trends Program: 2000-2006 Update. Silver Spring, MD. NOAA Technical

Memorandum NOS NCCOS 28. 17 pp.

EXTRACTION OF BIOLOGICAL TISSUES FOR AROMATIC AND CHLORINATED HYDROCARBONS AND POLYBROMINATED FLAME RETARDANTS 2000 – 2006

Donell. S. Frank, Bo Wang, Juan A. Ramirez, Susanne J. McDonald, Rebecca Price, and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Determining organic contaminant levels in tissues require extraction, isolation, and concentration of analytes from the matrix. Tissue extracts require extensive purification procedures to remove lipids, which cause analytical interferences. Bivalves are shucked and homogenized. Aliquots of homogenized sample are chemically dried using Hydromatrix® and extracted in dichloromethane using a Dionex Accelerated Solvent Extractor. The extracts are purified using alumina/silica gel chromatography columns. The volume of the resultant eluent is further purified using a gel permeation column coupled to a high performance liquid chromatograph. The volume of the resultant eluant is reduced and analyzed for aromatic and chlorinated hydrocarbons and polybrominated flame retardants by gas chromatography.

1.0 INTRODUCTION

The procedure described is used to extract, isolate, purify, and concentrate aromatic, chlorinated hydrocarbons, and polybrominated flame retardant contaminants from tissues. Shell length and shell volume are determined for specimens collected at each location. Bivalves are shucked and multiple organisms are processed as one sample to ensure the sample is representative of a population at a given site and to have sufficient sample to complete the analyses. Tissue samples are homogenized using a stainless steel blender with titanium blades. Aliquots of approximately 15 g of wet tissue are chemically dried with Hydromatix®. The tissue/Hydromatix® mixtures are extracted with 100% dichloromethane using a Dionex Accelerated Solvent Extractor (ASE200) operated at 100 °C and 2,000 psi. The extracts are reduced to 3 mL by evaporative solvent reduction. A 100 µL aliquot is removed and weighed to determine lipid weight (McDonald *et al.,* 2006). The remaining sample portion is purified using alumina/silica gel column chromatography and gel permeation column (GPC)/high performance liquid chromatography (HPLC). After HPLC purification, the eluents are reduced to 0.5 mL and analyzed for PAHs, PCBs, pesticides, PBBs, and PBDEs by gas chromatography.

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Dionex®, ASE200 Accelerated Solvent Extractor (ASE) with 33 mL extraction cells
Water bath, capable of maintaining a temperature of 55-60 °C
Balance, top loading, tare capacity to 300 g, capable of weighing to 1 mg
Microbalance, capable of weight to 1 μg
Calibrated weights, certified
Combustion furnace, electric, capable of combusting glassware at 400 °C for at least 4 hours
Oven capable of maintaining 40°C temperature maintenance
Conditioning oven, electric, gravity convection, capable of maintaining a stable temperature of up to 200 °C
Tumbler, Lortone rock tumbler or equivalent
HPLC system, Water Model 590 programmable solvent delivery module HPLC pump, Waters 717 plus autosampler, Waters UV absorbance detector, Waters 746 data module, Waters Fraction Collector, Phenogel 10μ GPC 100Å size exclusion columns and Phenogel 100Å guard column.
Glass fiber filter circles, 2.4 cm diameter
Collection vials, 60 mL certified pre-cleaned with open screw caps and Teflon lined septa
Micropipettors, calibrated, 1% accuracy, disposable tips
Zymark®, 50 mL concentration tubes
250 mL flat bottom, boiling flasks
Borosilicate glass chromatography columns, 300 mm x 19 mm, with Teflon stopcock
Kurderna-Danish (K-D) tubes, 25 mL, slow dry concentrator tubes
Synder columns, 3-ball
Boiling chips, Teflon
Glass wool

2.2 REAGENTS

Water (CAS 7732-18-5), gas chromatography/HPLC grade or equivalent purity
Acetone (CAS 67-64-1), pesticide grade or equivalent purity
Dichloromethane (CAS 75-09-2), pesticide grade or equivalent purity
Hexane (CAS 110-54-3), pesticide grade or equivalent purity
Pentane (CAS 109-66-0), pesticide grade or equivalent purity
Hydromatrix® (CAS 68855-54-9/14464-46-1), conditioned by combustion at 400 °C for at least 4 hours and stored at 120°C
Sodium sulfate (CAS 7757-82-6), anhydrous granular powder, ACS reagent grade, purified by combusting at 400°C for at least 4 hours and stored at 120°C.
Alumina (CAS 1344-98-2), 80-325 mesh, basic, purified by combusting at 400 °C for at least 4 hours and stored at 120 °C
Silica gel (CAS 1343-98-2), grade 923, 100-200 mesh, purified in an oven at 170 °C for at least 16 hours and store at 170°C
Nitrogen (CAS 7727-37-9), 99.8% purity

2.3 PROCEDURE

Shell length and volume are determined for all bivalves collected at each sampling site. The bivalves are then shucked and the soft tissue homogenized using a stainless steel Waring® blender. Homogenized tissue samples are frozen at –20 ºC until extraction. Prior to extraction, tissue samples are thawed and re-homogenized using a stainless steel spatula. A subsample is removed for percent moisture determination (McDonald *et al*., 2006).

Approximately 15 g of tissue are thoroughly mixed and ground with a sufficient quantity (approximately 40 g) of prepared (combusted) Hydromatrix® to "dry" the sample. The tissue samples must be thoroughly dry to optimize the extraction efficiency. Hydromatrix® chemically dries samples by binding moisture. The amount of Hydromatrix® necessary to dry a sample depends upon the amount of sample and the percent moisture in that sample.

Tissues are extracted with dichloromethane using an ASE200. The tissue/Hydromatix® mixture is loaded into 33 mL ASE extraction cells. Appropriate surrogates and spikes are added to the top of the samples. The ASE extractor tubes are sealed and placed in the ASE cell carousel. The ASE conditions are: 100% dichloromethane as the extraction solvent, 2,000-psi solvent pressure, 100 °C cell temperature, and 2 static cycles for 2 minutes each. Extracts are collected in 60 mL collection vials. The extracts are reduced to approximately 10 mL in the 60 mL collection vials in a 55-60 °C water bath. Extracts are then quantitatively transferred to Kurderna-Danish (K-D) tubes and the volume reduced to 3 mL in a 55-60 °C water bath. A 100 µL aliquot is removed and weighed to determine lipid content (McDonald *et al.*, 2006). Quality control samples (e.g., blanks, duplicates, matrix spikes and standard reference materials) are prepared and extracted in the same manner as samples.

Extracts are initially purified using alumina/silica gel chromatography columns. Combusted and cooled alumina is deactivated by adding 1% (w/w) HPLC water and tumbled for at least 1 hour using a Lortone rock tumbler. Combusted and cooled silica gel is deactivated by adding 5% (w/w) HPLC water and tumbling for at least 1 hour using a Lortone rock tumbler. Borosilicate glass columns (300 mm x 19 mm) are filled with dichloromethane and packed from the bottom with: glass wool, 1-2 g of sodium sulfate, 10 g of deactivated alumina, 20 g of deactivated silica gel, and another 1-2 g of sodium sulfate. The dichloromethane is drained to the top of the column followed by the addition of 50 mL of pentane. The pentane is drained from the top of the upper sodium sulfate layer and discarded. The sample extract (approximately 3 mL) is added to the top of the column and eluted with 200 mL of a 50:50 mixture of pentane and dichloromethane at a flow rate of 1 mL/min. The eluent is collected in a 250 mL flat-bottom flask. The eluent is reduced to approximately 10 mL in a 55-60°C water bath. The extract is transferred to 25 mL K-D tubes and reduced to1-2 mL. The concentrate is transferred to 4 mL amber HPLC vials and brought up to 4 mL with dichloromethane.

The extract is further purified using HPLC. The extract is injected using a Waters, Model 717 Plus autosampler and eluted through one Phenogel 100Å guard column and two Phenogel 10µ GPC 100Å size exclusion columns with 100% dichloromethane at a flow rate of 7 mL per minute. Elution times for compounds of interest are monitored using standards and a UV absorbance detector (254 nm). The appropriate fraction is collected using a Waters Fraction Collector. The sample is collected in 50 mL Zymark tubes and reduced to 10 mL in a 50-60 ºC water bath. The extract is transferred to K-D tubes and reduced to 1.0 mL. The dichloromethane is exchanged with hexane and reduced to a final volume of 0.5 mL. The concentrate is transferred to 2 mL amber vials and stored at 20 °C until analysis. Figure 3-1 shows a flow chart of the extraction and purification procedure.

1.0 QUALITY CONTROL (QC)

Solvents are verified to be contaminant-free by lot tests prior to use. All equipment and glassware used to extract samples are thoroughly cleaned by solvent rinsing or combustion at 400 °C. The calibration and accuracy of

balances, weights, pipettors and thermometers are checked daily using certified weights and thermometers with calibrations traced to the National Institute of Standards and Technology (NIST). The calibration and accuracy of balances, weight, pipettors and thermometers are verified yearly by an independent source. A series of quality control samples are processed with each batch of 20 samples or less. The following quality controls are used to ensure the accuracy and precision of tissue data.

- Surrogates. Solutions containing analytes that do not interfere with the analytes of interest are prepared at concentrations approximately 5 to 10 times the method detection limit (MDL). Specified surrogates are added to each sample extracted, including QC samples, at a specified volume (typically 100 μL) immediately prior to extraction.

- Method Blank. Method blanks are extractions of all support material used for extraction of samples, with the exception of tissue. A method blank is analyzed with each extraction batch of 20 or fewer samples. The method blank is extracted and analyzed in a manner identical to samples.

- Matrix Spike. Matrix spikes are extractions of sample matrix fortified with spikes of selected target analytes. Spikes are prepared at concentrations approximately 10 times the MDL. A matrix spike and matrix spike duplicate are analyzed with each extraction batch of 20 or fewer samples. Matrix spikes are extracted and analyzed in a manner identical to samples.

- Laboratory Duplicates. A sample is analyzed in duplicate with each extraction batch of 20 or fewer samples.

- Standard Reference Material (SRM). A standard reference material from the NIST (SRM 1974a) is analyzed with each extraction batch of 20 or fewer samples for aromatic and chlorinated hydrocarbons.

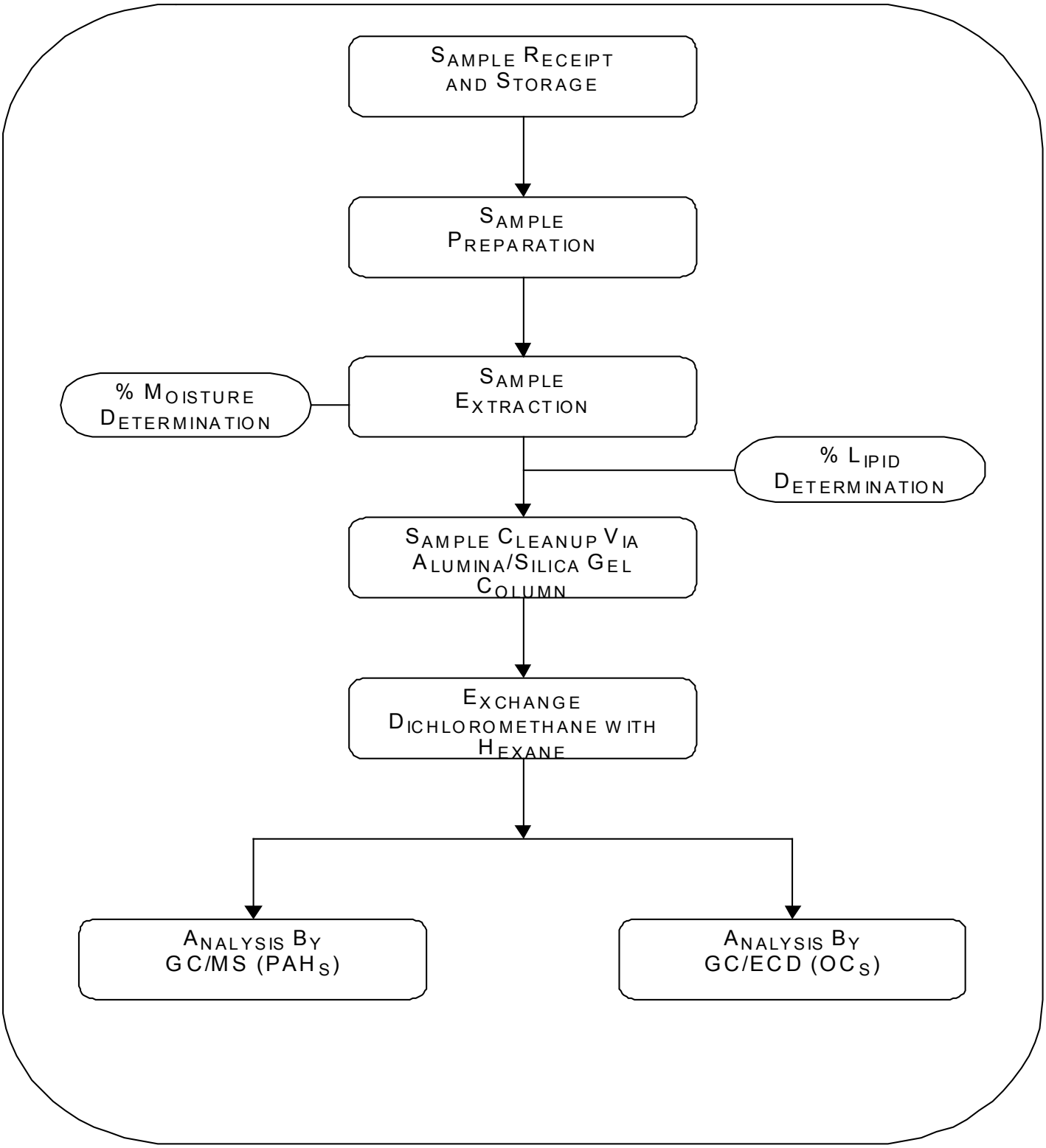


Figure 3-1. Methodology for extraction, isolation, and quantification of tissue samples for polycyclic aromatic hydrocarbons (PAHs) and organic contaminants (OC).

5.0 REFERENCES

McDonald, S. J., D. S. Frank, J. A. Ramirez, B. Wang, and J. M. Brooks. 2006. Ancillary Methods of the National Status and Trends Program: 2000-2006 Update. Silver Springs, MD. NOAA Technical Memorandums NOS NCCOS 28. 17 pp.

QUANTITATIVE DETERMINATION OF CHLORINATED HYDROCARBONS USING GAS CHROMATOGRAPHY/ELECTRON CAPTURE DETECTION 2000 – 2005

Juan A. Ramirez, Donell S. Frank, Susanne J. McDonald and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Selected chlorinated hydrocarbons, including polychlorinated biphenyls and pesticides, are detected using gas chromatograph/electron capture detector. This method is capable of detecting low concentration of chlorinated hydrocarbons in complex matrices such as tissues and sediments.

1.0 INTRODUCTION

A gas chromatograph/electron capture detector (GC/ECD), coupled to two capillary columns, is used to resolve and detect chlorinated hydrocarbons (polychlorinated biphenyls and pesticides) in tissues and sediments. Samples are injected into a temperature-programmed GC/ECD, operated in splitless mode. The capillary columns are DB-5 (30 m x 0.25 mm ID and 25 μm film thickness) and DB-17HT (30 m x 0.25 mm ID and 0.15 μm film thickness). The DB-17HT column is used for analyte confirmation. A data acquisition system continuously acquires and stores all data for quantitation. This method is capable of producing data at parts-per billion and parts-per trillion concentrations.

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Gas chromatograph, split/splitless injection port and electronic pressure control, dual electron capture detectors, Agilent Technologies 5890-II
Data acquisition system, Agilent Technologies ChemStation, capable of continuous acquisition and storage of all data during analysis
Autosampler, capable of making 1 to 5 μL injections
Capillary columns, J&W DB-5®(30 m x 0.25 mm ID and 0.25 μm film thickness) or equivalent, and J&W DB-17HT®(30 m X 0.25 mm ID and 0.15 μm film thickness)
Micropipetters, calibrated, 1% accuracy, disposable tips

1.2 REAGENTS

Hexane (CAS 110-54-3), pesticide grade or equivalent purity
Helium (CAS 7440-59-7), 99.8% purity
95% Argon/5% Methane, 99.8% purity

2.3 STANDARDS

2.3.1 SURROGATE SPIKING SOLUTION

A surrogate spiking solution is prepared from a commercially available solution (Ultra Scientific) that is diluted with hexane to a concentration of 1,000 pg/μL. The surrogate spiking solution includes 4,4'-dibromooctaflurobiphenyl (DBOFB), 2,2',4,5',6 pentachlorobiphenyl (PCB 103), and 2,2',3,3',4,5,5'6 octachlorobiphenyl (PCB 198). Surrogate solution (100 μL) is added to all samples and quality control samples prior to extraction. Surrogate compounds are resolved from, but elute in close proximity to, the analytes of interest. The recovery of PCB 103 is used to correct analyte concentrations.

2.3.2 INTERNAL STANDARD SOLUTION

The internal standard solution is prepared from a commercially available solution (Ultra Scientific) of tetrachloro-m-xylene (TCMX) diluted with hexane to a final concentration of 1,000 pg/μL. The internal standard compound is resolved from, but elutes in close proximity to, the analytes of interest. The internal standard solution (100 μL) is added to all samples and quality control samples just prior to analysis. Internal standards are used to calculate relative response factors and specific analyte concentrations based on retention time.

2.3.3 MATRIX SPIKING SOLUTION

To prepare the matrix spiking solution, a certified solution (Accustandard) containing analytes of interest is purchased from commercial vendors and diluted with hexane (Table 4-1). The matrix spike solution is diluted to a concentration approximately 10 times the MDL and is added to all matrix spike samples.

2.3.4 CALIBRATION SOLUTION

Calibration solutions are prepared at 5 concentrations ranging from approximately 5 to 200 pg/μL (Table 2) by diluting a commercially prepared solutions (Ultra Scientific and Accustandard) containing the analytes of interest.

Table 4-1. Chlorinated hydrocarbons contained in matrix spike solution.

Compound Name	CAS	Spiking Solution Concentration (pg/μL)
1,2,4,5-Tetrachlorobenzene	95-94-3	40
1,2,3,4-Tetrachlorobenzene	634-66-22	40
Pentachlorobenzene	608-93-5	40
Pentachloroanisole	1825-21-4	40
Chlorpyrifos	2921-88-2	40
Hexachlorobenzene	118-74-1	40
α-HCH	319-84-6	40
β-HCH	319-85-7	40
γ–HCH (Lindane)	55-89-9	40
δ-HCH	319-86-8	40
Heptachlor	76-44-8	40
Heptachlor epoxide	1024-57-3	40
α-Chlordane (cis-)	5103-71-9	40
γ -Chlordane (trans-)	5103-74-2	40
Trans-nonachlor	39765-80-5	40
Cis-nonachlor	5103-73-1	40
Aldrin	309-00-2	40
Dieldrin	60-57-1	40
Endrin	72-20-8	40
Mirex	2385-85-5	40
2,4’ DDE	3424-82-6	40
4,4’ DDE	72-55-9	40
2,4’ DDD	53-19-0	40
4,4’ DDD	72-54-8	40
2,4’ DDT	789-02-6	40
4,4’ DDT	50-29-3	40
Endosulfan II	33213-65-9	40
Oxychlordane	27304-13-8	40
Endosulfan sulfate	1031-07-8	40
PCB 8	34883-43-7	40
PCB 18	37680-65-2	40
PCB 28	7012-37-5	40
PCB 44	41464-39-5	40
PCB 52	35693-99-3	40
PCB 66	32598-10-0	40
PCB 101	37680-73-2	40
PCB 105	32598-14-4	40
PCB 118	31508-00-6	40
PCB 128	38380-07-3	40
PCB 138	35065-28-2	40
PCB 153	35065-27-1	40
PCB 170	35065-30-6	40
PCB 180	35065-29-3	40
PCB 187	52663-68-0	40
PCB 195	52663-78-2	40
PCB 206	40186-72-9	40
PCB 209	2051-24-3	40

Table 4-2. Chlorinated hydrocarbons contained in calibration solutions and their approximate concentrations.

Compounds Contained in Calibration Solutions	CAS	Level 1 (pg/μL)	Level 2 (pg/μL)	Level 3 (pg/μL)	Level 4 (pg/μL)	Level 5 (pg/μL)
Internal Standard						
TCMX	877-9-8	100	100	100	100	100
Surrogates						
DBOFB	10386-84-2	5	20	40	80	200
PCB 103	60145-21-3	5	20	40	80	200
PCB 198	68194-17-2	5	20	40	80	200
Analytes						
1,2,4,5-Tetrachlorobenzene	95-94-3	5	20	40	80	200
1,2,3,4-Tetrachlorobenzene	634-66-22	5	20	40	80	200
Pentachlorobenzene	608-93-5	5	20	40	80	200
Pentachloroanisole	1825-21-4	5	20	40	80	200
Chlorpyrifos	2921-88-2	5	20	40	80	200
Hexachlorobenzene	118-74-1	5	20	40	80	200
α-HCH	319-84-6	5	20	40	80	200
β-HCH	319-85-7	5	20	40	80	200
γ–HCH (Lindane)	319-86-6	5	20	40	80	200
δ-HCH	58-89-9	5	20	40	80	200
Heptachlor	76-44-8	5	20	40	80	200
Heptachlor epoxide	1024-57-3	5	20	40	80	200
Oxychlordane	27304-13-8	5	20	40	80	200
α-Chlordane (cis-)	5103-71-9	5	20	40	80	200
γ-Chlordane (trans-)	5103-74-2	5	20	40	80	200
Trans-nonachlor	39765-80-5	5	20	40	80	200
Cis-nonachlor	5103-73-1	5	20	40	80	200
Aldrin	309-00-2	5	20	40	80	200
Dieldrin	60-57-1	5	20	40	80	200
Endrin	72-20-8	5	20	40	80	200
Mirex	2385-85-5	5	20	40	80	200
2,4’-DDE	3424-82-6	5	20	40	80	200
4,4’-DDE	75-55-9	5	20	40	80	200
2,4’-DDD	53-19-0	5	20	40	80	200
4,4’-DDD	72-54-8	5	20	40	80	200
2,4’-DDT	789-02-6	5	20	40	80	200
4,4’-DDT	50-29-3	5	20	40	80	200
Endosulfan II	33213-65-9	5	20	40	80	200
Endosulfan sulfate	1031-07-8	5	20	40	80	200

Table 4-2 cont'd. Chlorinated hydrocarbons contained in calibration solutions and their approximate concentrations.

Compounds Contained in Calibration Solutions	CAS	Level 1 (pg/μL)	Level 2 (pg/μL)	Level 3 (pg/μL)	Level 4 (pg/μL)	Level 5 (pg/μL)
PCB 8	34883-43-7	5	20	40	80	200
PCB 18	37680-65-2	5	20	40	80	200
PCB 28	7012-37-5	5	20	40	80	200
PCB 44	41464-39-5	5	20	40	80	200
PCB 52	35693-99-3	5	20	40	80	200
PCB 66	32598-10-0	5	20	40	80	200
PCB 101	37680-73-2	5	20	40	80	200
PCB 105	32598-14-4	5	20	40	80	200
PCB 118	31508-00-6	5	20	40	80	200
PCB 128	38380-07-3	5	20	40	80	200
PCB 138	35065-28-2	5	20	40	80	200
PCB 153	35065-27-1	5	20	40	80	200
PCB 170	35065-30-6	5	20	40	80	200
PCB 180	35065-29-3	5	20	40	80	200
PCB 187	52663-68-0	5	20	40	80	200
PCB 195	52663-78-2	5	20	40	80	200
PCB 206	40186-72-9	5	20	40	80	200
PCB 209	2051-24-3	5	20	40	80	200

3.0 QUANTITATIVE DETERMINATION OF CHLORINATED HYDROCARBONS BY GC/ECD

3.1 CALIBRATION

An ECD exhibits limited linearity, particularly for low concentrations. Consequently, a calibration must be established for each analytical run. An analytical run consists of samples and 5 calibration standards (approximately 5 to 200 pg/μL or 5 to 200 ng/mL) that are interspersed throughout the run. A calibration curve is established by analyzing the 5 interspersed calibration standards and fitting the data to the following quadratic equation.

$$x = \frac{-b_1 + \sqrt{b_1^2 - 4b_2(b_0 - Y)}}{2b_2}$$

Where:

x = the concentration of the analyte (ng/mL)

Y = the ratio of the area of the analyte to the area of the internal standard multiplied by the amount of the internal standard (ng)

b_2, b_1, b_0 = the coefficients for the quadratic equation

The data generated for each analyte in the calibration standards are subjected to the method of least squares to determine the coefficients for the corresponding quadratic equation. Each analyte has different coefficients based on the relative response of the analyte compared to the internal standard, and as a function of the amount of the analyte. The injected concentration of the internal standard analyte is held constant for each set of calibration standards. In order for the calibration to be valid, each analyte must have a correlation coefficient greater than 0.997.

1.2 GC/ECD ANALYSIS

Sample analyses are completed only if the calibration meets previously described criteria. Samples are analyzed in analytical sets that consist of standards, samples, and quality control samples. Quality control (QC) samples are method blanks, laboratory duplicates, matrix spikes, and standard reference material (SRM). An autosampler is used to inject 1 or 5 µL of all samples, standards, and QC samples into the capillary column of the GC using the following instrument conditions. Slight modifications may be necessary depending upon the analysis.

Inlet: Splitless
Carrier gas: Helium, 1 mL/min

Temperatures:
- Injection port: 275 °C
- Detector: 325 °C

Oven program:
- Initial oven temp: 100 °C
- Initial hold time: 1 minute
- Ramp rate: 5 °C/min to 140 °C
- Hold time: 1 minute
- Ramp rate: 1.5 °C/min to 250 °C
- Hold time: 1 minute
- Final oven rate: 10 °C/min to 300 °C
- Final hold time: 5 minutes

1.3 ANALYTE IDENTIFICATION

The retention time of a sample analyte must fall within 15 seconds of the retention time for that analyte in a calibration standard or a retention index solution.

Chromatographic interferences may limit the ability to quantify peaks correctly and these data are reported but qualified to indicate interference.

4.0 QUANTITATION CALCULATIONS

Sample analyte concentrations are calculated based on the concentration and response of the internal standard (Table 4-2). The concentration (C) of each target analyte in the sample (ng/g) is calculated using the following equation:

$$C = \left(\frac{X}{W}\right)(V_e D)$$

Where:
- Ve = the final volume of the extract (mL)
- X = the concentration of the analyte (ng/mL) as found from solving the quadratic equation
- W = the sample weight (g)
- DF = the dilution factor

Analyte concentrations are reported as corrected for surrogate recoveries. Percent surrogate recoveries ($SU_{Recovery}$) for each surrogate are calculated using the following equation:

$$S_{Recovery} = \frac{C_{ESU}}{C_S} x100$$

Where:

C_{ESU} = calculated surrogate concentration in the extract

C_{SU} = known concentration of surrogate added to extract

Analyte concentration corrections ($C_{Corrected}$) for surrogate recovery are calculated using the following equation:

$$C_{Corrected} = \frac{C}{R_{Recovery}} \times 100$$

2.0 QUALITY CONTROL (QC)

Samples are analyzed in analytical batches consisting of 19 samples or fewer and QC samples. The QC samples are a method blank, laboratory duplicate, matrix spike, matrix spike duplicate, and SRM. A method blank is a reagent blank prepared in the laboratory. A duplicate is a sample for which a second aliquot is analyzed. Matrix spikes are samples that are spiked with known concentrations of known analytes. The SRM used depends upon availability, matrix, and analytes. All SRMs are certified and traceable to the National Institute of Standards and Testing (NIST).

The validity of the data is monitored using defined QC criteria. The following QC criteria are used to evaluate analytical batches:

1) Calibration

- The calibration criteria (Section 3.1) must be met prior to data analyses. If the calibration criteria are not met, then the run is aborted and the instrument re-calibrated before further sample analysis.

2) Method Blank

- No more than two target analytes may exceed 3 times the concentration of the MDL.
- Exceptions are that if an analyte detected in the method blank exceeds 3 times the concentration of the MDL, but is not present in the associated samples, or if a sample analyte concentration is greater than 10 times that analyte concentration in the method blank, the result is qualified and reported.
- If a method blank exceeds these criteria then the source of contamination is determined and corrective action is taken before further sample analysis.

3) Matrix Spikes

- Analytes spiked into a matrix are considered valid only if they are spiked at concentrations equivalent to levels found in the sample.
- The average recovery for all valid spiked analytes in a matrix spike is between 60% and 120%. No more than two individual spiked analyte (valid) recoveries may exceed 40% - 120%, with the exception of chlorpyrifos and endosulfan sulfate.
- If the QC criteria are not met then the matrix spike sample failing the criteria will be re-analyzed and if the re-analyzed spike meets the criteria then the data are reported.
- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.

- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the matrix-spike, another sample may be selected or a blank-spike may be substituted.

- The average relative percent difference (RPD) for a valid matrix spike/matrix spike duplicate or blank spike/blank spike duplicate pair is 30%. No more than two individual analyte RPDs may exceed 35%.

4) Duplicate

- The average RPD between the duplicate and original sample, for analytes greater than 10 times the concentration of the MDL is 30%. The RPD for no more than two individual analytes may exceed 35%.

- If the QC criteria are not met then the sample pair failing the criteria will be re-analyzed and if the re-analyzed samples meet the criteria then the data are reported.

- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.

- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the duplicate pair, another sample may be selected.

5). Standard Reference Material

- The average recovery for target analytes in a SRM should not exceed 30% of the upper and lower bounds of the mean certified values. No more than two target analytes should deviate more than 35% from the upper or lower bounds of the mean certified values.

- If the QC criteria are not met then the SRM failing the criteria will be re-analyzed and if the re-analyzed SRM meets the criteria then the data are reported.

- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.

- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed.

6). Surrogates

- The average recovery of surrogate compounds is between 50% and 150%.

- Exceptions are analytical interferences with the surrogates and diluted samples.

- If the average recovery of surrogates exceeds the criteria, and calculation and analytical errors are eliminated, the sample is re-analyzed. If sufficient sample is unavailable for re-extraction, the data are qualified and reported.

7). Method Detection Limit

- The method detection limit (MDL) is determined following the procedures outlined in the Federal Register (1984).

6.0 REFERENCES

Federal Registry (1984) Vol. 49, No. 209:198-199.

QUANTITATIVE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING SELECTED ION MONITORING GAS CHROMATOGRAPHY/MASS SPECTROMETRY 2000 – 2006

Thomas J. McDonald, Bo Wang, Susanne J. McDonald and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Selected polycyclic aromatic hydrocarbons (PAHs), including alkylated homologues, are detected using a gas chromatograph/mass spectrometer in selected ion monitoring mode. This method is capable of detecting low concentrations of PAHs in complex matrices such as tissues and sediments.

1.0 INTRODUCTION

A gas chromatograph/mass spectrometer (GC/MS) in selected ion mode (SIM), coupled to a capillary column, is used to resolve and detect polycyclic aromatic hydrocarbons (PAHs) in tissues and sediments. Samples are injected into a temperature-programmed GC/MS, operated in splitless mode. The capillary column is an HP-5MS (60 m x 0.25 mm ID and 0.25 μm film thickness). The mass spectrometer is capable of scanning from 35 to 500 AMU every second or less and uses 70 electron volts energy in electron impact ionization mode. A data acquisition system continuously acquires and stores all data for quantitation. This method is capable of producing data at parts-per-billion concentrations.

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Gas chromatograph, split/splitless injection port and electronic pressure control, Agilent Technologies 5890-II
Mass spectrometer, capable of scanning from 35 to 500 AMU, utilizing 70 electron volts of energy in impact ionization mode, Agilent Technologies 5972-MSD
Data acquisition system, Agilent Technologies ChemStation, capable of continuous acquisition and storage of all data during analysis
Autosampler, capable of making 1 to 5 μL injections
Capillary column, Agilent Technologies HP-5MS (60 m x 0.25 mm ID and 0.25 μm film thickness)
Micropipetters, calibrated, 1% accuracy, disposable tips

1.2 REAGENTS

Dichloromethane (CAS 75-09-02), pesticide grade or equivalent purity
Helium (CAS 7440-59-7), 99.8% purity

2.3 STANDARDS

SURROGATE SPIKING SOLUTION

The surrogate spiking solution is prepared from aliquots of pure compounds (Absolute Standards, Inc.) that are diluted with dichloromethane to a final concentration of 0.5 µg/mL. The surrogate spiking solution includes naphthalene-d_8, acenaphthalene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12}. Surrogate solution (100 µL) is added to all samples and quality control samples prior to extraction. Surrogate compounds are resolved from, but elute in close proximity to, the analytes of interest. Individual surrogate recoveries are used to correct specific analyte concentrations based on retention time.

INTERNAL STANDARD SOLUTION

The internal standard solution is made from aliquots of pure compounds (Cambridge Isotope Laboratories, Inc.) and diluted with dichloromethane to a final concentration of 0.5 µg/mL. The internal standard solution includes fluorine-d_{10}, pyrene-d_{10}, and benzo(a)pyrene-d_{12}. The internal standard compounds are resolved from, but elute in close proximity to, the analytes of interest. The internal standard solution (100 µL) is added to all samples and quality control samples just prior to instrument analysis. Internal standards are used to calculate relative response factors and specific analyte concentrations based on retention time.

MATRIX SPIKING SOLUTION

Certified solutions containing 2- to 5-ring PAH compounds are purchased from commercial vendors (Chiron, Aldrich and Absolute Standards) and diluted with dichloromethane to prepare the matrix spiking solution (Table 5-1). The matrix spiking solution is diluted to a concentration approximately 10 times the MDL and is added to all matrix spike samples.

CALIBRATION SOLUTION

Calibration solutions are prepared at 5 concentrations ranging from approximately 0.02 to 1 µg/mL (Table 5-2) by diluting commercially available certified solutions containing analytes of interest (Absolute Standards, Inc., Cambridge Isotope Laboratories, Inc., Chiron, Aldrich, and Absolute Standards).

RETENTION INDEX SOLUTIONS

The mid-level calibration standard, containing analytes at approximately 0.25 μg/mL, is used as a retention index solution to determine the retention times of unsubstituted compounds and certain substituted compounds. A crude oil/coal oil standard material is used as a retention index solution for the determination of retention times for the remaining alkyl homologues. The retention index solutions are also used to evaluate instrument retention time drift over time.

Table 5-1. Polycyclic aromatic hydrocarbons contained in the matrix-spiking solution.

Analyte	CAS	Spiking Solution Concentration (ng/mL)
Decalin	97-17-8	1.00
Naphthalene	91-20-3	1.06
2-Methylnaphthalene	91-57-6	1.05
1-Methylnaphthalene	90-12-0	1.06
Benzo[*b*]thiophene	95-15-8	1.86
Biphenyl	92-52-4	1.06
2,6-Dimethylnaphthalene	581-42-0	1.06
Acenaphthylene	208-96-8	1.02
Acenaphthene	83-32-9	1.10
1,6,7-Trimethylnaphthalene	2245-38-7	0.940
Dibenzofuran	132-64-9	1.00
Fluorene	86-73-7	1.05
Pentachlorophenol	87-86-5	4.00
Carbazole	86-74-8	1.00
Anthracene	120-12-7	0.801
1-Methylphenanthrene	832-69-9	1.05
Phenanthrene	85-07-8	1.06
Dibenzothiophene	132-65-01	1.00
Fluoranthene	206-44-0	1.06
Pyrene	129-00-0	1.06
Naphthobenzothiophene	239-35-0	1.00
Benz[*a*]anthracene	56-55-3	0.919
Chrysene	218-01-9	1.07
C30-Hopane	13849-96-2	1.00
Benzo[*b*]fluoranthene	200-99-2	1.06
Benzo[k]fluoranthene	207-08-9	1.05
Benzo[*e*]pyrene	192-97-2	1.06
Benzo[*a*]pyrene	50-32-8	0.955
Perylene	198-55-0	0.800
Indeno[*1,2,3-c,d*]pyrene	193-39-5	0.938
Dibenzo[*a,h*]anthracene	53-70-3	0.794
Benzo[*g,h,i*]perylene	191-24-2	0.945

Table 5-2. Polycyclic aromatic hydrocarbons contained in calibration solutions and their approximate concentrations.

Compounds Contained in Calibration Solutions	CAS	Level 1 (μg/mL)	Level 2 (μg/mL)	Level 3 (μg/mL)	Level 4 (μg/mL)	Level 5 (μg/mL)
Internal Standards						
Fluorene-d_{10}	NA	0.05	0.05	0.05	0.05	0.05
Pyrene-d_{10}	NA	0.05	0.05	0.05	0.05	0.05
Benzo[*a*]pyrene-d_{12}	NA	0.05	0.05	0.05	0.05	0.05
Surrogates						
Naphthalene-d_8	NA	0.02	0.10	0.25	0.50	1.00
Acenaphthene-d_{10}	NA	0.02	0.10	0.25	0.50	1.00
Phenanthrene-d_{10}	NA	0.02	0.10	0.25	0.50	1.00
Chrysene-d_{12}	NA	0.02	0.10	0.25	0.50	1.00
Perylene-d_{12}	NA	0.02	0.10	0.25	0.50	1.00
5β(H)-Cholane	80373-86-0	0.02	0.10	0.25	0.50	1.00
Analytes						
Naphthalene	91-20-3	0.021	0.106	0.267	0.531	1.06
2-Methylnaphthalene	91-57-6	0.021	0.104	0.263	0.524	1.05
1-Methylnaphthalene	90-12-0	0.021	0.105	0.265	0.527	1.05
Benzo[*b*]thiophene	95-15-8	0.037	0.184	0.464	0.928	1.86
Biphenyl	92-52-4	0.021	0.105	0.266	0.529	1.06
2,6-Dimethylnaphthalene	581-42-0	0.021	0.105	0.265	0.527	1.06
Acenaphthylene	208-96-8	0.020	0.101	0.255	0.508	1.02
Acenaphthene	83-32-9	0.022	0.109	0.276	0.548	1.10
1,6,7-Trimethylnaphthalene	2245-38-7	0.019	0.093	0.236	0.470	0.939
Dibenzofuran	132-64-9	0.020	0.100	0.250	0.500	1.00
Fluorene	86-73-7	0.021	0.105	0.264	0.526	1.05
Pentachlorophenol	87-86-5	0.250	0.500	1.000	1.500	2.00
Carbazole	86-74-8	0.020	0.100	0.250	0.500	1.00
Dibenzothiophene	132-65-01	0.020	0.100	0.250	0.500	1.00
Phenanthrene	85-01-8	0.021	0.105	0.266	0.528	1.06
Anthracene	120-12-7	0.016	0.080	0.201	0.400	0.800
1-Methylphenanthrene	832-69-9	0.021	0.104	0.263	0.523	1.05
Fluoranthene	206-44-0	0.021	0.106	0.267	0.530	1.06
Pyrene	129-00-0	0.021	0.105	0.266	0.530	1.06
Naphthobenzothiophene	239-35-0	0.020	0.099	0.250	0.500	1.00
Benz[*a*]anthracene	56-55-3	0.018	0.091	0.231	0.459	0.918
Chrysene	218-01-9	0.021	0.106	0.267	0.532	1.06
C_{30}-Hopane	13849-96-2	0.020	0.100	0.250	0.500	1.00
Benzo[*b*]fluoranthene	200-99-2	0.021	0.105	0.265	0.528	1.06
Benzo[*k*]fluoranthene	207-08-9	0.021	0.105	0.264	0.526	1.05
Benzo[*e*]pyrene	192-97-2	0.021	0.105	0.265	0.528	1.06
Benzo[*a*]pyrene	50-32-8	0.019	0.095	0.238	0.477	0.954
Perylene	198-55-0	0.016	0.080	0.201	0.400	0.799
Indeno[*1,2,3-cd*]pyrene	193-39-5	0.019	0.093	0.236	0.469	0.937
Dibenzo[*a,h*]anthracene	53-70-3	0.016	0.079	0.199	0.396	0.793
Benzo[*g,h,i*]perylene	191-24-2	0.019	0.094	0.237	0.472	0.944

3.0 QUANTITATIVE DETERMINATION OF PAHS BY GC/MS-SIM

3.1 MASS SPECTROMETER TUNING

Prior to calibration, the MS is autotuned using perfluorotributylamine (PFTBA) to criteria established by the instrument manufacturer.

3.2 INITIAL CALIBRATION

A 5-point relative response factor (RRF) calibration curve is established for analytes of interest prior to the analysis of samples and quality control (QC) samples (Table 5-2). A RRF is determined for each analyte for each calibration level using the following equation:

$$RRF = \frac{(A_A)(C_B)}{(A_B)(C_A)}$$

Where:
A_A = the area of the characteristic ion for the analyte to be measured
A_{IS} = the area of the characteristic ion for the specific internal standard
C_A = the known concentration of the analyte in the calibration solution (µg/mL)
C_{IS} = the known concentration of the internal standard in the calibration solution (µg/mg)

The response factors determined for each calibration level are averaged to produce a mean relative response factor ($\overline{RRF_i}$) for each analyte. The percent relative standard deviation (%RSD) for the 5 response factors must be less than or equal to 15%, for each analyte.

$$\%RSD = \frac{\text{Standard Deviation of the RRFs}}{\text{Average of the RRFs}} \times 100$$

Where:

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{(n-1)}}$$

Where:
x_i = each RRF value used to calculate the mean RRF
$\bar{x}$ = the mean of n values
n = total number of values (5)

3.3 CONTINUING CALIBRATION

A mid-level calibration standard is analyzed at the beginning and end of each analytical set or every 10 samples (whichever is more frequent). The daily relative response factor for each compound is compared to the mean relative response factor from the initial calibration curve and the average relative percent difference (RPD) of all analytes must be less than 25%. If the calibration check does not meet this criterion then the initial five-point calibration is repeated.

$$RPD = \frac{RRF_c - \overline{RRF_i}}{\overline{RRF_i}} x\,100$$

Where:

$\overline{RRF_i}$ = mean relative response factor from the most recent initial calibration (meeting technical acceptance criteria)

RRF_c = relative response factor from the continuing calibration standard

1.4 GC/MS-SIM ANALYSIS

The initial calibration of the GC/MS must meet the previously described criteria prior to sample analysis. Samples are analyzed in analytical sets that consist of standards, samples, and QC samples. Quality control samples are method blanks, laboratory duplicates, matrix spikes, and standard reference materials. An autosampler is used to inject 1 or 2 μL of all samples, standards, and QC samples into the capillary column of the GC using the following instrument conditions. Slight modifications may be necessary depending upon the analysis.

Inlet: Splitless
Carrier gas: Helium, 1 mL/min

Temperatures:
- Injection port: 300 °C
- Transfer line: 290 °C

Oven program:
- Initial oven temp: 60 °C
- Initial hold time: 0 minutes
- Ramp rate: 7 °C/min
- Final oven temp: 315 °C
- Final hold time: 22 minutes
- Total run time: 56 minutes

The effluent from the GC capillary column is routed directly into the ion source of the MS. The MS is operated in the selected ion monitoring mode (SIM) and includes the quantitation and confirmation masses for the PAHs listed in Table 5-3. For all compounds detected at a concentration above the MDL, the confirmation ion is checked to confirm the analyte's presence.

1.5 ANALYTE IDENTIFICATION

The extracted ion current profiles of the primary m/z and the confirmatory ion for each analyte must meet the following criteria:

- The characteristic masses of each analyte of interest must be in the same scan or within one scan of each other. The retention time must fall within ±5 seconds of the retention time of the authentic compound or alkyl homologue grouping determined by the analysis of the daily calibration check or reference oil standard.

- The alkylated PAH homologue groupings (e.g. C4-naphthalene) appear as a group of isomers. The pattern of each group and the retention time window for the group is established by the analysis of a reference oil standard. Each group of alkylated homologues is integrated in its entirety and the total area response is used to determine the concentration of the entire group.

- The relative peak heights of the primary mass ion, compared to the confirmation or secondary mass ion, must fall within ±30 percent of the relative intensities of these masses in a reference mass spectrum (Table 5-3). The reference mass spectrum is obtained from the continuing calibration solution or the reference oil standard for the parent compounds and alkylated homologues, respectively. In some instances, a compound that does not meet secondary ion confirmation criteria may still be determined to be present in a sample after close inspection of the data by a qualified mass spectrometrist. Supportive data includes the presence of the confirmation ion, but at a ratio different then that indicated in Table 5-3.

- Data not meeting the criteria established in this section are appropriately qualified or re-analyzed.

Table 5-3. Target analyte parameters.

Analyte	CAS	Reference to Internal Standard and Surrogate	Quantitation Ion	Confirmation Ion	% Relative Abundance of Confirmation Ion
Fluorene-d_{10} (I -1)	NA		176	174	85
Naphthalene-d_8 (S-1)	NA	I-1	136	134	11
Decalin	91-17-8	I-1, S-1	138	96	90
C1-Decalins	NA	I-1, S-1	152	ND	ND
C2-Decalins	NA	I-1, S-1	166	ND	ND
C3-Decalins	NA	I-1, S-1	180	ND	ND
C4-Decalins	NA	I-1, S-1	180	ND	ND
Naphthalene	91-20-30	I-1, S-1	128	127	13
2-Methylnaphthalene	91-57-6	I-1, S-1	142	141	80
1-Methylnaphthalene	90-12-0	I-1, S-1	142	141	80
C1-Naphthalenes	NA	I-1, S-1	142	141	ND
Acenaphthene-d_{10} (S-2)	NA	I-1	164	162	89
2,6-Dimethylnaphthalene	581-42-0	I-1, S-2	156	141	90
1,6,7-Trimethylnaphthalene	2245-38-7	I-1, S-2	170	155	102
C2-Naphthalenes	NA	I-1, S-2	156	141	ND
C3-Naphthalenes	NA	I-1, S-2	170	155	ND
C4-Naphthalenes	NA	I-1, S-2	184	169	ND
Benzothiophene	11095-43-5	I-1, S-2	134	89	10
C1-Benzothiophenes	NA	I-1, S-2	148	ND	ND
C2-Benzothiophenes	NA	I-1, S-2	162	ND	ND
C3-Benzothiophenes	NA	I-1, S-2	176	ND	ND
Biphenyl	92-52-4	I-1, S-2	154	152	30
Acenaphthylene	208-96-8	I-1, S-2	152	153	15
Acenaphthene	83-32-9	I-1, S-2	154	153	98
Dibenzofuran	132-64-9	I-1, S-2	168	139	25
Fluorene	86-73-7	I-1, S-2	166	165	95
C1-Fluorenes	NA	I-1, S-2	180	165	ND
C2-Fluorenes	NA	I-1, S-2	194	179	ND
C3-Fluorenes	NA	I-1, S-2	208	193	ND
Pyrene-d_{10} (I -2)	NA		212	210	15
Phenanthrene-d_{10} (S-3)	NA	I-2	188	184	15
Pentachlorophenol	87-86-5	I-2, S-3	266	268	70
Carbazole	86-74-8	I-2, S-3	167	139	10
Dibenzothiophene	132-65-01	I-2, S-3	184	152	18
C1-Dibenzothiophenes	NA	I-2, S-3	198	184	ND
C2-Dibenzothiophenes	NA	I-2, S-3	212	197	ND
C3-Dibenzothiophenes	NA	I-2, S-3	226	211	ND
C4-Dibenzothiophenes	NA	I-2, S-3	240	ND	ND
Phenanthrene	85-01-8	I-2, S-3	178	176	20
Anthracene	120-12-7	I-2, S-3	178	176	20
C1-Phenanthrene/anthracenes	NA	I-2, S-3	192	191	ND
C2-Phenanthrene/anthracenes	NA	I-2, S-3	206	191	ND
C3-Phenanthrene/anthracenes	NA	I-2, S-3	220	205	ND
C4-Phenanthrene/anthracenes	NA	I-2, S-3	234	219	ND
Naphthobenzothiophene	NA	I-2, S-3	234	ND	ND
C1-Naphthobenzothiophenes	NA	I-2, S-3	248	ND	ND
C2-Naphthobenzothiophenes	NA	I-2, S-3	262	ND	ND
C3-Naphthobenzothiophenes	NA	I-2, S-3	276	ND	ND
Fluoranthene	206-44-0	I-2, S-3	202	101	15
Pyrene	129-00-0	I-2, S-3	202	101	15
C1-Fluoranthene/pyrenes	NA	I-2, S-3	216	215	ND
C2-Fluoranthene/pyrenes	NA	I-2, S-3	230	ND	ND

Table 5-3. Target analyte parameters (cont'd).

Analyte	CAS	Reference to Internal Standard and Surrogate	Quantitation Ion	Confirmation Ion	% Relative Abundance of Confirmation Ion
C3-Fluoranthene/pyrenes	NA	I-2, S-3	244	ND	ND
Chrysene-d_{12} (S-4)	NA	I-2	240	236	20
Benz[*a*]anthracene	56-55-3	I-2, S-4	228	226	30
Chrysene	218-01-9	I-2, S-4	228	226	30
C1-Chrysenes/Benzo[*a*]anthracenes	NA	I-2, S-4	242	241	ND
C2-Chrysenes/Benzo[*a*]anthracenes	NA	I-2, S-4	256	241	ND
C3-Chrysenes/Benzo[*a*]anthracenes	NA	I-2, S-4	270	255	ND
C4-Chrysenes/Benzo[*a*]anthracenes	NA	I-2, S-4	284	269	ND
Benzo(a)pyrene-d_{12} (I-3)	NA		264	260	20
5β(H)-Cholane(S-6)	80373-86-0	I-3	217	ND	ND
C29-Hopane	53584-60-4	I-3, S-3	191	398	5
18α–Oleanane	30759-92-3	I-3, S-3	191	412	5
C30-Hopane	13849-96-2	I-3, S-3	191	412	5
Benzo[*b*]fluoranthene	200-99-2	I-3, S-4	252	253	30,
Benzo[*k*]fluoranthene	207-08-9	I-3, S-4	252	253	30,
Benzo[*e*]pyrene	192-97-2	I-3, S-4	252	253	30
Benzo[*a*]pyrene	50-32-8	I-3, S-4	252	253	30
Indeno[*1,2,3-cd*]pyrene	193-39-5	I-3, S-4	276	277	25,
Dibenzo[*a,h*]anthracene	53-70-3	I-3, S-4	278	279	25,
C1-Dibenzo[*a,h*]anthracenes	NA	I-3, S-4	292	ND	ND
C2-Dibenzo[*a,h*]anthracenes	NA	I-3, S-4	306	ND	ND
C3-Dibenzo[*a,h*]anthracenes	NA	I-3, S-4	320	ND	ND
Benzo[*g,h,i*]perylene	191-24-2	I-3, S-4	276	277	25,
Perylene-d_{12} (S-5)	NA	I-3	264	260	20
Perylene	198-55-0	I-3, S-5	252	253	20

ND = Not determined
(I-#) = Internal standard reference number

(S-#) = Surrogate reference number

4.0 QUANTITATION CALCULATIONS

Sample analyte concentrations are calculated based on the concentration and response of the internal standard compounds (Table 5-2). The equations in Section 3.2 are used to calculate the RRF of each analyte relative to the concentration and area of the internal standard in the initial calibration. Response factors for alkyl homologues are presumed equal to the response factor of the respective unsubstituted (parent) compound.

The mass (M_A) of each target analyte (ng), including alkyl homologues, is calculated using the following equation:

$$M_A = \frac{(A_A M_{IS})}{(A_{IS} \overline{RRF_i})}$$

Where:

A_A = the area of the characteristic ion for the analyte measured

A_{IS} = the area of the characteristic ion for the specific internal standard

M_{IS} = mass of internal standard added to the extract (ng)

$\overline{RRF_i}$ = the average relative response factor for the analyte from the current calibration

The concentration (C) of each target analyte in a sample (ng/g) is calculated using the following equation:

$$C = \frac{(M_A DF)}{(W)}$$

Where:

DF = the dilution factor applied to the extract

W = the sample weight (g)

$$DF = \frac{\text{Volume of Extract } (\mu L)}{\text{Volume of extract used to make dilution } (\mu L)}$$

Analyte concentrations are reported as corrected for individual surrogate recoveries. The corrections for each compound are based on the surrogates referenced in Table 5-3. Percent surrogate recoveries ($SU_{Recovery}$) for each surrogate are calculated using the following equation:

$$SU_{Recovery} = \frac{C_{ESU}}{C_{SU}} x100$$

Where:

C_{ESU} = calculated surrogate concentration in the extract

C_{SU} = known concentration of surrogate added to extract

Analyte concentration corrections ($C_{corrected}$) for surrogate recovery are calculated using the following equation:

$$C_{Corrected} \frac{C}{SU_{Recovery}} x\,100$$

5.0 QUALITY CONTROL (QC)

Samples are analyzed in analytical batches consisting of 19 samples or fewer and QC samples. The QC samples are a method blank, laboratory duplicate, matrix spike, matrix spike duplicate, and standard reference material (SRM). A method blank is a reagent blank prepared in the laboratory. A duplicate is a sample for which a second aliquot is analyzed. Matrix spikes are samples that are spiked with known analyte concentrations. The SRM is a reference material with known analyte concentrations. All SRMs are certified and traceable to National Institute of Standards and Technology (NIST). The SRM used depends upon availability, matrix, and analytes.

The validity of the data is monitored using defined QC criteria. The following QC criteria are used to evaluate analytical batches:

1) Calibration

- The calibration criteria (Section 3.2) must be met prior to data analyses. If the calibration criteria are not met, then the run is aborted and the instrument re-calibrated before further sample analysis.

2) Method Blank

- No more than two target analytes may exceed 3 times the concentration of the MDL.
- Exceptions are that if an analyte detected in the method blank exceeds 3 times the concentration of the MDL but is not present in the associated samples, or if a sample analyte concentration is greater than 10 times that analyte concentration in the method blank, the result is qualified and reported.
- If a method blank exceeds these criteria then the source of contamination is determined and corrective action is taken before further sample analysis.

3) Matrix Spikes

    - Analytes spiked into a matrix are considered valid only if they are spiked at concentrations equivalent to levels found in the sample.
    - The average recovery for all valid spiked analytes in a matrix spike is between 60% and 120%. No more than two individual spiked analyte (valid) recoveries may exceed 40%-120%, with the exception of decalin and biphenyl.
    - If the QC criteria are not met then the matrix spike sample failing the criteria will be re-analyzed and if the re-analyzed spike meets the criteria then the data are reported.
    - If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
    - If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the matrix-spike, another sample may be selected or a blank-spike may be substituted.

- The average RPD for a valid matrix spike/matrix spike duplicate or blank spike/blank spike duplicate pair is 30%. No more than two individual analyte RPDs may exceed 35%.

4) Duplicate

- The average RPD between the duplicate and original sample, for analytes greater than 10 times the concentration of the MDL, is 30%. The RPD for no more than two individual analytes may exceed 35%.
    - If the QC criteria are not met then the sample pair failing the criteria will be re-analyzed and if the re-analyzed samples meet the criteria then the data are reported.
    - If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
    - If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the duplicate pair, another sample may be selected.

5) Standard Reference Material

- The average recovery for target analytes in a SRM should not exceed 30% of the upper and lower bounds of the mean certified values. No more than two target analytes should deviate more than 35% from the upper or lower bounds of the mean certified values.
    - If the QC criteria are not met then the SRM failing the criteria will be re-analyzed and if the re-analyzed SRM meets the criteria then the data are reported.
    - If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
    - If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed.

6) Surrogates

- The average recovery of surrogate compounds is between 50% and 150%.
- Exceptions are analytical interferences with the surrogates and diluted samples.
- If the average recovery of surrogates exceeds the criteria, and calculation and analytical errors are eliminated, the sample is re-analyzed. If sufficient sample is unavailable for re-extraction, the data are qualified and reported.

7) Method Detection Limit

    - The method detection limit (MDL) is determined following the procedures outlined in Federal Register (1984).

6.0 REFERENCES

Federal Registry (1984) Vol. 49, No. 209:198-199.

EXTRACTION OF SEDIMENTS FOR BUTYLTINS

Juan A. Ramirez, Donell S. Frank, Susanne J. McDonald, and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Determining organic contaminant levels in sediments require extraction, isolation, and concentration of analytes from the matrix. Sediments are dried, pulverized, and homogenized prior to extraction. Sediments are extracted in a tropolone/hexane or tropolone/dichloromethane mixture using sonication. The extracts are reduced in volume and hexylmagnesium bromide (Grignard reagent) is added, followed by neutralization with hydrochloric acid. The organic fraction is reduced in volume and purified using silica gel/florisil chromatography columns. The volume of the resultant eluent is reduced and analyzed for butyltins by gas chromatography/flame photometric detection.

1.0 INTRODUCTION

Butyltins (BTs), including monobutyltin, dibutyltin, tributyltin, and tetrabutyltin are contaminants of concern in the estuarine environment. The procedure described is used to extract, isolate, purify, and concentrate BT contaminants from sediments. Contaminant concentrations in parts per billion can be resolved in sediments. Sediment samples are homogenized, dried, and ground using a mortar and pestle. Approximately 10 g of dry sediment are extracted in 60 mL of 0.1% tropolone/hexane or 0.1% tropolone/dichloromethane using a sonic probe. The liquid is decanted and the extraction procedure is repeated twice more. The combined extracts are reduced in volume. The samples are treated with hexylmagnesium bromide (Grignard reagent) and then neutralized with hydrochloric acid (HCl). The organic layer is drawn off, reduced in volume, and then purified using silica gel/florisil columns. The eluent is reduced to 10 mL from which 2 mL is prepared for the analysis of BTs by gas chromatography/flame photometric detection (GC/FPD).

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Solvent reduction apparatus, Zymark TurboVap LV concentration workstation
Sonicator, Tekmar TMX500 sonicator
Balance, top loading, tare capacity to 300 g, capable of weighing to 1 mg
Microbalance, capable of weight to 1 µg
Calibrated weights, certified
Combustion furnace, electric capable of combusting glassware at 400 °C for at least 4 hr
Oven capable of maintaining 40 °C temperature
Conditioning oven, electric, gravity convection, capable of maintaining a stable temperature of up to 200 °C
Concentration tubes, Zymark® 60 mL borosilicate glass
Concentration tubes, 60 mL certified pre-cleaned with open screw caps and Teflon lined septa
Micropipettors, calibrated, 1% accuracy, disposable tips
Beakers, 150 mL
Erlenmeyer flasks, 250 mL
Amber extract vials, 2.0 mL, with teflon-lined screw caps
Glass wool

2.2 REAGENTS

Silica gel (CAS 1343-98-2)/Florisil (CAS 1343-88-0) columns, Resteck, 16 g florisil and 5 g silica gel
Dichloromethane (CAS 75-09-2), pesticide grade or equivalent purity
Hexane (CAS 110-54-3), pesticide grade or equivalent purity
Hydrochloric acid (CAS 7647-01-0), Tracepur® Plus or equivalent purity
Copper (CAS 7440-50-8), granular, 20-30 mesh, ACS reagent grade, purified
Tropolone (CAS 533-75-5), 98% purity
Grignard reagent, hexylmagnesium bromide (CAS 3761-92-0)
Nitrogen (CAS 7727-37-9), 99.8% purity

3.0 PROCEDURE

Sediment samples are frozen upon receipt at –20 °C until extraction. Prior to extraction, sediment samples are thawed and homogenized using a stainless steel spatula. A subsample is removed for percent moisture determination (McDonald *et al.,* 2006). At least 10 g of sample are dried in an oven at 40 °C and then ground and homogenized using a mortar and pestle. Approximately 10 g of the dried sediment are extracted.

Dried samples are weighed into 150 mL beakers to which 60 mL of 0.1% tropolone in either hexane or dichloromethane is added. Appropriate surrogates and spikes are added to beakers prior to extraction. The sediment/tropolone mixture is sonicated at 50% power, 1 second on and 0.5 second off, for a total of 3 minutes. The extract is decanted into a 250 mL Erlenmeyer flask. The extraction is repeated two more times. Quality control (QC) samples (e.g., blanks, duplicates, matrix spikes, and certified reference materials) are prepared in the same manner as samples.

The combined extracts are reduced to 10 mL using a Zymark TurboVap LV concentration station set at 40 °C and 15 psi. An aliquot of extract (between 20 and 45 mL) is added to a 60 mL concentration tube and reduced in volume. Additional aliquots of extract are added to the tube as the volume is decreased until the sample extract has been reduced to approximately 10 mL. Purified copper granules are carefully mixed with concentrated hydrochloric acid, followed by thorough rinsing with HPCL grade water. Copper is added to the concentration tube to remove sulfur. Copper is added until it no longer turns black, indicating that all sulfur has been bound. Samples extracted in the tropolone/dichloromethane mixture are back-extracted into hexane. The samples are quantitatively transferred to a clean 60 mL concentration tube and reduced to 10 mL. The butyltin in the sample extracts are then hexylated by adding 1 mL of hexylmagnesium bromide (Grignard reagent). The sample headspace is purged using nitrogen, the tubes are capped and shaken for 1 hour on a shaker table. After shaking, the Grignard reagent is neutralized by adding 40 mL of 10% HCl to each tube in an ice bath. The tubes are shaken and the upper organic layer is transferred to a 50 mL Zymark concentration tube. The acid fraction is rinsed twice more using 10 mL of hexane, each time transferring the organic layer to the concentration tube. The extract is then reduced to 2 – 4 mL using a Zymark TurboVap LV set at 40 °C and 15 psi.

The concentrated extract is purified using silica gel/florisil chromatography columns. The chromatography columns contain 16 g of florisil and 5 g silica gel and are conditioned by flushing with 30 mL of hexane. The sample and solvent rinses are added to the top of column and 125 mL of hexane is added and eluted until the column is dry. The eluent is collected in 250 mL Erlenmeyer flasks. The eluent is quantitatively transferred to a Zymark concentration tube and reduced to a final volume of 10 mL. Approximately 2 mL of each extract is transferred to a clean 2 mL amber vial and stored at –20 °C until analysis. Internal standard is added immediately prior to instrument analysis. Figure 9-1 shows a flow chart of the extraction and purification procedure.

4.0 QUALITY CONTROL (QC)

Solvents are verified to be contaminant-free by lot tests prior to use. All equipment and glassware used to extract samples are thoroughly cleaned by solvent rinsing or combustion at 400 °C. The calibration and accuracy of balances, weights, pipettors, and thermometers are checked daily using certified weights and thermometers with calibrations traced to the National Institute of Standards and Technology (NIST). The calibration and accuracy of balances, weight, pipettors, and thermometers are verified yearly by independent sources. A series of quality control samples are processed with each batch of 20 samples or less. The following quality controls are used to ensure the accuracy and precision of tissue data.

- Surrogates. Solutions containing analytes that do not interfere with the analytes of interest are prepared at concentrations approximately 5 to 10 times the method detection limit (MDL). Specified surrogates are added to each sample extracted, including QC samples, at a specified volume (typically 100 µL), immediately prior to extraction.

- Method Blank. Method blanks are extractions of all support material used for extraction of samples, with the exception of sediment. A method blank is analyzed with each extraction batch of 20 or fewer samples. The method blank is extracted and analyzed in a manner identical to samples.

- Matrix Spike. Matrix spikes are extractions of sample matrix fortified with spikes of selected target analytes. Spikes are prepared at concentrations approximately 10 times the MDL. A matrix spike and matrix spike duplicate are analyzed with each extraction batch of 20 or fewer samples. Matrix spikes are extracted and analyzed in a manner identical to samples.

- Laboratory Duplicates. A sample is analyzed in duplicate with each extraction batch of 20 or fewer samples.

- Certified Reference Material (CRM). A standard reference material (CRM PACS-2) is analyzed with each extraction batch of 20 or fewer samples.

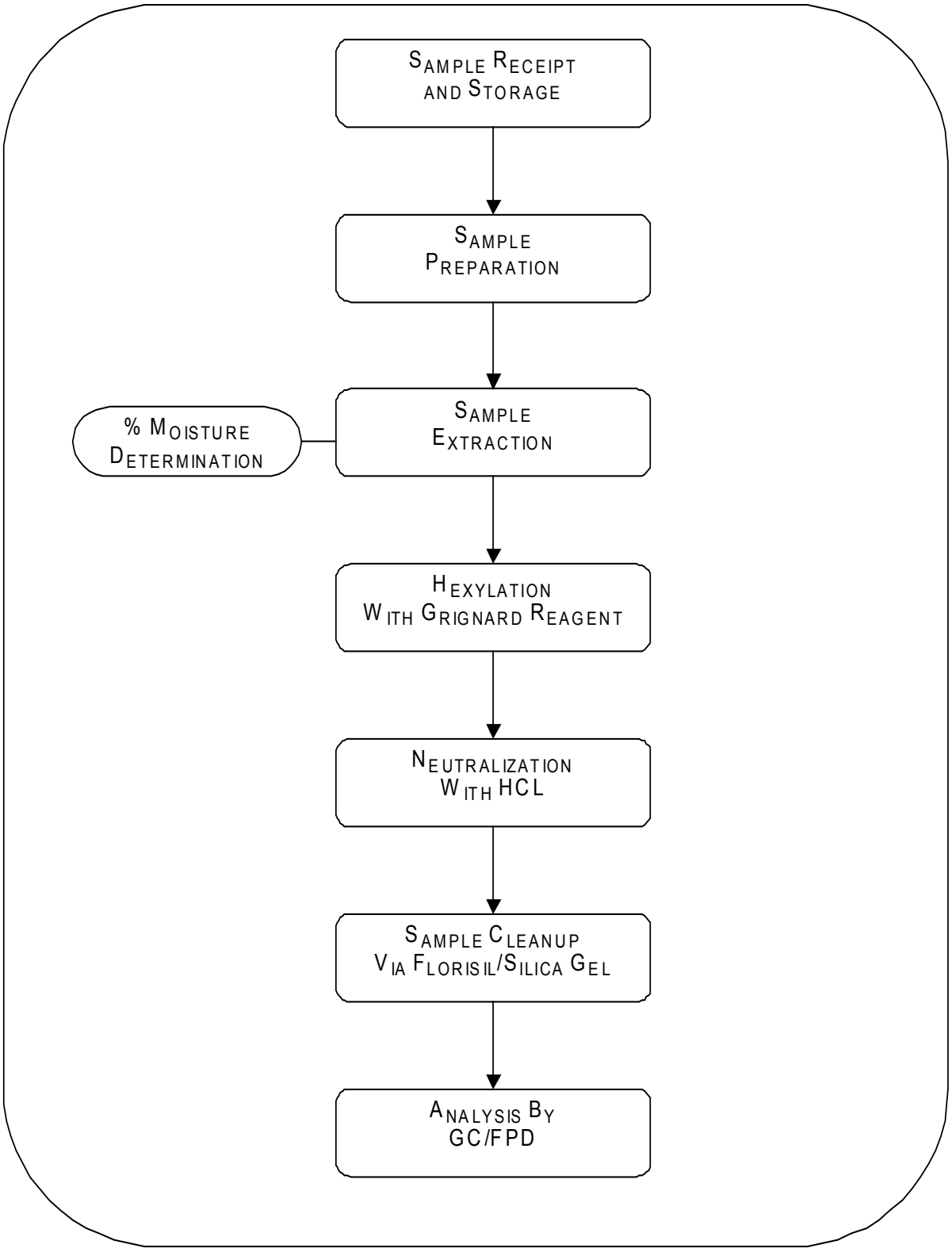


Figure 6-1. Methodology for extraction, isolation, and quantification of sediment samples for butyltins.

EXTRACTION OF BIOLOGICAL TISSUES FOR BUTYLTINS

Juan A. Ramirez, Donell S Frank, Susanne J. McDonald, and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Determining organic contaminant levels in tissues require extraction, isolation, and concentration of analytes from the matrix. Bivalves are shucked and homogenized. Aliquots of homogenized sample are chemically dried using Hydromatrix® and extracted in a tropolone/ hexane or tropolone/dichloromethane mixture using a tissuemizer. The extracts are reduced in volume and hexylmagnesium bromide (Grignard reagent) is added, followed by neutralization with hydrochloric acid. The organic fraction is reduced in volume and purified using silica gel/ florisil chromatography columns. The volume of the resultant eluent is reduced and analyzed for butyltins by gas chromatography/flame photometric detection.

1.0 INTRODUCTION

Butyltins (BTs), including monobutyltin, dibutyltin, tributyltin, and tetrabutyltin are contaminants of concern in the estuarine environment. The determination of these compounds at low concentrations in tissue is necessary to accurately monitor spatial and temporal changes in U.S. coastal waters. The procedure described is used to extract, isolate, purify, and concentrate BT contaminants from tissues. Contaminant concentrations in parts-per billion can be resolved in lipid-rich tissues. Shell length and shell volume are determined for specimens collected at each location. Bivalves are then shucked and multiple organisms are processed as one sample to ensure the sample is representative of a population at a given site and to have sufficient sample to complete the analyses. Tissue samples are homogenized using a stainless steel blender outfitted with titanium blades. Aliquots of approximately 10 g of wet tissue are chemically dried with Hydromatrix® or sodium sulfate. The tissue/drying agent mixtures are extracted in 60 mL of 0.1% tropolone in either hexane or dichloromethane using a Tekmar® tissumizer. The liquid is decanted and the extraction procedure is repeated twice more. The combined extracts are reduced in volume. The samples are treated with hexyl magnesium bromide (Grignard reagent) and then neutralized with hydrochloric acid (HCl). The organic layer is drawn off, reduced in volume, and then purified using silica/florisil columns. The eluent is reduced to 10 mL, from which 2 mL is prepared for the analysis of BTs by gas chromatography/flame photometric detection (GC/FPD).

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Solvent reduction apparatus, Zymark TurboVap LV concentration workstation
Tissumizer, Tekmar®, with stainless steel probes
Balance, top loading, tare capacity to 300 g, capable of weighing to 1 mg
Microbalance, capable of weight to 1 µg
Calibrated weights, certified
Combustion furnace, electric, capable of combusting glassware at 400 °C for at least 4 hours
Oven capable of 40 °C temperature maintenance
Conditioning oven, electric, gravity convection, capable of maintaining a stable temperature of up to 200 °C
Concentration tubes, Zymark® 60 mL borosilicate glass
Concentration tubes, 60 mL certified pre-cleaned tubes with open screw caps and Teflon lined septa.
Micropipettors, calibrated to 100 µL, 1% accuracy, disposable tips
Centrifuge bottles, 200 mL
Erlenmeyer flasks, 250 mL
2 mL amber extract vials with teflon-lines screw caps
Glass wool

2.2 REAGENTS

Silica gel (CAS 7343-88-0)/Florisil (CAS 1343-88-0) columns, Resteck, 16 g florisil and 5 g silica gel
Dichloromethane (CAS 75-09-2), pesticide grade or equivalent purity
Hexane (CAS 110-54-3), pesticide grade or equivalent purity
Hydrochloric acid (CAS 7647-01-0), Tracepur® Plus or equivalent purity
Hydromatrix® (CAS 68855-54-9/14464-46-1), conditioned by combustion at 400 °C for at least 4 hours and stored at 120 °C
Sodium sulfate (CAS 7757-82-6), anhydrous granular powder, ACS reagent grade, purified by combusting at 400°C for at least 4 hours and stored at 120 °C.
Hydrochloric acid (CAS 7647-07-0)
Topolone (CAS 5533-75-5), 98% purity
Grignard reagent, hexylmagnesium bromide (CAS 3761-92-0)
Nitrogen, (CAS 7727-37-9) 99.8% purity

3.0 PROCEDURE

Shell length and volume are determined for all bivalves collected at each sampling site. The bivalves are then shucked and the soft tissue is homogenized using a stainless steel Waring® blender with titanium blades. Homogenized tissue samples are frozen at –20 °C until extraction. Prior to extraction, tissue samples are thawed and re-homogenized using a stainless steel spatula. A subsample is removed for percent moisture determination (McDonald *et al*., 2006).

Approximately 10 g wet tissue and 20 g of prepared Hydromatrix® or prepared sodium sulfate are weighed into a 200 mL centrifuge bottle. The tissues samples must be thoroughly dry to optimize the extraction efficiency. Hydromatrix® and sodium sulfate chemically dry samples by binding moisture. Appropriate surrogates and spikes are added to the centrifuge bottle prior to extraction. The samples are extracted in 60 mL of 0.1% tropolone, in

hexane or dichloromethane, using a Tekmar® tissumizer. The samples are macerated for 2 min. The liquid is decanted into a 250 mL Erlenmeyer flask. The extraction is repeated two more times. Quality control (QC) samples (e.g., blanks, duplicate, matrix spikes) are prepared in the same manner as samples.

The combined extracts are reduced to 10 mL using a Zymark TurboVap LV concentration station set at 40 °C and 15 psi. An aliquot of extract (between 20 and 45 mL) is added to a 60 mL concentration tube and reduced in volume. Additional aliquots of extract are added to the tube as the extract volume is decreased until the sample extract has been reduced to approximately 10 mL. Samples extracted in the tropolone/dichloromethane mixture are back extracted into hexane. Samples are quantitatively transferred to a clean 60 mL concentration tube and reduced to 10 mL. BTs in the sample extracts are then hexylated by adding 1 mL of hexylmagnesium bromide (Grignard reagent). The sample headspace is purged using nitrogen and then the tubes are capped and shaken for 1 hour on a shaker table. After shaking the Grignard reagent is neutralized by adding 40 mL of 10% HCl to each tube, in an ice bath. The tubes are shaken and the upper organic layer is transferred to a 50 mL Zymark concentration tube. The acid fraction is rinsed twice more using 10 mL of hexane, each time transferring the organic layer to the concentration tube. The extract is then reduced to 2 – 4 mL using a Zymark TurboVap LV set at 40 °C and 15 psi.

The concentrated extract is purified using silica gel/florisil chromatography columns. The chromatography columns contain 16 g of florisil and 5 g silica gel and are conditioned by flushing with 30 mL of hexane. The sample and solvent rinses are added to the top of the column and 125 mL of hexane is added and eluted until the column is dry. The eluent is collected in 250 mL Erlenmeyer flasks. The eluent is quantitatively transferred to a Zymark concentration tube and reduced to a final volume of 10 mL. Approximately 2 mL of each extract is transferred to a clean 2 mL amber vial and stored at –20 °C until analysis. Internal standard is added immediately prior to instrument analysis. Figure 10-1 shows a flow chart of the extraction and purification procedure.

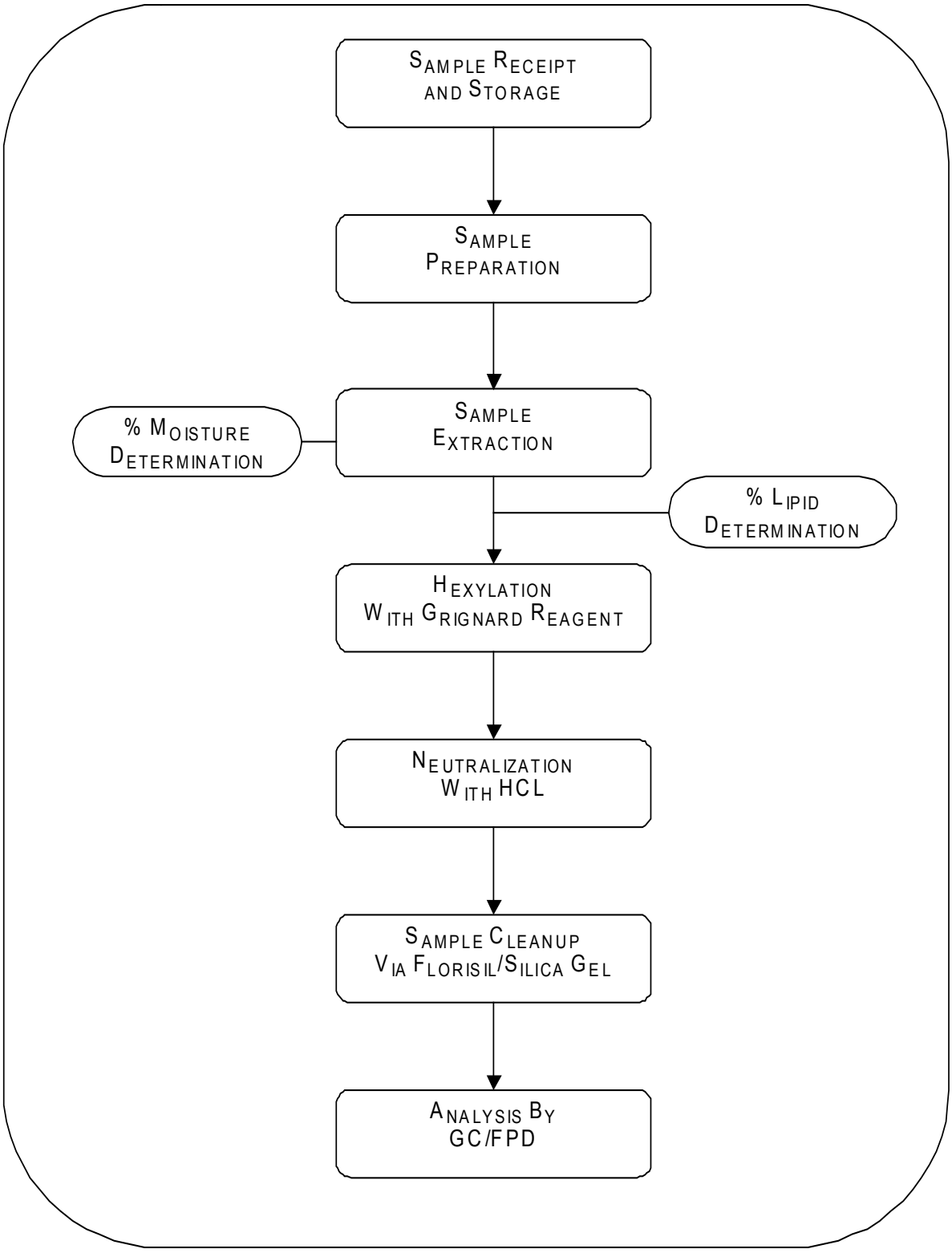


Figure 7-1. Methodology for extraction, isolation, and quantification of tissue samples for butyltins.

4.0 QUALITY CONTROL (QC)

Solvents are verified to be contaminant-free by lot tests prior to use. All equipment and glassware used to extract samples are thoroughly cleaned by solvent rinsing or combustion at 400 °C. The calibration and accuracy of balances, weights, pipettors, and thermometers are checked daily using certified weights and thermometers with calibrations traced to the National Institute of Standards and Technology (NIST). The calibration and accuracy

of balances, weight, pipettors, and thermometers are verified yearly by an independent source. All samples are shipped and received under chain-of-custody. A series of quality control samples are processed with each batch of 20 samples or less. The following quality controls are used to ensure the accuracy and precision of tissue data.

- Surrogates. Solutions containing analytes that do not interfere with the analytes of interest are prepared at concentrations approximately 5 to 10 times the method detection limit (MDL). Specified surrogates are added to each sample extracted, including QC samples, at a specified volume (typically 100 μL) immediately prior to extraction.

- Method Blank. Method blanks are extractions of all support material used for extraction of samples, with the exception of tissue. A method blank is analyzed with each extraction batch of 20 or fewer samples. The method blank is extracted and analyzed in a manner identical to samples.

- Matrix Spike. Matrix spikes are extractions of sample matrix fortified with spikes of selected target analytes. Spikes are prepared at concentrations approximately 10 times the MDL. A matrix spike and matrix spike duplicate are analyzed with each extraction batch of 20 or fewer samples. Matrix spikes are extracted and analyzed in a manner identical to samples.

- Laboratory Duplicates. A sample is analyzed in duplicate with each extraction batch of 20 or fewer samples.

QUANTITATIVE DETERMINATION OF BUTYLTINS USING GAS CHROMATOGRAPHY/FLAME PHOTOMETRIC DETECTION 1999 -2006

Donell S. Frank, Susanne J. McDonald, Juan A. Ramirez and James M. Brooks
TDI-Brooks International/B&B Laboratories Inc.
College Station, Texas 77845

ABSTRACT

Selected butyltins (BTs) are detected using a gas chromatograph/flame photometric detector. This method is capable of detecting low concentrations of monobutyltin, dibutyltin, tributyltin, and tetrabutyltin in complex matrices such as tissues and sediments.

1.0 INTRODUCTION

A gas chromatograph/flame photometric detector (GC/FPD), coupled to a capillary column, is used to resolve and detect butyltins (BTs) in tissues and sediments. Samples are injected into a temperature-programmed GC/FPD. The capillary column is a DB-5 (30 m x 0.25 mm ID and 0.25 μm film thickness). The data acquisition system continuously acquires and stores all data for quantitation. This method is capable of producing data at parts per-billion-concentrations.

2.0 APPARATUS AND MATERIALS

2.1 EQUIPMENT

Gas chromatograph equipped with large volume injector, electronic pressure control, and a flame photometric detector with a tin selective 610 nm filter, Thermoquest 2000 Series Trace GC
Data acquisition system, Thermoquest Chromquest software, capable of continuous acquisition and storage of all data during analysis
Autosampler capable of making 1 to 250 μL injections
Capillary column, J&W Scientific DB-5 (30 m x 0.25 mm ID and 0.25 μm film thickness)
Desolvation column, Supelco fused silica intermediate polarity (15 m long by 0.53 mm ID)
Micropipetters, calibrated, 1% accuracy, disposable tips

2.2 REAGENTS

Hexane (CAS 110-54-3), pesticide grade or equivalent purity
Helium (CAS 7440-59-7), 99.8% purity
Hydrogen (CAS1333-74-0), 99.8% purity
Nitrogen (CAS 7727-37-9), 99.8% purity
Air (CAS 132259-10-0), 99.8% purity

2.3 STANDARDS

SURROGATE SPIKING SOLUTION

The surrogate spiking solution is prepared with aliquots of pure compounds (Restek) diluted with hexane to a final concentration of 646 ng Sn/mL. The surrogate spiking solution includes tripentyltin chloride. The surrogate solution (100 µL) is added to all samples and quality control (QC) samples prior to extraction. The surrogate is resolved from, but elutes in close proximity to, the analytes of interest. The recovery of the surrogate is used to correct analyte concentrations based on retention time.

INTERNAL STANDARD SOLUTION

The internal standard solution is made from an aliquot of pure compound (Restek) and diluted with hexane to a final concentration of 1,015 ng Sn/mL. The internal standard compound is tetra-n-propyltin. The internal standard compound is resolved from, but elutes in close proximity to, the analytes of interest. The internal standard solution (100 µL) is added to all samples and QC samples just prior to analysis. The internal standard is used to calculate relative response factors and specific analyte concentrations based on retention time.

MATRIX SPIKING SOLUTION

A certified solution containing monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT) and tetrabutyltin (TeBT) is purchased from a commercial vendor (Restek**)** and diluted with hexane to prepare the matrix spiking solution (Table 8-1). The matrix spike solution is diluted to approximately 10 times the MDL and is added to all matrix spike samples.

CALIBRATION SOLUTION

Calibrations solutions are prepared at 5 concentrations ranging from approximately 0.5 to 50 ng Sn/mL (Table 8-2) by diluting a commercially prepared solution (Restek) containing the analytes of interest.

RETENTION INDEX SOLUTIONS

The mid-level calibration standard is used as a retention index solution to determine the retention times of analytes of interest.

Table 8-1. Butyltins Contained in the Matrix-Spiking Solution.

Analyte	CAS	Spiking Solution Concentration (ng Sn/mL)
Tetrabutyltin	1461-25-2	683.9
Tributyltin chloride	1461-22-9	729.4
Dibutyltin dichloride	683-18-1	781.4
Butyltin trichloride	1118-46-3	841.4

Table 8-2. Butyltins Contained in Calibration Solutions and their Approximate Concentrations.

Compounds Contained in Calibration Solutions	CAS	Level 1 (ng Sn/mL)	Level 2 (ng Sn/mL)	Level 3 (ng Sn/mL)	Level 4 (ng Sn/mL)	Level 5 (ngSnmL)
Internal Standard						
Tetra-n-propyltin	2176-98-9	10.15	10.15	10.15	10.15	10.15
Surrogates						
Tripentyltin chloride	3342-67-4	0.5	2.5	5.0	25	50
Tri-n-propyltin	995-25-5	0.5	2.5	5.0	25	50
Analytes						
Tetrabutyltin	1461-25-2	0.5	2.5	5.0	25	50
Tributyltin chloride	1461-22-9	0.5	2.5	5.0	25	50
Dibutyltin dichloride	683-18-1	0.5	2.5	5.0	25	50
Butyltin trichloride	1118-46-3	0.5	2.5	5.0	25	50

3.0 QUANTITATIVE DETERMINATION OF BUTYLTINS BY GC/FPD

3.1 INITIAL CALIBRATION

A 5-point relative response factor (RRF) calibration curve is established for analytes of interest prior to the analysis of samples and quality control (QC) samples. An RRF is determined for each analyte for each calibration level using the following equation:

$$RRF = \frac{(A_A)(C_B)}{(A_B)(C_A)}$$

Where:

A_A = the area of the analyte to be measured
A_{IS} = the area of the specific internal standard
C_A = the known concentration of the analyte in the calibration solution (ng Sn/mL)
C_{IS} = the known concentration of the internal standard in the calibration solution (ng Sn/mL)

The response factors determined for each calibration level are averaged to produce a mean relative response factor ($\overline{RRF_i}$) for each analyte. The percent relative standard deviation (%RSD) for the 5 response factors must be less than or equal to 15% for each analyte.

$$\%RSD = \frac{\text{Standard Deviation of the RRFs}}{\text{Average of the RRFs}} \times 100$$

Where:

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{(n-1)}}$$

Where:

x_i = each RRF value used to calculate the mean RRF
$\bar{x}$ = the mean of n values
n = total number of values (5)

3.2 CONTINUING CALIBRATION

A mid-level calibration standard is analyzed at the beginning and end of each analytical set, or every 10 samples (whichever is more frequent). The daily relative response factor for each compound is compared to the mean relative response factor from the initial calibration curve and the average percent difference (RPD) of all analytes must be less than 15%. If the calibration check does not meet this criterion then the initial five-point calibration is repeated.

$$RPD = \frac{RRF_c - \overline{RRF_i}}{\overline{RRF_i}} \times 100$$

Where:

$\overline{RRF_i}$ = mean relative response factor from the most recent initial calibration (meeting technical acceptance criteria)
RRF_c = relative response factor from the continuing calibration standard

3.3 GC/FPD ANALYSIS

The initial calibration of the GC/FPD must meet the previously described criteria prior to sample analysis. Samples are analyzed in analytical sets that consist of standards, samples, and QC samples. Quality control samples are method blanks, laboratory duplicates, blank spikes, matrix spikes, and standard reference materials. An autosampler is used to inject 50 µL of all samples, standards, and QC samples into the capillary column of the GC using the following instrument conditions. Slight modifications may be necessary depending upon the analysis.

Inlet: Large volume with solvent vent
Carrier gas: Helium, 1.9 mL/min
Detector gas: Hydrogen 90 mL/min:Air 105 mL/min
Make up gas: Nitrogen 20 mL/min

Temperatures:
- Base Temp: 250°C
- FPD: 160°C

Oven program:
- Initial oven temp: 65°C
- Initial hold time: 0.18 minutes
- Ramp rate: 10°C/min
- Final oven temp: 240°C
- Final hold time: 4 minutes

3.4 ANALYTE IDENTIFICATION

The retention time of a sample analyte must fall within 15 seconds of the retention time for the analyte in a calibration standard or retention index solution.

Data not meeting established criteria are appropriately qualified or are re-analyzed.

4.0 QUANTITATION CALCULATIONS

Sample analyte concentrations are calculated based on the concentration and response of the internal standard. The equations in section 3.1 are used to calculate the RRF of each analyte relative to the concentration and area of the internal standard in the initial calibration.

The mass (M_A) of each target analyte (ng), is calculated using the following equation:

$$M_A = \frac{(A_A M_B)}{(A_B \overline{RRF_i})}$$

Where:

A_A = the area of the analyte measured
A_{IS} = the area of the specific internal standard
M_{IS} = mass of internal standard added to the extract (ng)
$\overline{RRF_i}$ = the average relative response factor for the analyte from the current calibration

The concentration (C) of each target analyte in a sample (in ng Sn/g) is calculated using the following equation:

$$C = \frac{(M_A V_E D)}{(V_{inj} W)}$$

Where:

Ve = final volume of the extract (µL)
Vinj = volume of the sample injected onto the column (µL)
W = the weight of the sample (g)
DF = the dilution factor applied to the extract

$$D = \frac{\text{Volume of Extract } (\mu L)}{\text{Volume of extract used to make dilution } (\mu L)}$$

Analyte concentrations are reported as corrected for surrogate recovery. Percent surrogate recovery ($SU_{Recovery}$) is calculated using the following:

$$SU_{Recovery} = \frac{C_{ESU}}{C_{SU}} x100$$

Where:

C_{ESU} = calculated surrogate concentration in the extract (ng Sn/mL)
C_{SU} = known concentration of surrogate added to extract (ng Sn/mL)

Analyte concentration corrections ($C_{corrected}$) for surrogate recovery are calculated using the following equation:

$$C_{Corrected} \frac{C}{SU_{Recovery}} x\,100$$

5.0 QUALITY CONTROL (QC)

Samples are analyzed in analytical batches consisting of 19 samples or fewer and QC samples. The QC samples are a method blank, laboratory duplicate, matrix spike, matrix spike duplicate, and standard reference material (SRM), if available. A method blank is a reagent blank prepared in the laboratory. A duplicate is a sample for which a second aliquot is analyzed. Matrix spikes are samples that are spiked with known concentrations of known analytes. The SRM is a reference material with known analyte concentrations. All SRMs are certified and traceable to the Canadian Research Council (NRC). A SRM is not available for tissues. The validity of the data is monitored using defined QC criteria. The following QC criteria are used to evaluate analytical batches:

1). Calibration

- The calibration criteria (Section 3.2) must be met prior to data analyses. If the calibration criteria are not met, then the run is aborted and the instrument re-calibrated before further sample analysis.

2). Method Blank

- No more than one target analyte may exceed 3 times the concentration of the MDL.
- Exceptions are that if an analyte detected in the method blank exceeds 3 times the concentration of the MDL but is not present in the associated samples, or if a sample analyte concentration is greater than 10 times that analyte concentration in the method blank, the result is qualified and reported.
- If a method blank exceeds these criteria then the source of contamination is determined and corrective action is taken before further sample analysis.

3). Matrix Spikes

- Analytes spiked into a matrix are considered valid only if they are spiked at concentrations equivalent to levels found in the sample.
- The average recovery for all valid spiked analytes in a matrix spike is between 60% and 120%. No more than one individual spiked analyte (valid) recoveries may exceed 40%-120%, with the exception of monobutyltin.
- If the QC criteria are not met then the matrix spike sample failing the criteria will be re-analyzed and if the re-analyzed spike meets the criteria then the data are reported.
- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the matrix-spike, another sample may be selected or a blank-spike may be substituted.
- The average RPD for a valid matrix spike/matrix spike duplicate or blank spike/blank spike duplicate pair is 30%. No more than two individual analyte RPDs may exceed 35%.

4). Duplicate

- The average RPD between the duplicate and original sample, for analytes greater than 10 times the concentration of the MDL, is 30%. The RPD for no more than two individual analytes may exceed 35%.
- If the QC criteria are not met then the sample pair failing the criteria will be re-analyzed and if the re-analyzed samples meet the criteria then the data are reported.
- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed. If sufficient sample is unavailable to re-extract the duplicate pair, another sample may be selected.

5). Standard Reference Material

- The average recovery for target analytes in a SRM should not exceed 30% of the upper and lower bounds of the mean certified values. No more than two target analytes should deviate more than 35% from the upper or lower bounds of the mean certified values.
- If the QC criteria are not met then the SRM failing the criteria will be re-analyzed and if the re-analyzed SRM meets the criteria then the data are reported.
- If an analyte exceeds the criteria and is not present in the associated samples analyzed with the analytical batch, the result is qualified and reported.
- If upon re-analysis, QC criteria are still not met, the entire batch of samples is reanalyzed.

6). Surrogates

- The average recovery of surrogate compounds is between 50% and 150% with the exception of monobutyltim.
- Exceptions are analytical interferences with the surrogates and diluted samples.
- If the average recovery of surrogates exceeds the criteria, and calculation and analytical errors are eliminated, the sample is re-analyzed. If sufficient sample is unavailable for re-extraction, the data are qualified and reported.

7). Method Detection Limit

- The method detection limit (MDL) is determined following the procedures outlined in Federal Register (1984).

6.0 REFERENCES

Federal Registry (1984) Vol. 49, No. 209:198-199.

METHOD DETECTION LIMITS

Table A-1. Reporting units for organic contamiants measured by the Mussel Watch Project.

Analysis	Reporting Unit
BT Analysis by GC/FPD	ng Sn/dry g
PAH Analysis by GC/MS	ng/dry g
PCB and PEST Analysis by GC/ECD	ng/dry g

Table A-2. Mussel Watch Project tissue butyltin method limits of detection (ng Sn/g dry weight).

Sample size	2000 1.15 g	2001 1.15 g	2002 1.15 g	2003 1.15 g	2004 1.15 g
Monobutyltin	1.95	1.95	1.95	0.56	0.56
Dibutyltin	3.6	3.6	3.6	4.88	4.88
Tributyltin	4.83	4.83	4.83	6.82	6.82
Tetrabutyltin	2.73	2.73	2.73	6.53	6.53

Table A-3. Mussel Watch Project sediment butyltin method limits of detection (ng Sn/g dry weight).

Sample size	2003 1.15 g	2004 15.0 g
Monobutyltin	2.54	0.71
Dibutyltin	0.40	0.41
Tributyltin	0.27	0.56
Tetrabutyltin	0.21	0.49

Table A-4. Mussel Watch Project tissue polycyclic aromatic hydrocarbon method limits of detection (ng/g dry weight).

	2000	2001	2002	2003	2004
Sample size	**3 g**	**2.5 g**	**2.5 g**	**2.1 g**	**2.1 g**
Decalin				5.9	5.9
C1-Decalin				5.9	5.9
C2-Decalin				5.9	5.9
C3-Decalin				5.9	5.9
C4-Decalin				5.9	5.9
Naphthalene	2.2	2.3	2.3	9.03	9.03
C1-Naphthalenes	1.6	4.6	4.6	9.03	9.03
C2-Naphthalenes	1.4	4.6	4.6	9.03	9.03
C3-Naphthalenes	1.4	4.6	4.6	9.03	9.03
C4-Naphthalenes	1.4	4.6	4.6	9.03	9.03
Benzothiophene				3.94	3.94
C1-Benzothiophene				3.94	3.94
C2-Benzothiophene				3.94	3.94
C3-Benzothiophene				3.94	3.94
Biphenyl	1.5	2	2	2.45	2.45
Acenaphthylene	0.7	0.5	0.5	2.22	2.22
Acenaphthene	0.6	0.4	0.4	2.12	2.12
Dibenzofuran				2.22	2.22
Fluorene	0.7	0.6	0.6	2.48	2.48
C1-Fluorenes	1.5	1.1	1.1	2.48	2.48
C2-Fluorenes	1.5	1.1	1.1	2.48	2.48
C3-Fluorenes	1.5	1.1	1.1	2.48	2.48
Anthracene	2	1.7	1.7	1.18	1.18
Phenanthrene	2.2	1.5	1.5	3.63	3.63
C1-Phenanthrenes/Anthracenes	4.4	2.9	2.9	3.63	3.63
C2-Phenanthrenes/Anthracenes	4.4	2.9	2.9	3.63	3.63
C3-Phenanthrenes/Anthracenes	4.4	2.9	2.9	3.63	3.63
C4-Phenanthrenes/Anthracenes	4.4	2.9	2.9	3.63	3.63
Dibenzothiophene	3.5	0.5	0.5	1.78	1.78
C1-Dibenzothiophenes	7.1	1.1	1.1	1.78	1.78
C2-Dibenzothiophenes	7.1	1.1	1.1	1.78	1.78
C3-Dibenzothiophenes	7.1	1.1	1.1	1.78	1.78
Fluoranthene	2.1	0.8	0.8	9.02	9.02
Pyrene	1.9	1.4	1.4	5.71	5.71
C1-Fluoranthenes/Pyrenes	4.1	1.6	1.6	9.02	9.02
C2-Fluoranthenes/Pyrenes				9.02	9.02
C3-Fluoranthenes/Pyrenes				9.02	9.02

Table A-4 (cont'd). Mussel Watch Project tissue polycyclic aromatic hydrocarbon method limits of detection (ng/g dry weight).

	2000	2001	2002	2003	2004
Sample size	**3 g**	**2.5 g**	**2.5 g**	**2.1 g**	**2.1 g**
Naphthobenzothiophene				2.94	2.94
C1-Naphthobenzothiophene			2.94	2.94	5.9
C2-Naphthobenzothiophene			2.94	2.94	5.9
C3-Naphthobenzothiophene			2.94	2.94	5.9
Benz[*a*]anthracene	3.3	1	1	3.16	3.16
Chrysene	3.8	0.9	0.9	5.08	5.08
C1-Chrysenes	6.6	1.8	1.8	5.08	5.08
C2-Chrysenes	6.6	1.8	1.8	5.08	5.08
C3-Chrysenes	6.6	1.8	1.8	5.08	5.08
C4-Chrysenes	6.6	1.8	1.8	5.08	5.08
Benzo[*b*]fluoranthene	1.6	0.8	0.8	3.83	3.83
Benzo[*k*]fluoranthene	1.2	0.7	0.7	2.82	2.82
Benzo[*e*]pyrene	1.6	1.9	1.9	2.68	2.68
Benzo[*a*]pyrene	1.2	1.3	1.3	1.63	1.63
Perylene	1.5	2	2	5.35	5.35
Indeno[*1,2,3-c,d*]pyrene	1.2	1	1	3.34	3.34
Dibenzo[*a,h*]anthracene	1	0.4	0.4	2.36	2.36
C1-Dibenzo[*a,h*]anthracene				2.36	2.36
C2-Dibenzo[*a,h*]anthracene				2.36	2.36
C3-Dibenzo[*a,h*]anthracene				2.36	2.36
Benzo[*g,h,i*]perylene	1.4	0.7	0.7	2.39	2.39
2-Methylnaphthalene	1.1	0.6	0.6		
1-Methylnaphthalene	0.7	0.3	0.3		
2,6-Dimethylnaphthalene	0.7	0.5	0.5		
1,6,7-Trimethylnaphthalene	1.2	1	1		
1-Methylphenanthrene	1.2	1.3	1.3		

	2003	2004
Compound	15.0 g	15.0 g
Decalin	0.17	0.17
C1-Decalin	0.35	0.35
C2-Decalin	0.35	0.35
C3-Decalin	0.35	0.35
C4-Decalin	0.35	0.35
Naphthalene	0.17	0.17
C1-Naphthalenes	0.33	0.33
C2-Naphthalenes	0.35	0.35
C3-Naphthalenes	0.35	0.35
C4-Naphthalenes	0.35	0.35
Benzothiophene	0.17	0.17
C1-Benzothiophene	0.35	0.35
C2-Benzothiophene	0.35	0.35
C3-Benzothiophene	0.35	0.35
Biphenyl	0.14	0.14
Acenaphthylene	0.19	0.19
Acenaphthene	0.13	0.13
Dibenzofuran	0.2	0.2
Fluorene	0.19	0.19
C1-Fluorenes	0.39	0.39
C2-Fluorenes	0.39	0.39
C3-Fluorenes	0.39	0.39
Carbazole	0.33	0.33
Anthracene	0.19	0.19
Phenanthrene	0.14	0.14
C1-Phenanthrenes/Anthracenes	0.29	0.29
C2-Phenanthrenes/Anthracenes	0.29	0.29
C3-Phenanthrenes/Anthracenes	0.29	0.29
C4-Phenanthrenes/Anthracenes	0.29	0.29
Dibenzothiophene	0.15	0.15
C1-Dibenzothiophenes	0.31	0.31
C2-Dibenzothiophenes	0.31	0.31
C3-Dibenzothiophenes	0.31	0.31
Fluoranthene	0.21	0.21
Pyrene	0.19	0.19
C1-Fluoranthenes/Pyrenes	0.39	0.39
C2-Fluoranthenes/Pyrenes	0.39	0.39
C3-Fluoranthenes/Pyrenes	0.39	0.39

	2003	2004
Compound	15.0 g	15.0 g
Naphthobenzothiophene	0.2	0.2
C1-Naphthobenzothiophene	0.41	0.41
C2-Naphthobenzothiophene	0.41	0.41
C3-Naphthobenzothiophene	0.41	0.41
Benz[a]anthracene	0.13	0.13
Chrysene	0.17	0.17
C1-Chrysenes	0.35	0.35
C2-Chrysenes	0.35	0.35
C3-Chrysenes	0.35	0.35
C4-Chrysenes	0.35	0.35
Benzo(b)fluoranthene	0.29	0.29
Benzo(k)fluoranthene	0.23	0.23
Benzo(e)pyrene	0.31	0.31
Benzo(a)pyrene	0.22	0.22
Perylene	1.38	1.38
Indeno[1,2,3-c,d]pyrene	0.28	0.28
Dibenzo(a,h)anthracene	0.15	0.15
C1-Dibenzo[a,h]anthracene	0.31	0.31
C2-Dibenzo[a,h]anthracene	0.31	0.31
C3-Dibenzo[a,h]anthracene	0.31	0.31
Benzo[g,h,i]perylene	0.14	0.14
2-Methylnaphthalene		
1-Methylnaphthalene		
2,6-Dimethylnaphthalene		
1,6,7-Trimethylnaphthalene		
1-Methylphenanthrene		

Table A-6. Mussel Watch Project pesticide tissue method limits of detection (ng/g dry weight).

Sample size	2000 3 g	2001 2.5 g	2002 2.5 g	2003 2.1 g	2004 2.1 g
Aldrin	0.23	0.25	0.25	0.24	0.24
Dieldrin	0.27	0.37	0.37	0.22	0.22
Endrin	0.38	0.24	0.24	0.21	0.21
Heptachlor	0.33	0.28	0.28	0.25	0.25
Heptachlor-Epoxide	0.28	0.3	0.3	0.23	0.23
Oxychlordane	0.52	0.27	0.27	0.28	0.28
Alpha-Chlordane	0.59	0.29	0.29	0.23	0.23
Gamma-Chlordane	0.71	0.29	0.29	0.27	0.27
Trans-Nonachlor	0.44	0.22	0.22	0.22	0.22
Cis-Nonachlor	0.4	0.26	0.26	0.24	0.24
Alpha-HCH	0.43	0.25	0.25	0.23	0.23
Beta-HCH	0.27	0.26	0.26	0.23	0.23
Delta-HCH	0.43	0.23	0.23	0.23	0.23
Gamma-HCH	0.37	0.25	0.25	0.22	0.22
2,4'-DDD	0.34	0.29	0.29	0.22	0.22
4,4'-DDD	0.57	0.34	0.34	0.2	0.2
2,4'-DDE	0.28	0.25	0.25	0.21	0.21
4,4'-DDE	0.51	0.24	0.24	0.22	0.22
2,4'-DDT	0.3	0.25	0.25	0.25	0.25
4,4'-DDT	0.47	0.25	0.25	0.21	0.21
1,2,3,4-Tetrachlorobenzene	0.26	0.29	0.29	0.33	0.33
1,2,4,5-Tetrachlorobenzene	0.38	0.27	0.27	0.3	0.3
Hexachlorobenzene	0.51	0.24	0.24	0.25	0.25
Pentachloroanisole	0.32	0.28	0.28	0.18	0.18
Pentachlorobenzene	0.28	0.26	0.26	0.22	0.22
Endosulfan II	0.72	0.26	0.26	0.25	0.25
Endosulfan I	0.72	0.26	0.26	0.25	0.25
Endosulfan Sulfate	0.72	0.29	0.29	0.27	0.27
Mirex	0.42	0.28	0.28	0.23	0.23
Chlorpyrifos	0.47	0.28	0.28	0.25	0.25

Table A-7. Mussel Watch Project pesticide sediment method limits of detection (ng/g dry weight).

	2003	2004
Sample size	**15.0 g**	**15.0 g**
Aldrin	0.1	0.1
Dieldrin	0.06	0.06
Endrin	0.12	0.12
Heptachlor	0.09	0.09
Heptachlor-Epoxide	0.18	0.18
Oxychlordane	0.05	0.05
Alpha-Chlordane	0.04	0.04
Gamma-Chlordane	0.05	0.05
Trans-Nonachlor	0.04	0.04
Cis-Nonachlor	0.07	0.07
Alpha-HCH	0.09	0.09
Beta-HCH	0.07	0.07
Delta-HCH	0.08	0.08
Gamma-HCH	0.05	0.05
2,4'-DDD	0.07	0.07
4,4'-DDD	0.11	0.11
2,4'-DDE	0.05	0.05
4,4'-DDE	0.04	0.04
2,4'-DDT	0.1	0.1
4,4'-DDT	0.08	0.08
1,2,3,4-Tetrachlorobenzene	0.12	0.12
1,2,4,5-Tetrachlorobenzene	0.08	0.08
Hexachlorobenzene	0.05	0.05
Pentachloroanisole	0.06	0.06
Pentachlorobenzene	0.06	0.06
Endosulfan II	0.1	0.1
Endosulfan I	0.1	0.1
Endosulfan Sulfate	0.11	0.11
Mirex	0.04	0.04
Chlorpyrifos	0.1	0.1

Table A-8. Mussel Watch Project polychlorinated biphenyl tissue method limits of detection (ng/g dry weight).

	2000	2001	2002	2003	2004
Sample size	**3 g**	**2.5 g**	**2.5 g**	**2.1 g**	**2.1 g**
PCB8	0.77	0.39	0.39	0.36	0.36
PCB18	0.8	0.52	0.52	0.44	0.44
PCB28	0.54	0.45	0.45	0.22	0.22
PCB44	0.63	0.35	0.35	0.4	0.4
PCB52	0.26	0.48	0.48	0.24	0.24
PCB66	0.67	0.43	0.43	0.34	0.34
PCB101	0.43	0.66	0.66	0.32	0.32
PCB105	0.85	0.41	0.41	0.33	0.33
PCB118	0.35	0.67	0.67	0.25	0.25
PCB128	0.83	0.43	0.43	0.54	0.54
PCB138	0.58	0.87	0.87	0.43	0.43
PCB153	0.97	0.67	0.67	0.49	0.49
PCB170	0.51	0.81	0.81	0.32	0.32
PCB180	0.84	0.58	0.58	0.24	0.24
PCB187	0.45	0.62	0.62	0.31	0.31
PCB195	0.34	0.33	0.33	0.27	0.27
PCB206	0.33	0.31	0.31	0.29	0.29
PCB209	0.32	0.36	0.36	0.25	0.25

Table A-9. Mussel Watch Project polychlorinated biphenyl sediment method limits of detection (ng/g dry weight).

	2003	2004
Sample size	**15.0 g**	**15.0 g**
PCB8	0.1	0.1
PCB18	0.06	0.06
PCB28	0.05	0.05
PCB44	0.1	0.1
PCB52	0.05	0.05
PCB66	0.04	0.04
PCB101	0.04	0.04
PCB105	0.1	0.1
PCB118	0.06	0.06
PCB128	0.11	0.11
PCB138	0.06	0.06
PCB153	0.06	0.06
PCB170	0.06	0.06
PCB180	0.06	0.06
PCB187	0.04	0.04
PCB195	0.04	0.04
PCB206	0.04	0.04
PCB209	0.07	0.07

Appendix 3: Biological Methods

GONADAL ANALYSIS

Y. Kim, K. A. Ashton-Alcox, and E. N. Powell
Haskin Shellfish Research Laboratory
Rutgers University
Port Norris, NJ 08349

ABSTRACT
This chapter describes the procedures for determining the reproductive stage of oysters, mytilid mussels, and dreissenid mussels collected for NOAA's National Status and Trends Mussel Watch Project. Analyses are conducted on paraffin-embedded tissues sectioned at a 5-µm thickness and stained using a pentachrome staining procedure. Each slide is examined microscopically to determine the animal's sex and stage of gonadal development. A semi-quantitative ranking is assigned.

INTRODUCTION
Assessment of the physiological state of bivalve populations requires an analysis of the state of gonadal development. Determination of reproductive stage is included as part of the Mussel Watch Project to give an indication of the amount of gametic material in bivalve tissues at the time of chemical analysis. Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Cunningham and Tripp 1975; Mo and Neilson, 1993). Because gametic material can account for 20% to 50% of body weight in target species of oysters and mussels (Sprung, 1991; Choi *et al.*, 1993), the relative proportion of gonadal to somatic tissue and the timing of spawns (an important depuration route for some contaminants) can significantly impact the body burden of contaminants.

This description updates the methods presented in Ellis *et al.* (1998b) and Powell *et al*. (1993). The original intent of the determination of reproductive stage was to assure that sampling was conducted at the same stage of the reproductive cycle so that analyses of the lipophilic organic contaminants and the trace element contaminants were not influenced by reproductive state. Unfortunately, the time required for sampling and the wide latitudinal range encompassed by the sites did not permit consistent recovery of individuals in similar stages of reproductive development at all sites. For example, typically oysters are undifferentiated in the winter. Gonads begin to develop in early spring and spawning occurs late spring through early fall. Most Gulf Coast oysters spawn at least twice during this time period. Single spawns tend to occur in the shorter summers of the mid-Atlantic region (e.g., Dittman *et al*., 2001). The timing of the last spawn varies with latitude and with yearly variations in climate (e.g., Wilson *et al.*, 1990, 1992). Southeast Atlantic and Southern Gulf sites, for example, routinely yield oysters in reproductive development or that are ready to spawn in mid-winter during the period when Mussel Watch sampling occurs. Mid-Atlantic sites are typically characterized by individuals in an undifferentiated state and thus contain significantly less lipid-rich gametic tissue than the southern animals. Mytilid mussels and dreissenid mussels have the same assortment of problems relating to latitude and interannual changes in climate (Newell, 1989; Seed and Suchanek, 1992; Borcherding, 1991). In addition, dreissenid mussels are typically collected during late August-September whereas the remaining Mussel Watch species are collected during winter. Thus, analysis of reproductive stage has proved important in identifying differences in tissue composition that might affect comparisons of contaminant data among sites and among years. Wilson *et al.* (1990, 1992) and Kim *et al*. (1999, 2001)

discuss the influence of climate on reproductive stage and contaminant body burden in the Mussel Watch Project in more detail.

Oyster gonadal tissue is distributed around the body mass (Morales-Alamo and Mann, 1989). Gonads of dreissenid mussels also develop within the body, around the periphery of the viscera. In contrast to oysters and dreissenid mussels, gonadal follicles develop primarily within the mantle of mytilid mussels. Nevertheless, in none of these cases can the gonad be easily excised and weighed. Consequently, virtually all assays of reproductive stage use histological methods to recognize the changes in the germinal epithelium and germinal products that identify stages in gonadal development. The histological approach uses a semiquantitative numerical assignment to rank reproductive stage. Quantitative measures, such as egg protein content (Choi and Powell, 1993, Choi *et al*., 1993, 1994), remain expensive and time consuming, and do not permit a concomitant histopathological analysis. Therefore, a histological examination is still the single method of choice when only one method can be used.

For oysters, a dorsal-ventral slice of tissue is taken and fixed in Davidson's fixative. Dreissenid and mytilid mussels are preserved whole, and a dorsal-ventral slice is taken after fixation. Tissue slices are embedded in paraffin, sectioned, and stained using a pentachrome staining protocol. Stained sections are examined under a compound microscope, and sex and the state of gonadal development determined. Fixation follows the method described in Preece (1972). The staining procedure is an adaptation of Masson's (1928) trichrome procedure (Ellis *et al*., 1998b). Reproductive stage in oysters is determined using a semiquantitative scale adapted from Ford and Figueras (1988). The scale developed by Seed (1975, 1976) for determining gonad index in mussels was adopted for mytilid mussels and dreissenid mussels.

2.0 EQUIPMENT, REAGENTS, SOLUTIONS, AND SAMPLE PREPARATION

2.1. EQUIPMENT

Cover slips - various sizes. Fisher Scientific, Pittsburgh, PA.
Disposable cassettes - HistoPrep tissue capsules, 15-182-218. Fisher Scientific, Pittsburgh, PA.
Disposable microtome blades, 12-634-11. Fisher Scientific, Pittsburgh, PA.
Drying oven, 13-254-1. Fisher Scientific, Pittsburgh, PA.
Embedding rings - HistoPrep embedding rings, 12-652-1OB. Fisher Scientific, Pittsburgh, PA.
Frosted microslides, 12-552. Fisher Scientific, Pittsburgh, PA.
Microscope, compound, Zeiss, 12-070-20. Fisher Scientific, Pittsburgh, PA.
Microtome, HM330. Microm GmbH. D-6900 Heidelberg, Germany.
Slide staining set, Tissue-Tek II. Miles Laboratories, Inc. Westmont, IL.
Slide staining holder, Tissue-Tek, 4466. Miles Laboratories, Inc. Westmont, IL.
Stainless steel molds, various sizes. Fisher Scientific, Pittsburgh, PA.
Tissue embedding system, Histocentre 2, 6400012. Shandon, Inc., Pittsburgh, PA.
Tissue processor, Citadel 2000, 69800006. Shandon, Inc., Pittsburgh, PA.
Vacuum infiltrator, Tissue-Tek II, 4613. Miles Laboratories, Inc. Westmont, IL.
Water bath - Fisher Tissue PrepModel 134 Flotation Bath, 15-464. Fisher Scientific, Pittsburgh, PA.

2.2. REAGENTS

Acetone HistoPrep (CH_3COCH_3), [67-64-1], HC300-1GAL. Fisher Scientific, Pittsburgh, PA.
Acid fuchsin, certified stain ($C_{20}H_{17}N_3O_9S_3Ca$) [136132-76-8], A3908. Sigma Chemical Co.,St. Louis, MO.
Ammonium hydroxide (NH_4OH) [1336-21-6], A6899. Sigma Chemical Co., St. Louis, MO.
Aniline blue powder, certified stain [28631-66-5], A967-25. Fisher Scientific, Pittsburgh, PA.
Chromotrope powder 2R, ($C_{16}H_{10}N_2Na_2O_8S_2$) [4197-07-3], C3143. Sigma Chemical Co., St. Louis, MO.
Ethanol (C_2H_5OH) [64-17-5], R8382. Sigma Chemical Co., St. Louis, MO.
Fast green FCF, certified stain ($C_{37}H_{34}N_2O_{10}S_3Na_2$) [2353-45-9], F7252. Sigma Chemical Co., St. Louis, MO.
Ferric ammonium sulfate ($Fe\ NH_4(SO_4)_2 \cdot 12H_2O$) [7783-83-7], F1018. Sigma Chemical Co., St. Louis, MO.
Formaldehyde, 37% solution (CH_2O) [50-00-0], F1635. Sigma Chemical Co., St. Louis, MO.
Glacial acetic acid ($C_2H_4O_2$) [64-19-7], A0808. Sigma Chemical Co., St. Louis, MO.
Glycerin ($C_3H_8O_3$) [56-81-5], G7893. Sigma Chemical Co., St. Louis, MO.
Hematoxylin powder, certified stain ($C_{16}H_{14}O_6$) [517-28-2], H3136. Sigma Chemical Co., St. Louis, MO.
Orange G powder, certified stain ($C_{16}H_{10}N_2O_7S_2Na_2$) [1936-15-8], O7252. Sigma Chemical Co., St. Louis, MO.
Paraffin - Paraplast tissue embedding media (melting pt. 56 °C), 12-646-111, Fisher Scientific, Pittsburgh, PA.
Permount mounting media, SP15-500. Fisher Scientific, Pittsburgh, PA.
Phosphomolybdic acid ($20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) [51429-74-4], P7390. Sigma Chemical Co., St. Louis, MO.
Phosphotungstic acid ($12WO_3 \cdot H_3PO_4 \cdot H_2O$) [12501-23-4], P4006. Sigma Chemical Co., St. Louis, MO.
Sodium chloride (NaCl) [7647-14-5], S9625. Sigma Chemical Co., St. Louis, MO.
Sulfuric acid (H_2SO_4) [7664-93-9], S1526. Sigma Chemical Co., St. Louis, MO.
Tissue Clear III, SH3-4. Fisher Scientific, Pittsburgh, PA.
Tissue Dry, SH5-20. Fisher Scientific, Pittsburgh, PA.
Xyleness, histological grade ($C_6H_4(CH_3)_2$), X3S-4. Fisher Scientific, Pittsburgh, PA.

SOLUTIONS

Ferric alum mordant: 25 g ferric ammonium sulfate dissolved in 500 mL distilled water.
Basic ethanol: 26 mL ammonium hydroxide in a solution of 3370 mL 95% ethanol and 630 mL distilled water.
Phosphomolybdic acid solution: 5 g phosphomolybdic acid crystals dissolved in 495 mL distilled water.
1% acetic acid: 20 mL glacial acetic acid in 1980 mL distilled water.
1% acid acetone: 20 mL glacial acetic acid in 1980 mL acetone.

Groat/Weigert hematoxylin working solution: 245 mL distilled water, 5 mL sulfuric acid, 5 g ferric ammonium sulfate, 245 mL 95% ethanol, and 2.5 g hematoxylin powder.
Acid fuchsin working solution: 1.5 g acid fuchsin powder dissolved in 495 mL distilled water, to which is added 5 mL glacial acetic acid.
Phosphotungstic acid solution: 10 g phosphotungstic acid crystals dissolved in 490 mL distilled water.
Orange G/Chromotrope: 4 g orange G powder and 1 g chromotrope powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.
Fast green/Aniline blue working solution: 5 g fast green FCF, 4 g aniline blue powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.
Davidson's fixative solution: 1 part glycerin, 1 part glacial acetic acid, 2 parts 37% formaldehyde, 3 parts 95% ethanol, and 3 parts isotonic sodium chloride (usually 20 - 30‰).

3.0 SAMPLE COLLECTION AND FIXATION

3.1. SAMPLING

From 1986 to 1994, the same oysters were used for organic contaminant analysis and gonadal analysis at all Gulf coast sites (e.g., Powell *et al.*, 1993). The use of the same animals for gonadal analysis and for analysis of contaminant body burden potentially biases the latter analyses because digestive gland tissue and gonadal tissue, that contribute disproportionately to the tissue taken for histological analysis, may contain a higher than average body burden of certain contaminants. Sericano *et al.* (1993) showed that this source of error resulted in an underestimation of true body burden no greater than 10% when a 5-mm slice was removed from the large oysters normally sampled in the Mussel Watch Project. This potential error would be much larger for the smaller mytilids and dreissenids. To avoid this error, separate samples have always been obtained for gonadal analysis of mussels and East Coast oysters. Beginning in 1995, the same protocol was adopted for Gulf Coast oysters. The number of animals analyzed per site has also changed. Through 1994, 15 animals were analyzed per site. Thereafter, five were analyzed per site. Thus, the present sampling method requires the assumption that individuals collected from a common collection area will have experienced similar chemical loading and that the five animals analyzed for gonadal analysis are representative of the animals pooled for contaminant analysis.

3.2. TISSUE PREPARATION

3.2.1. Oyster tissue preparation

Five of the 12 oysters processed for Dermo analysis (Ashton-Alcox *et al*., this volume) are chosen randomly for gonadal analysis. A 5-mm thick cross-section of tissue is removed from the oyster using a scalpel or scissors. The determination of reproductive stage is based on a histological evaluation of the maturation stage of oyster gonads located within/around the visceral mass. The tissue section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gill tissue just posterior to the palps (Figure 1 in Ellis *et al*., 1998). This aspect provides example sections of most oyster tissues for histopathological analysis (Kim *et al.*, this volume), while also providing a representative cross-section of oyster gonad. Each section is immediately placed in a tissue cassette and the cassette placed in a jar filled with Davidson's fixative for 48 hr. After 48 hr, the fixative is decanted, 70% ethanol added and the tissues are allowed to stand until processing.

3.2.2. Mytilid mussel tissue preparation

Determination of reproductive stage for mussels is based on a histological evaluation of the maturation stages of mussel gonads, most of which are located in the mantle (Newell, 1989). Five animals are

analyzed but a few more are preserved for reasons discussed subsequently. The tip of a sharp knife is carefully inserted between the shells at the ventral lip and run dorsally between the shells until the posterior adductor muscle is cut so that the shells remain in an open position. Care is taken to cut no further than the adductor muscle to avoid cutting into the digestive gland immediately below the adductor muscle. Shucking of fresh mussels usually results in severe damage to the mantle tissue lying next to the shell. Therefore the mussels are placed whole in a wide-mouth jar filled with Davidson's fixative after the adductor muscle has been cut. Because the entire animal is being preserved, the specimens are left in fixative for at least a week to ensure preservation of all tissues. After this time, the fixative is decanted and 70% ethanol is added for storage until processing.

Once preserved, the tissue hardens and becomes easier to detach from the shell. To excise the preserved mussel meat from its shell, a knife is carefully run between the mantle and the lip of each valve, detaching the mantle from the shell. At this time, byssal threads are completely removed from the byssal gland to avoid later difficulties in tissue sectioning. Five specimens are chosen from each site and their anterior-posterior lengths are measured using a ruler. A 5-mm thick cross-section is then removed using a scalpel. The cross-section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gills at an angle across the body and such that ventral edge of the cross-section is slightly towards the posterior-ventral margin. Each cross-section is placed in a tissue cassette and processed immediately after dissection. If the mantle tissue is damaged during the shucking procedure, the specimen is replaced by one of the additional specimens preserved from the same site because the wound could result in the loss of gametic material and lead to an erroneous evaluation.

3.2.3. Dreissenid mussel tissue preparation

Most of the gonad of a dreissenid mussel is concentrated within the visceral mass (Borcherding, 1990). Due to their small size however, dissection of living tissue without destroying the gonads is difficult. Therefore, dreissenid mussels collected from each site are preserved whole in Davidson's fixative, without cutting the adductor muscle. They are left in fixative for one week to allow adequate time for tissue fixation. After this time, 20 to 30 mL of acetic acid is added to enhance decalcification of the shell. The shell is properly decalcified when it is no longer hard.

After decalcification, the Davidson's fixative is replaced with 70% ethanol according to the procedure followed for mytilid mussels and stored for later embedding. Prior to embedding, byssal threads are cut away from the byssal gland to minimize difficulty in sectioning the tissue. A 5-mm thick cross-section is taken from five individuals as described for mytilid mussels. Each section is placed in a tissue cassette and processed for embedding immediately thereafter.

4.0 SLIDE PREPARATION

4.1. TISSUE EMBEDDING

Individual tissue samples are prepared for embedding in paraffin using an established dehydration protocol (Table 1-1). The solutions used for dehydration, clearing, and infiltration are changed frequently to maintain solution purity. The tissue embedding sequence uses an automated tissue processor that processes tissue in plastic cassettes through the dehydration-clearing series and into paraffin. Embedding can also be done manually by moving the tissues through the sequence. The paraffin is melted in an embedding center with temperature set at 60°C. Newly melted paraffin should always be used in the final infiltration and embedding steps.

After the tissues are infiltrated with paraffin (Table 1-1), they are transferred to a vacuum infiltrator set at 60°C and placed under a vacuum for 30 min. Tissues are transferred to a holding tank of melted paraffin

and removed singly to stainless steel molds. The tissues are oriented with the cross-sectional face down for sectioning, and a plastic mold embedding ring is placed on top. The ring is filled with paraffin and the mold moved to a cold plate of the embedding system. As the tissue/paraffin cools and hardens, the paraffin shrinks. Care must be taken to use sufficient paraffin to cover the tissue after this shrinkage. The mold is left on the cold plate until the tissue-paraffin block is removed. The block is then placed in a freezer until sectioning.

4.2. TISSUE SECTIONING

The paraffin blocks are first cut at 20 µm to expose an entire tissue cross-section and then sliced at 5 µm using a microtome. Tissue sections may be cut singly or into contiguous sections. The sections are placed on the surface of a water bath maintained at 45-50°C and allowed to expand. Once the sections expand to full size, a microscope slide is held at an angle and slid under one or more of the tissue sections. The sections are then lifted out of the water and onto the slide. The sections are positioned on the slide in the orientation in which they will be stained and read. The slide is allowed to air dry until it can be placed in a slide rack. The slide rack is placed in a drying oven at 40°C. After drying overnight or longer, the slides are ready to stain.

Table 1-1. Tissue embedding sequence*.

Dehydration		**Clearing**	
Tissue Dry	60 min	Tissue Clear	60 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min		
Tissue Dry	120 min	**Infiltration**	
Tissue Dry	120 min		
Tissue Dry	60 min	paraffin	120 min
		paraffin	120 min
		paraffin (in vacuum infiltrator)	30min

* In cases where the sequential solutions are the same, each transfer is a transfer to a fresh solution.

4.3. TISSUE STAINING

Sections are deparaffinized and hydrated using a xylenes-ethanol series (Table 1-2). Following hydration, slides are stained in a pentachrome series, dehydrated in a series of acetic acid dips followed by acetone, cleared in xyleness and mounted in Permount (Table 1-2). The pentachrome staining procedure is an adaptation of the trichrome stain of Masson (1928) as modified by Gurr (1956) (Ellis *et al*., 1998b). The modifications include the addition of aniline blue to the fast green working solution, substitution of chromotrope 2R/orange G for Ponceau de Xylidene, and the addition of phosphotungstic acid prior to the orange G/chromotrope stain; the procedure is now a pentachrome technique. The addition of these stains yields better differentiation of tissue types and mucins. Times required for each step are flexible in both the staining procedures discussed here and in the previous embedding protocol. Different tissue types may require different times. All solutions, especially the xyleness and ethanol ones, should be changed frequently. Slides should not be allowed to dry during transfers. Solutions to

common embedding, sectioning, and staining problems are discussed in Preece (1972) and most other manuals of histological technique.

5.0 ANALYSIS

Each slide is examined microscopically to determine sex and stage of gonadal development. A histopathological examination can also be made at this time (Kim *et al*., this volume). Careful examination of early developmental stages is needed to positively distinguish males or females in early stages of development from individuals as yet undifferentiated. Occasional hermaphrodites will also be found (all target species normally have separate sexes). The stage in the gametogenic cycle is assigned based on the maturity of the follicles and gametes and a numerical value is assigned as described in Tables 1-3 and 1-4.

Cases of renewed gonadal development following spawning are common in oysters (stage 7), particularly along the Gulf of Mexico coast (Supan and Wilson, 2001). These animals typically have a few remaining large, mature ova and many developing ova that would normally be found in stages 3 or 4. Accordingly, for oysters, further data reduction can better be achieved by comparing the number of individuals with substantial gonadal development with those having little gonadal volume using an egg/eggless ratio, calculated as:

$$\text{Oyster egg/eggless ratio} = \frac{\text{the number of individuals at stages 3, 4, 5, 6 and 7}}{\text{the number of individuals at stages 1, 2 and 8}}$$

For mytilids and dreissenids, the analogous ratio is calculated as:

$$\text{Mussel egg/eggless ratio} = \frac{\text{the number of individuals at stages 3, 4 and 5}}{\text{the number of individuals at stages 0, 1 and 2}}$$

Abnormal gonadal development is commonly observed in mytilid mussels. This is often characterized by unusual development of gametes at the base of the follicles. The cells resemble those of a germinoma (Peters *et al.* 1994) and are differentiated from normal cells by being either enlarged or by appearing to have an enlarged nucleus. In other cases, underdeveloped, small gonadal follicles are observed. These occupy a smaller portion of the mantle tissue. Follicles may be filled with cellular debris (Figure 1-1; see also Figure 10 in Ellis *et al*., 1998a). Sometimes cells adhere to each other, forming accumulations and empty spaces among developing cells. Occasionally, fibrosis occurs, with proliferation of fibroblasts inside the follicles and in the interfollicular connective tissue. Abnormal gonadal development is often associated with degeneration of Leydig tissue around the follicles and hemocytic infiltration into the surrounding tissues (Figure 11 in Ellis *et al*., 1998). The approach used to score instances of abnormal gonadal development uses a scale that rates the spatial coverage of the condition (e.g., fraction of follicles affected), but not the degree of effect in each follicle (Table 1-5). Normally, the entire follicle is completely affected or unaffected.

Table 1-2. Tissue staining sequence.

Deparaffinization	
xylenes	5 min
xylenes	5 min
xylenes	5 min
100% ethanol	3 min
100% ethanol	2 min
Hydration	
95% ethanol	2 min
10% ethanol	2 min
distilled water	2 min
Staining series	
Ferric alum mordant	10 min
Running tap water	quick dip
Groat/Weigert Hematoxylin*	30-45 min
Running tap water	5 min
Acid Fuchsin stain	1.5 min
Running tap water**	5 min
Phosphotungstic acid	2 min
Orange G/Chromotrope stain	1.5 min
Running tap water**	5 min
Phosphomolybdic acid	2 min
Fast Green/Aniline Blue stain	3 min
Dehydration	
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips
Clearing	
xylenes	5 min
xylenes	5 min
Mounting	
Mounting in Permount	24 hr to dry

* A basic ethanol dip can be used to blue the hematoxylin, if necessary.

** At these steps, no stain should remain between the slides and holding grooves in the slide rack.

Table 1-3. Oyster development stages adapted from Ford and Figueras (1988) by Powell *et al*. (1993).

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

Table 1-4. Mytilid and dreissenid development stages adapted from Seed (1975, 1976) by Hillman (1993).

Reproductive stage	Description
Resting/spent gonad	
Stage 0	Inactive or undifferentiated
Developing gonad	
Stage 1	Gametogenesis has begun; no ripe gametes visible
Stage 2	Ripe gametes present; gonad developed to about one-third of its final size
Stage 3	Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes
Stage 4	Gametogenesis still progressing, follicles contain mainly ripe gametes
Ripe gonad	
Stage 5	Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails
Spawning gonad	
Stage 4	Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced
Stage 3	Gonad about half empty
Stage 2	Gonadal area reduced; follicles about one-third full of ripe gametes
Stage 1	Only residual gametes remain; some may be undergoing cytolysis

Table 1-5. Semi-quantitative scale for abnormal gonadal development in mytilid mussels.

Score	Description
0	Normal gonad
1	Less than half the follicles are affected
2	About half the follicles are affected
3	More than half the follicles are affected
4	All follicles affected

6.0 CONCLUSIONS

The procedures described provide a semiquantitative ranking of reproductive stage but no quantification of the amount of gametic tissue present. The strengths of this approach are that it provides an assessment of sexual stage in the gametogenic cycle and allows for a concomitant histopathological analysis, with a single sample preparation protocol. The procedure cannot be performed on pooled samples. Thus, a direct correspondence between, for example, hydrocarbon body burden and stage in the gametogenic cycle may be difficult, because subsampling of individual animals will result in a certain degree of bias in the measurement of contaminant body burden, normally around 10% in adult oysters (Sericano *et al.*, 1993) and more for smaller individuals and species. This bias, therefore, will be size, contaminant, and time-of-year dependent**.**

If a quantitative gonadal/somatic index is desired, the technique of Choi and Powell (1993) should be used. The latter technique measures the concentration of egg protein present; however, it is not compatible with a concomitant histopathological analysis in that the standard histological preparation for assessing reproductive stage is not used in the quantitative analysis and tissue subsampling for histology cannot be done on the same individuals to be analyzed quantitatively for gonadal-somatic index. Choi *et al.* (1993) have further discussed the relative value of various approaches to gonadal evaluation. Overall, these authors found that the same general trends could be identified using either the semiquantitative or quantitative technique in most species, because normally an increase in gonadal volume occurs more or less simultaneously with advancement in reproductive stage and because gamete maturation occurs more or less evenly throughout the entire gonad.

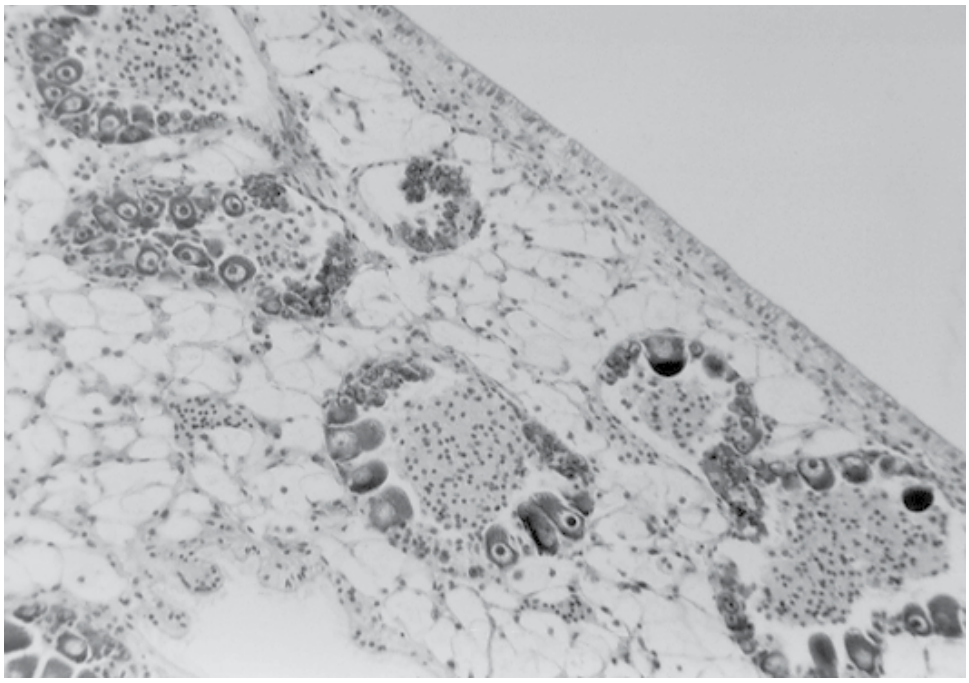


Figure 1-1. *Mytilus edulis* follicle with abnormal gametic tissue infiltrated with hemocytes.

REFERENCES

Abbe, G. R., J. G. Sanders and G. F. Riedel (1994) Silver uptake by the oyster (*Crassostrea virginica*): effect of organisms size and storage sites. Estuarine Coastal Shelf Sci., 39:249-60.

Borcherding, J. (1990) Die Reproduktionsleistungen der Wandermuschel *Dreissena polymorpha*. Ph.D. Dissertation, Universität zu Köln, Germany. 168 pp.

Borcherding, J. (1991) The annual reproductive cycle of the freshwater mussel *Dreissena polymorpha* Pallas in lakes. Oecologia (Berl.), 87:208-18.

Choi, K-S. and E. N. Powell (1993) Development of and immunological probe for quantification of oyster gonad. In: Sampling and analytical methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992. Vol. II Comprehensive descriptions of complementary measurements. NOAA Tech. Mem. NOS ORCA 71. NOAA/NOS/ORCA, Silver Spring, MD. 85-102.

Choi, K-S., D. H. Lewis, E. N. Powell and S. M. Ray (1993) Quantitative measurement of reproductive output in the American oyster, *Crassostrea virginica* (Gmelin), using an enzyme-linked immunosorbent assay (ELISA). Aquacult. Fish. Management, 24:299-322.

Choi, K-S., E. N. Powell, D. H. Lewis and S. M. Ray (1994) Instantaneous reproductive effort in female American oysters, *Crassostrea virginica*, measured by a new Immunoprecipitation Assay. Biol. Bull., 186:41-61.

Cunningham, P. A. and M. R. Tripp (1975) Accumulation, tissue distribution and elimination of $^{203}HgCl_2$ and $CH_3{}^{203}HgCl$ in the tissues of the American oyster *Crassostrea virginica.* Mar. Ecol., 31:321-34.

Dittman, D. E., S. E. Ford and D. K. Padilla (2001) Effects of *Perkinsus marinus* on reproduction and condition of the eastern oyster, *Crassostrea virginica*, depend on timing. J. Shellfish Res., 20:1025-34.

Ellis, M. S., R. D. Barber, R. E. Hillman, Y. Kim and E. N. Powell (1998a) Histopathology analysis. In: Sampling and Analytical Methods of the National Status and Trends Program Mussel Watch Project: 1993-1996 Update. NOAA Tech. Memo. NOS ORCA 130. NOAA/NOS/ORCA Silver Spring, MD. 198-215.

Ellis, M. S., R. D. Barber, R. E. Hillman and E. N. Powell (1998b) Gonadal analysis. In: Sampling and Analytical Methods of the National Status and Trends Program Mussel Watch Project: 1993-1996 Update. NOAA Tech. Memo. NOS ORCA 130. NOAA/NOS/ORCA Silver Spring, MD. 216-227.

Ford, S. E. and A. J. Figueras (1988) Effects of sublethal infection by the parasite *Haplosporidium nelsoni* (MSX) on gametogenesis, spawning, and sex ratios of oysters in Delaware Bay, USA. Dis. Aquat. Org., 4:121-33.

Gurr, E. (1956) A Practical Manual of Medical and Biological Staining Techniques. Interscience, New York. 320 pp.

Hillman, R. E. (1993) Gonadal index and histopathology for the East and West coasts used in the National Status and Trends Mussel Watch Project. In: Sampling and Analytical Methods of the National

Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992. Vol. II Comprehensive descriptions of complementary measurements. NOAA Tech. Mem. NOS ORCA 71. NOAA/NOS/ORCA, Silver Spring, MD. 45-53.

Kim, Y., E. N. Powell, T.L. Wade, B.J. Presley and J.M. Brooks (1999) Influence of climate change on interannual variation in contaminant body burden in Gulf of Mexico oysters. Mar. Environ. Res., 48:459-88.

Kim, Y., E.N. Powell, T.L. Wade, B.J. Presley and J.M. Brooks (2001) The geographic distribution of population health and contaminant body burden in Gulf of Mexico oysters. Arch. Environ. Contam. Toxicol., 41:30-46.

Lee, R. F. (1993) Passage of xenobiotics and their metabolites from hepatopancreas into ovary and oocytes of blue crabs, *Callinectes sapidus*: possible implications for vitellogenesis. Mar. Environ. Res., 35:181-7.

Masson, P. (1928) Carcinoids (argentaffin cell tumors) and nerve hyperplasia of the appendicular mucosa. Am. J. Pathol., 4:181-211.

Mo, C. and B. Neilson (1993) Weight and salinity effects on zinc uptake and accumulation for the American oyster (*Crassostrea virginica* Gmelin). Environ. Pollut., 82:191-6.

Morales-Alamo, R. and R. Mann (1989) Anatomical features in histological sections of *Crassostrea virginica* (Gmelin, 1791) as an aid in measurements of gonad area for reproductive assessment. J. Shellfish Res., 8:71-82.

Newell, R. I. E. (1989) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North and Mid-Atlantic) - blue mussel. U.S. Fish. Wildl. Serv. Biol. Rep. 82(11.102). U.S. Army Corps of Engineers, TR E1-82-4. 25 pp.

Peters, E. C., P. P. Yevich, J. C. Harshbarger and G. E. Zaroogian (1994) Comparative histopathology of gonadal neoplasms in marine bivalve molluscs. Dis. Aquat. Org., 20:59-76.

Powell, E. N., E. A. Wilson-Ormond and K-S. Choi (1993) Gonadal analysis - *Crassostrea virginica*. In: Sampling and Analytical Methods of the National Status and Trends Program National Benthic Surveillance and Mussel Watch Projects 1984-1992. Vol. II Comprehensive descriptions of complementary measurements. NOAA Tech. Mem. NOS ORCA 71. NOAA/NOS/ORCA, Silver Spring, MD. 55-62.

Preece, A. (1972) A Manual for Histologic Technicians. Little, Brown, and Company, Boston. 428 pp.

Seed, R. (1975) Reproduction in *Mytilus* (Mollusca: Bivalvia) in European waters. Pubblicazioni della Stazione Zoologica di Napoli (Milan), 39(Suppl. 1):317-334.

Seed, R. (1976) Ecology. In: Marine Mussels: Their Ecology and Physiology. B. L. Bayne (ed.). Cambridge University Press, New York, NY. 13-66.

Seed, R. and T. H. Suchanek (1992) Population and community ecology of *Mytilus*. In: The Mussel *Mytilus*: Ecology, Physiology, Genetics and Culture. E. Gosling (Ed.). Developments in Aquaculture and Fisheries Science, 25:87-169.

Sericano, J. L., T. L. Wade, E. N. Powell and J. M. Brooks (1993) Concurrent chemical and histological analyses: are they compatible? Chem. Ecol., 8:41-7.

Sprung, M. (1991) Costs of reproduction: a study on metabolic requirements of the gonads and fecundity of the bivalve *Dreissena polymorpha*. Malacologia, 33:63-70.

Supan, J. E. and C. A. Wilson (2001) Analyses of gonadal cycling by oyster broodstock, *Crassostrea virginica* (Gmelin), in Louisiana. J. Shellfish Res., 20:215-20.

Wilson, E. A., E. N. Powell, M. A. Craig, T. L. Wade and J. M. Brooks (1990) The distribution of *Perkinsus marinus* in Gulf coast oysters: its relationship with temperature, reproduction, and pollutant body burden. Int. Rev. Gesamten Hydrobiol., 75:533-50.

Wilson, E. A., E. N. Powell, T. L. Wade, R. J. Taylor, B. J. Presley and J. M. Brooks (1992) Spatial and temporal distributions of contaminant body burden and disease in Gulf of Mexico oyster populations: the role of local and large-scale climatic controls. Helgol. Meeresunters, 46:201-35.

HISTOPATHOLOGY ANALYSIS

Y. Kim, E. N. Powell, and K. A. Ashton-Alcox
Haskin Shellfish Research Laboratory
Rutgers University
Port Norris, NJ 08349

ABSTRACT
This chapter describes the procedures followed for histopathological analysis of oysters, mytilid mussels, and dreissenid mussels collected for NOAA's National Status and Trends Mussel Watch Project. Analyses are conducted on paraffin-embedded tissues sectioned at a 5-µm thickness and stained using a pentachrome staining procedure. The infection intensity of parasites, the occurrence and extent of tissue pathologies, and the intensity of diseases are recorded using quantitative or semi-quantitative measures.

1.0 INTRODUCTION

The use of bivalves in the National Status and Trends (NS&T) Program is predicated upon their reliability as environmental integrators of contamination. The influence of population health on body burden and, in turn, the influence of contaminant exposure on population health are, as yet, poorly understood. Clearly, certain diseases (normally caused by viruses or single-celled prokaryotes and eukaryotes) and non-disease causing parasites (mostly ciliates and multi-cellular parasites) produce tissue level changes that might be expected to affect contaminant body burden. Gonadal quantity, for example, can be dramatically altered by disease (Hofmann *et al.*, 1995; Barber, 1996; Ford and Figueras, 1988) and by parasites (Hopkins, 1957; Yoo and Kajihara, 1985). Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Mo and Neilson, 1993; Cunningham and Tripp, 1975). Evidence, mostly by correlation, continues to mount for a relationship between certain tissue pathologies and contaminant exposure (Bowmer *et al.*, 1991; Weis *et al.*, 1995; Johnson *et al.*, 1992; MacKenzie *et al*., 1995; Heinonen *et al*., 1999) and the influence of contaminant exposure on the bivalve immune system has been described (Anderson *et al.*, 1992; Cheng, 1988; Winstead and Couch, 1988; Ashton-Alcox *et al*., 2000). Besides contaminants, other environmental factors may facilitate disease or trigger the development of pathologies (e.g., Lee *et al.*, 1996; Landsberg, 1996; Zander, 1998) with significant consequences to tissue composition and, in all likelihood, subsequent contaminant retention. Thus, contaminant exposure and health, as they feed back upon each other, may have dramatic effects on monitoring programs that use sentinel organisms to define spatial and temporal trends in contaminant loading and contaminant gradients.

Evaluation of the health of bivalves collected as a part of the Mussel Watch Project necessitates determining the prevalence and intensity of diseases, parasites and pathologies by histological examination. Certain pathological conditions recognized by shell condition (Warburton, 1958; Lawler and Aldrich, 1987), ligament degradation (Dungan *et al.*, 1989), or periostracal abnormalities (Davis and Barber, 1994) will not be identified using this approach and some ectoparasites are lost during collection (e.g., odostomians, White *et al.*, 1985, 1989). However most common diseases, parasites, and pathologies can be evaluated by this method. Specific assays are available now for some organisms (e.g., Ko *et al*., 1999; Stokes and Burreson, 2001), but a histological examination remains the best general approach for simultaneously evaluating a wide range of parasites, disease and pathologies (Ford, 2002).

Classically, histological examination involves evaluating samples for parasite prevalence and the occurrence of pathologies. Measures of prevalence or occurrence, however, do not give a true indication of the health of an organism. Wilson-Ormond *et al*. (2000) compared the usefulness of prevalence to semi-quantitative and quantitative measures of intensity in the Gulf of Mexico Offshore Operations Monitoring Experiment and found that most significant trends were observed from intensity data rather than prevalence. One reason for this is that prevalence depends on transmission, and transmission rate may be controlled by biological factors such as population density, encounter rates, and inherent differences in susceptibility, as well as factors acting as stressors directly on the individuals in the population (Kermack and McKendrick, 1991; Ackerman *et al.*, 1984; Hofmann *et al.*, 1995; Powell *et al.*, 1996). Thus, measures of intensity or extent of tissue alteration may more reliably correspond to measures of exposure. Accordingly, beginning in 1995, a histopathological analysis designed to evaluate population health was included in the Mussel Watch Project. The approach taken was to evaluate the intensity of diseases and parasites, and the extent of tissue pathologies, rather than simply prevalence, to better assess the health of sampled populations. This description updates protocols described earlier by Ellis *et al*. (1998).

A measure of overall health has not been applied, although a number of these have been suggested, based on tissue appearance (Quick and Mackin, 1971), histological grading (Bowmer *et al.*, 1991), or summation of total parasite load (Wilson-Ormond *et al*., 2000). Although Laird (1961) argues on theoretical grounds for an underlying relationship between total parasite body burden and environmental quality, generally, these overall measures of health have not proven efficacious because the various parasites, diseases and pathologies originate in different ways and certain parasites, even if abundant, may not have a large impact on organism health.

2.0 EQUIPMENT, REAGENTS, AND SOLUTIONS

Preparation of samples for histopathological analysis follows the protocols established for gonadal analysis (see Kim *et al*., this volume).

ANALYSIS

Prepared slides are examined individually under the microscope using a 10X ocular and a 10X objective. If any tissue needs to be examined more closely, a 25X or 40X objective may be used for closer examination of suspected pathologies or parasites. Major tissue types examined include gill, mantle, gonad and gonoducts, digestive gland tubules, stomach/digestive gland ducts, and connective tissue. Thus, a proper tissue cross-section is essential (Kim *et al*., this volume). As the histopathological analysis is done in concert with gonadal analysis (Kim *et al*., this volume), mytilid mussels are usually examined beginning with the gonads and mantle tissue to determine sex and stage of gonadal development. The gills and the visceral mass are then examined. The gonads of oysters and dreissenid mussels are located within/around the visceral mass, so gonads are examined first, followed by scanning of the visceral mass and gills.

Parasites, diseases, or tissue pathologies are scored for intensity using either a quantitative or semi-quantitative scale, as described subsequently (Table 2-1). Conditions scored quantitatively are evaluated by keeping a running count of incidences of the condition as the slide is scanned to avoid re-examining each slide multiple times for each category. Conditions scored semi-quantitatively may require re-scanning portions of the tissue for each category to fully evaluate the scale of infection.

Listed subsequently are the common parasites and pathologies encountered during histopathological analysis including, in each case, the method of analysis, a short description, and a reference to published

figures of the condition. The list is not intended to be inclusive of all known parasites and pathologies for Mussel Watch species or all conditions encountered in specimens obtained during the Mussel Watch Project. Frequently, in routine examination, we do not attempt to differentiate at a low taxonomic level between related parasites because infection intensities are low for the majority of parasite species and so, the information gained from taxonomic analysis does not warrant the time spent in identification. Rather, we have lumped the various species into higher categories (e.g., all cestodes, all ciliates). When further differentiation is needed, we first differentiate by tissue of occurrence because most species have distinct tissue preferences. In nearly all cases, this level of differentiation has been adequate for estimates of prevalence and infection intensity.

3.1. QUANTITATIVE CATEGORIES

Most parasites are counted quantitatively (Table 2-1). These include prokaryotic inclusion bodies, gregarines, ciliates of various types, *Pseudoklossia* (a coccidian), cestodes, trematode metacercariae, worms ectoparasitic or commensal on the gills, nematodes, copepods, pinnotherid crabs, and worms in the gonoducts. We also evaluate a number of tissue conditions quantitatively, including the number of ceroid bodies, incidences of tissue inflammation, and suspected neoplasms and tumors.

Table 2-1. List of quantitative and semi-quantitative categories for each bivalve taxon. In a number of cases, e.g., gregarines and ciliates, subcategories by tissue type and organism morphology are individually tallied, as described in the text.

	Oyster	Mytilid Mussel	Dreissenid Mussel
Quantitative Category			
Prokaryote inclusions	X	X	
Gregarines	X	X	
Ciliates	X	X	
Xenomas	X	X	
Coccidians		X	
Cestodes	X		
Trematode metacercariae	X	X	
Turbellarians and nemerteans	X	X	
Nematodes	X		X
Copepods	X	X	
Pinnotherid crabs	X	X	
Echinostomes	X		
Unidentified organisms	X	X	X
Ceroid bodies	X	X	X
Neoplasms		X	
Tissue inflammation	X	X	X
Tissue necrosis	X	X	X
Semi-quantitative Category			
Haplosporidium nelsoni (MSX)	X		
Trematode sporocysts	X	X	
Digestive tubule atrophy	X	X	X
Gonadal abnormalities (Kim *et al*., this volume)	X	X	
Unusual digestive tubules	X	X	X

Prokaryotic inclusions (Figures 2-1 – 2-2) [Additional photographs: Otto *et al*. (1979), p. 295, Figs. 2-7; Gulka and Chang (1984), p. 320, Fig. 1; Couch (1985), p. 63, Fig. 2; Gauthier *et al*. (1990), p. 112, Fig. 7; Murchelano and MacLean (1990), p. 9, Figs. 1-5, 1-6; Figueras *et al*. (1991b), p. 20, Fig. 2; Harshbarger *et al*. (1977), p. 667, Fig. 1; Robledo *et al*. (1994), p. 291 Fig. 3, p. 292 Fig. 4; Villalba *et al*. (1997), p. 130, Figs. 2-3; Powell *et al*. (1999), p. 2059 Fig. 2, p. 2060 Fig. 4], variously referred to as rickettsial bodies, chlamydial bodies or mycoplasms, are normally observed in the duct and tubule walls of the digestive gland. In Mussel Watch sites, prokaryote inclusions have been recorded in both mytilid mussels and oysters from the East, West, and Gulf coasts, but have not been observed in dreissenids from the Great Lakes area. Prokaryotic inclusions similar to those described by Harshbarger *et al*. (1977) have been observed within the epithelial cells of the digestive system and also occasionally occur in the lumen of the digestive tract in mytilids from West coast Mussel Watch sites and in oysters from East and Gulf coast Mussel Watch sites. In some cases, cysts containing prokaryotic inclusions are associated with the gill and the renal tissues in mytilids. Inclusions found in the digestive tract are usually roundish, whereas those in the gill and kidney are rather amorphous in shape. No apparent pathological effects or host responses to prokaryote infection have been detected, as is typical for most bivalves (Otto *et al*., 1979; Figueras *et al*., 1991a; Villalba *et al*., 1997). Each individual inclusion is counted.

Gregarines in the genus *Nematopsis* (Figures 2-3 - 2-5) [Additional photographs: Cheng (1967), p. 148, Fig. 29; Ford (1988), p. 218, Fig. 6G; Friedman *et al*. (1989), p. 136, Fig. 3; Gauthier *et al*. (1990), p. 110, Figs. 2, 3] are sporozoan parasites frequently found in oysters, and occasionally in mytilid mussels. Different *Nematopsis* species often show a tissue preference for either mantle or gill (Sprague and Orr, 1952). Kim *et al*. (1998) noted that gregarines were common in oysters from the southeastern and Gulf of Mexico coasts in Mussel Watch samples, and also observed gregarines in mytilid mussels from the West coast. Mud and stone crabs are known to be final hosts (Prytherch, 1940). Although gregarine infections are known to have low pathogenicity, mechanical interference by heavy infections has been suggested to have some harmful effects on the host (e.g., oyster) physiology (Sindermann, 1990). In Mussel Watch samples, gregarine spores typically occur in the connective tissue around the visceral mass of the body, in the gills, and in the mantle connective tissues of oysters and West coast mytilids. No host tissue reaction or discernible pathological effects have been observed, in agreement with Cheng (1967). Although, species identifications are not made, gregarines are routinely scored according to tissue occurrence; body, gill, or mantle, following Landau and Galtsoff (1951). Quantification is obtained by counting each individual in each tissue within one representative tissue section.

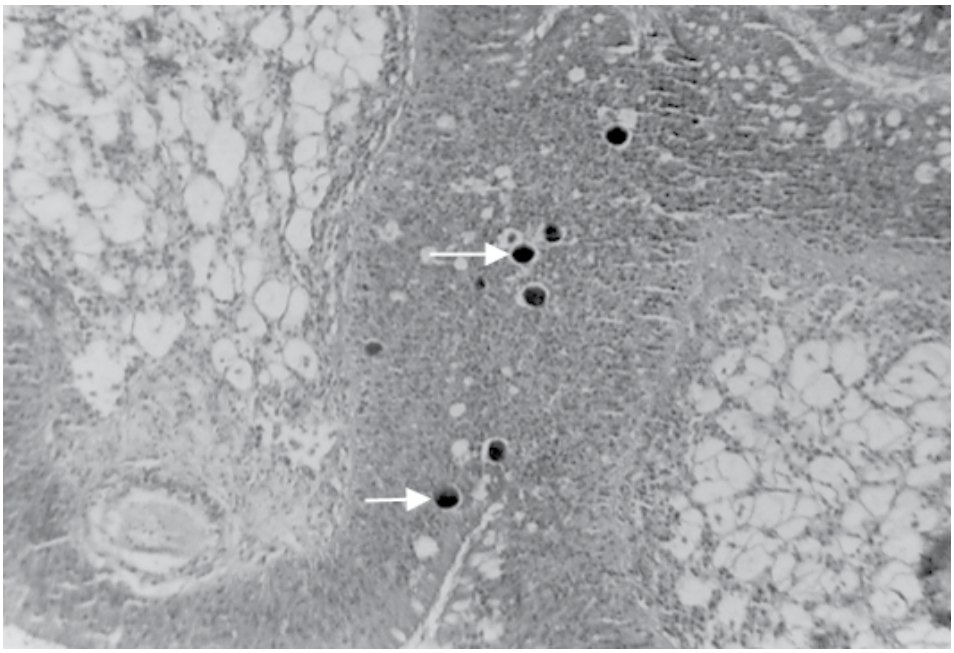


Figure 2-1. Prokaryotic inclusions present in digestive tract epithelium of an oyster, *Crassostrea virginica*. Arrows indicate examples. x 100.

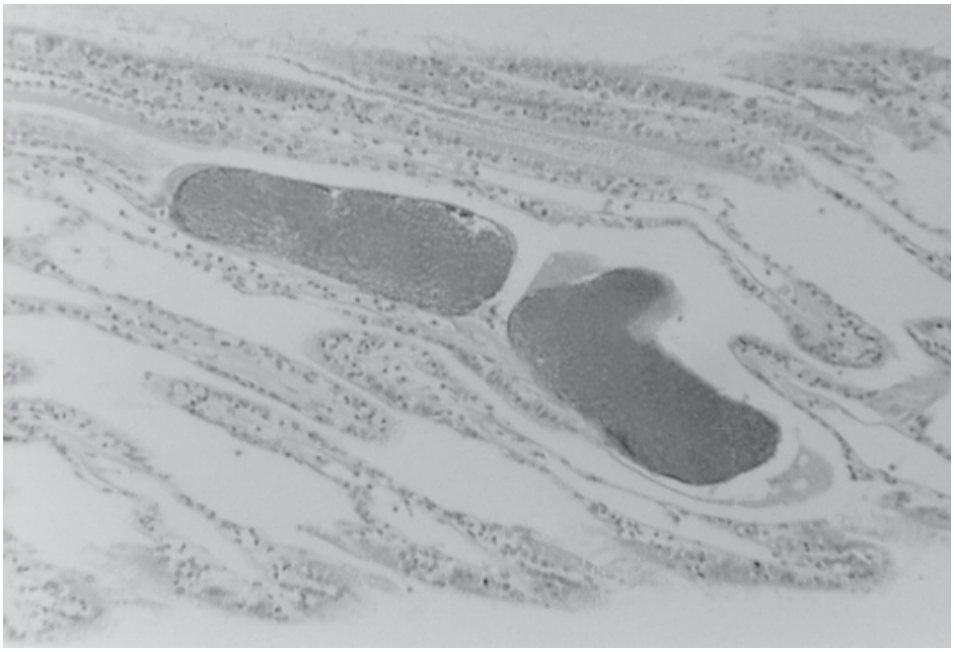


Figure 2-2. Cyst-like encapsulations of prokaryotic microorganism in gills of a mytilid mussel, *Mytilus edulis*. x 100.

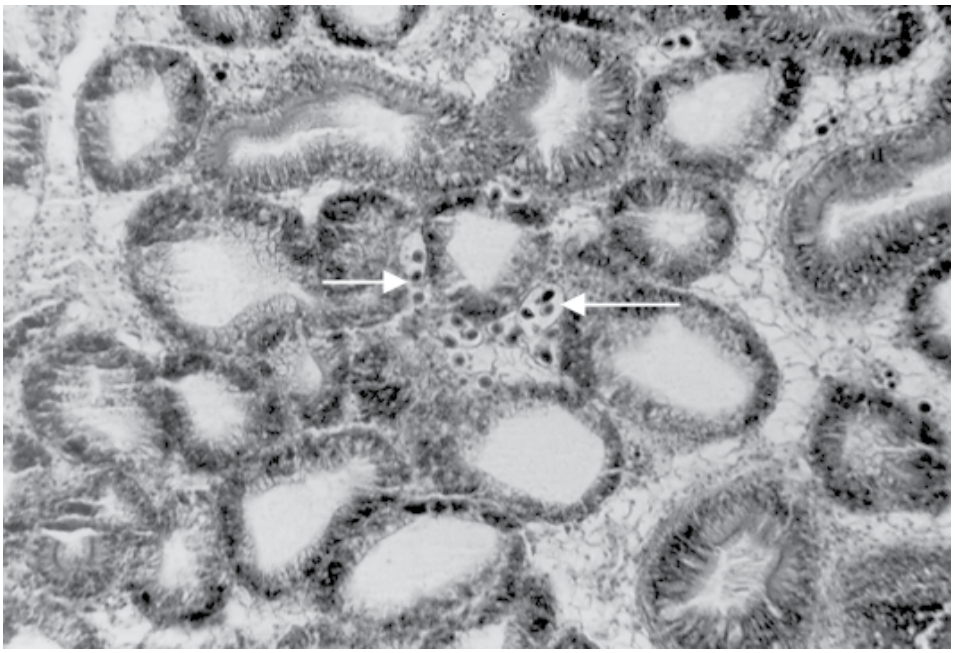


Figure 2-3. *Nematopsis* sp. spores in connective tissue between digestive tubules of an oyster, *C. virginica*. Arrows indicate examples. x 100.

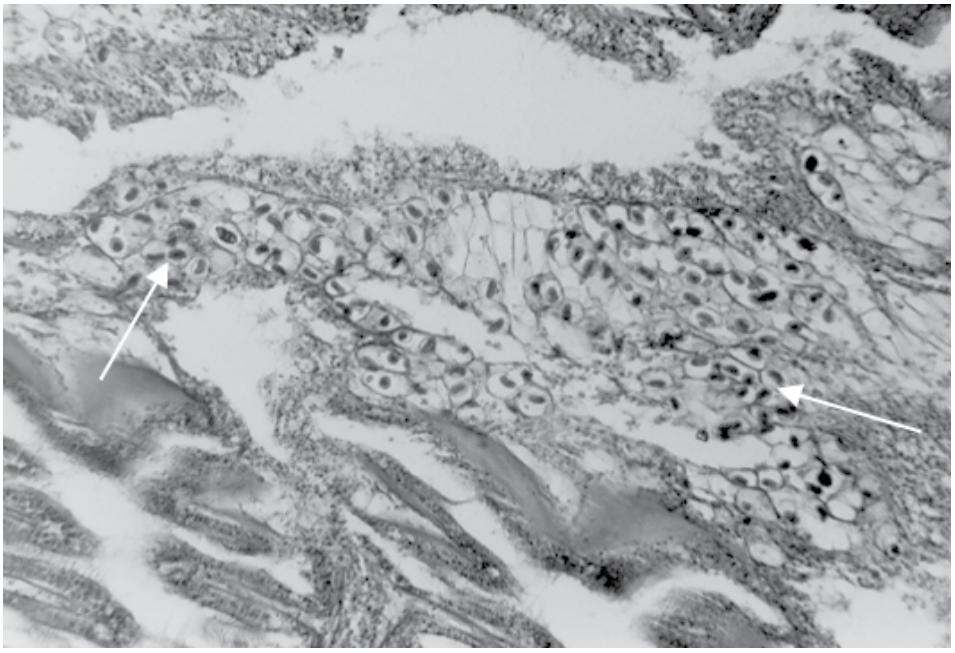


Figure 2-4. Numerous *Nematopsis* sp. spores in the gills of an oyster, *C. virginica*. Arrows indicate examples. x 100.

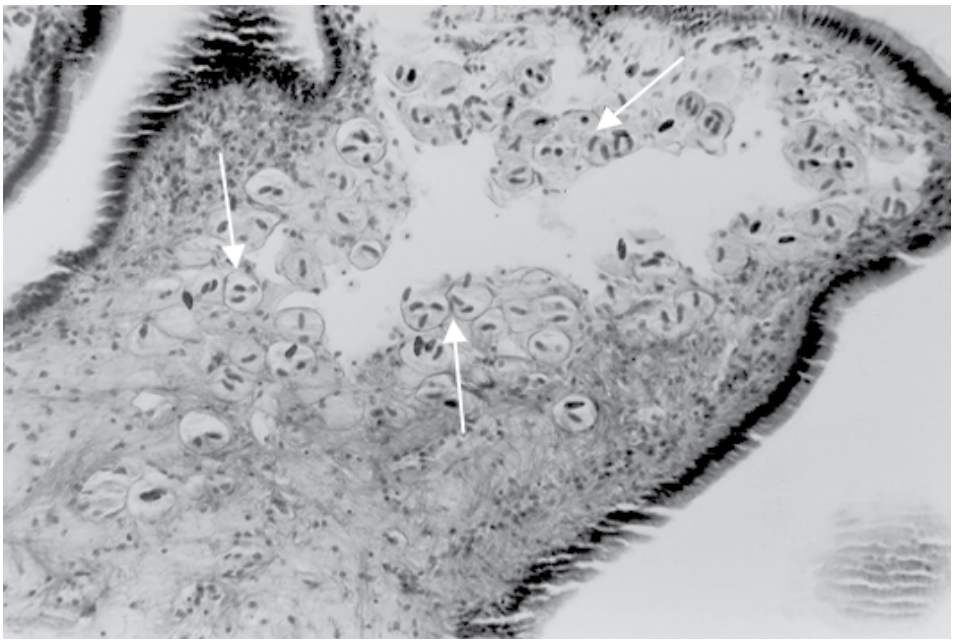


Figure 2-5. Gregarine-like spores near the tip of the mantle of a mytilid mussel, *M. californianus*. Arrows indicate examples. x 100.

A variety of ciliate types (Figures 2-6 - 2-7) [Additional photographs: Cheng (1967), p. 184, Fig. 88, p. 193, Fig. 102; Murchelano and MacLean (1990), p. 11 Fig. 1-11, p. 15 Figs. 1-19, 1-20; Figueras *et al.* (1991a), p. 91, Fig. 2; Gauthier *et al.* (1990), p. 110, Figs. 4-6; Villalba *et al*. (1997), p. 132, Figs. 10-11; Laruelle *et al*. (1999), p. 254-256, Figs. 1-3; Moret *et al*. (1999), p. 36, Fig. 1] have been observed in bivalves from Mussel Watch sites (Kim *et al*., 1998), normally at low prevalence and more frequently in mytilid mussels than in oysters. Ciliate infections are observed in mytilids from the East and West coasts and in oysters from the East and Gulf coasts. Gill ciliates are one of the most commonly observed parasites. Ciliates occur in between gill filaments or are attached to gill surfaces of mytilids and oysters. Ciliates are also found in the gut lumen or attached to the digestive tract epithelia. Otto *et al*. (1979), Figueras *et al*. (1991a) and Villalba *et al*. (1997) reported no notable pathology in bivalves parasitized by ciliates and ciliate infections do not appear to elicit any obvious pathological conditions or host responses in Mussel Watch samples. Ciliates are quantitated by tissue type (e.g., gut, digestive gland, gill). Xenomas (Figure 2-8), cells distended with maturing ciliates, are tabulated separately. If a large xenoma has burst, the individual small ciliates are counted.

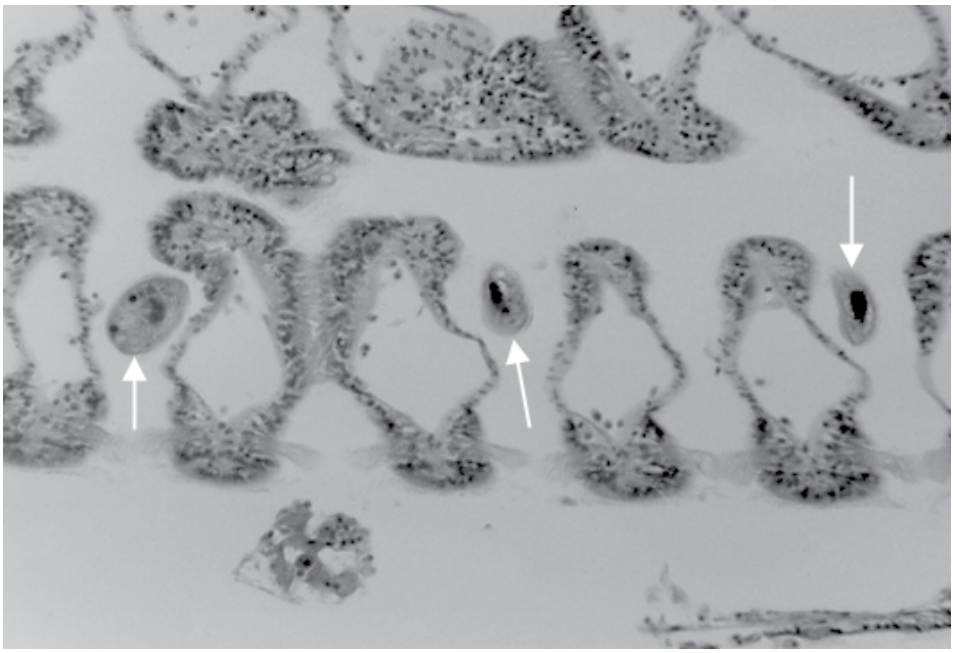


Figure 2-6. Ciliates between the gill filaments of a mytilid mussel, *M. edulis*. Arrows indicate examples. x 100.

Coccidians of the genus *Pseudoklossia* (Apicomplexa) [Photographs: Morado *et al*. (1984), p. 212 Figs. 7-10, p. 213 Figs. 11-16; Friedman *et al*. (1995), p. 35, Figs. 3-11; Villalba *et al*. (1997), p. 130, Figs. 5-6] are another protozoan parasite and are occasionally observed in the kidney of mytilid mussels at Mussel Watch sites. Each parasite is counted.

A variety of encysted larval cestodes (Figures 2-9 – 2-10) [Additional photographs: Cheng (1966a), p. 248 Fig. 6, p. 252 Figs. 1-6, p. 254 Figs. 1-2; Murchelano and MacLean (1990), p. 17, Fig. 1-21; Sindermann (1970), p. 128, Fig. 42] (for examples, see Cake, 1977; Cake and Menzel, 1980) have been observed. Encysted cestodes have been observed in either connective tissue around the digestive gland and gut or in the gills of oysters in Mussel Watch samples. None have been observed in mytilid mussels or dreissenids. From histological examination of their tissue location in oysters, cestodes presumably penetrate the gill or digestive epithelium of the host bivalve. Cestode infection does not seem to significantly damage the oyster. Cellular reaction to cestode cysts, characterized by encapsulation of larval cestodes by layers of connective tissue fibers (Cheng, 1966a; Sindermann, 1970), is routinely observed. Encapsulated larval cestodes normally appear to be disintegrating and to be in the process of resorption. Cestodes are quantified by tissue location (e.g., body, gill, mantle). Each occurrence observed is counted separately.

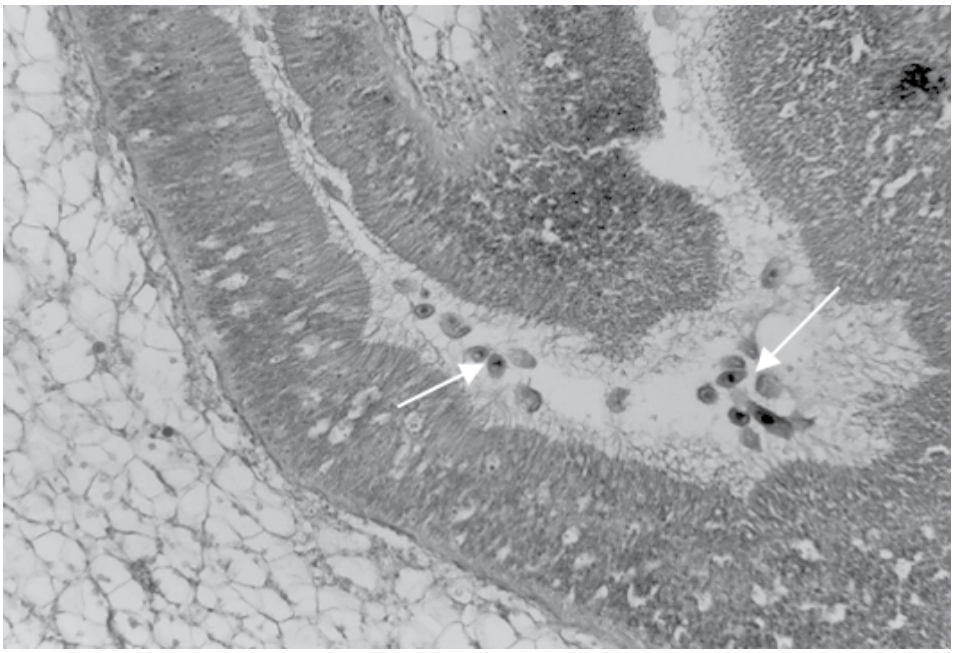


Figure 2-7. Ciliates in the lumen and attached to the epithelium of the intestine of an oyster, *C. virginica*. Arrows indicate examples. x 100.

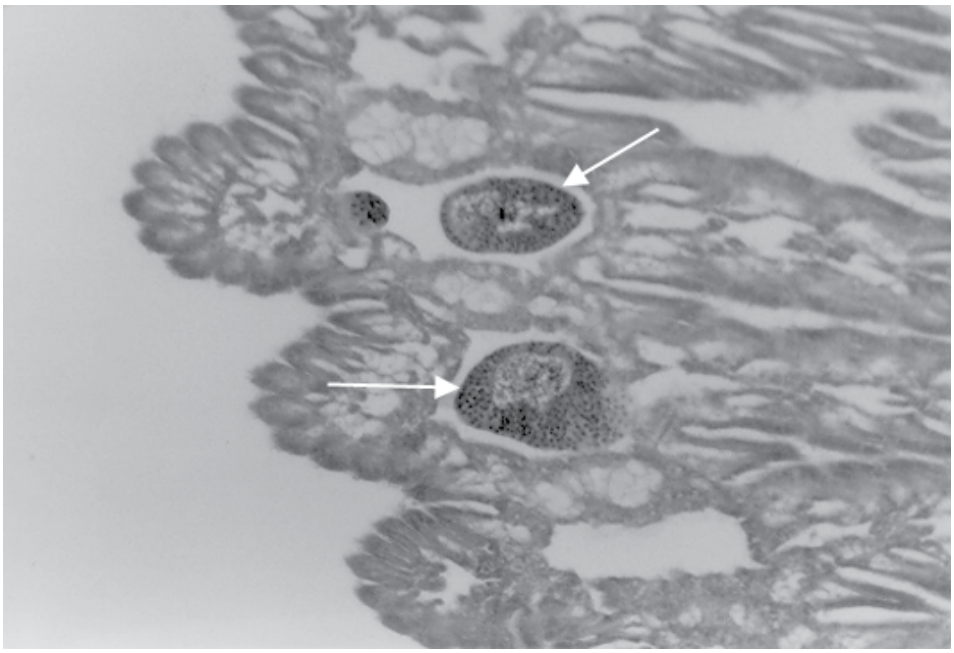


Figure 2-8. Ciliated xenomas on the gill surface of an oyster, *C. virginica*. Arrows indicate examples. x 100.

Metacercariae of trematodes (nearly all *Proctoeces* sp.) [Photographs: Little *et al*. (1969), p. 455, Fig. 1; Wolf *et al*. (1987), p. 380, Fig. 1; Tripp and Turner (1978), p. 77 Fig. 4, p. 79 Figs. 5-8, p. 81 Figs. 9-12; Winstead and Couch (1981), p. 297 Fig. 1, p. 299 Figs. 2-3] have been observed, normally at low prevalence, and occur in the mantle, foot, gonad/gonoduct and pericardial cavity of mytilid mussels and in the gonoduct of oysters at Mussel Watch sites. *Proctoeces* and occasional nematodes also found in the gonoduct should be distinguished from the echinostome metacercariae [Photograph: Ellis *et al*. (1998), p. 201, Fig. 1] observed in the gonoducts of oysters from the Gulf of Mexico (Winstead *et al*., 1998). Encysted metacercariae (Figure 2-11) (presumably gymnophallids) [Additional photograph: Bower *et al.* (1994), p. 74, Fig. 62] are frequently observed in all tissues of mytilids: mantle, visceral connective tissue, foot, byssal gland and gill. In most cases, no conspicuous host response is observed. Hemocytes, however, occasionally infiltrate and surround the worms, especially those that are dead or dying. Each trematode occurrence is counted separately.

Gill nemerteans and turbellarians (Brun *et al*., 1999) [Photographs: Villalba *et al*. (1997), p. 132, Figs. 12-13; Cáceres-Martínez *et al*. (1998), p. 218, Figs. 3-4] are occasionally seen between gill filaments. Whether these are commensal or ectoparasitic is unknown. Each cross-section observed is counted although one individual may be responsible for a number of tissue cross-sections. However, this method of quantification has proven effective even at high infection intensities (Wilson-Ormond *et al*., 2000).

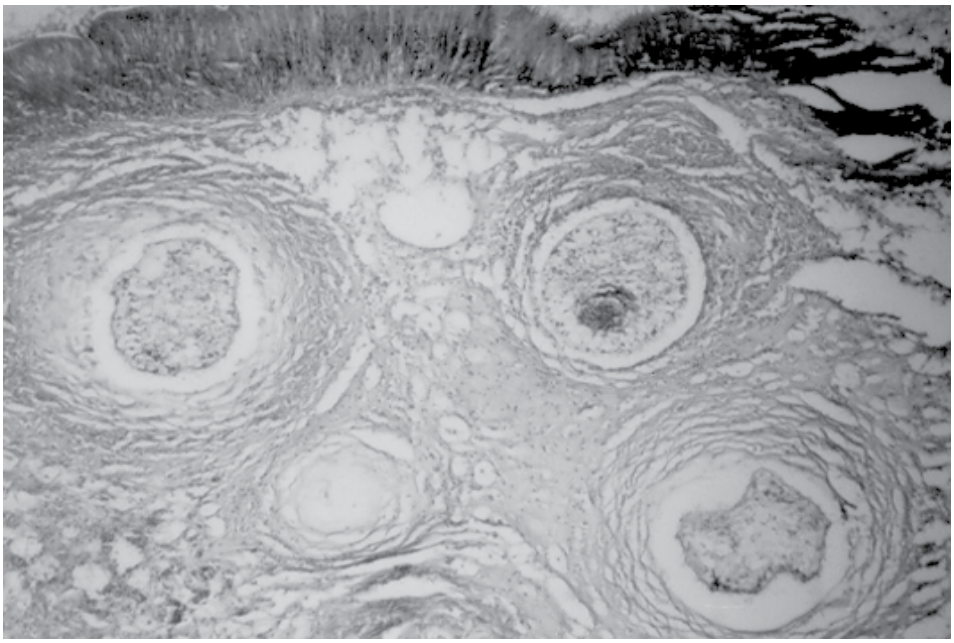


Figure 2-9. Encapsulated larval cestodes in the vesicular connective tissue surrounding the stomach of an oyster, *C. virginica*. x 63.

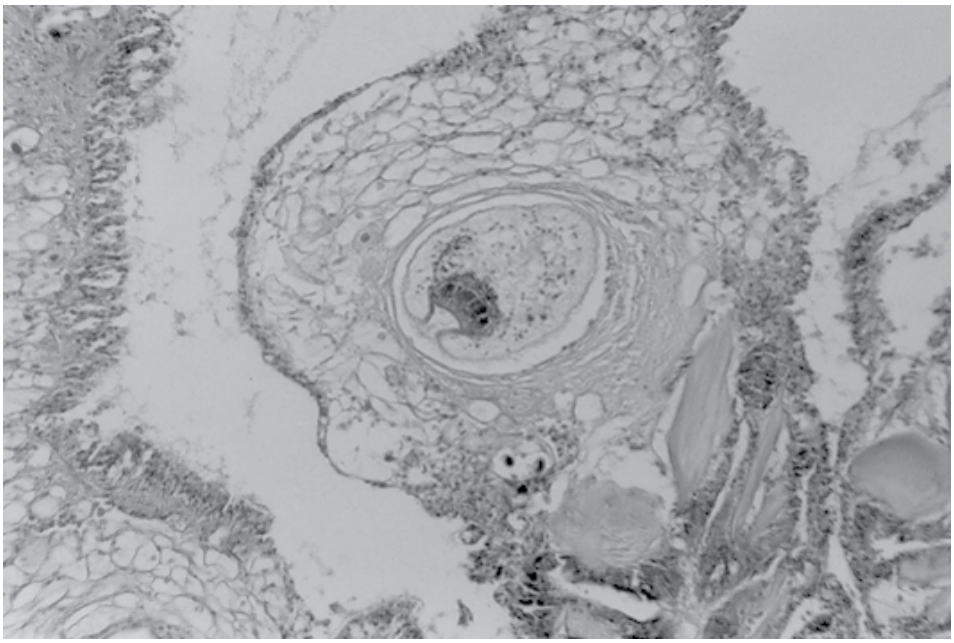


Figure 2-10. A larval cestode in the gill connective tissue of an oyster, *C. virginica*. x 100.

Nematodes (Figure 2-12) [Additional photographs: Cheng (1967), p. 264, Fig. 172; Lowe and Moore (1979), p. 140, Fig. 8; Gauthier *et al.* (1990), p. 112, Fig. 9; Murchelano and MacLean (1990), p. 19, Fig. 1-25; Sparks (1985), p. 375-376, Figs. 10-16] are occasionally observed parasitizing oysters from the East and Gulf coasts, and also dreissenids in Mussel Watch samples (Kim *et al*., 1998). Nematodes reported in molluscs are usually larval stages (Cheng 1978; Lichtenfels *et al*., 1980). Adults are found in predators of molluscs (Cheng, 1978), such as elasmobranchs (Millemann, 1963) and sea turtles (Berry and Cannon, 1981). Cheng (1966b) suggested that larval nematodes invade oysters via the digestive tract and migrate through tissues by way of blood vessels. In Mussel Watch samples, larval nematodes in oysters have been found localized in vesicular connective tissues around the region of the digestive gland, as described by Burton (1963) and Couch (1985), destroying adjacent host tissues. In some cases, a host cellular response, infiltration of hemocytes, is observed in association with the worm, as was reported by Couch (1985). Each individual cross-section is counted separately, although, like the nemerteans, a single individual may be responsible for a number of cross-sections.

Parasitic copepods [Photographs: Lowe and Moore (1979), p. 140, Fig. 7; Murchelano and MacLean (1990), p. 19, Fig. 1-26; Figueras *et al*. (1991b), p. 27, Fig. 6; Robledo *et al*. (1994), p. 295 Fig. 8; Villalba *et al*. (1997), p. 132, Fig. 15] are occasionally found in the gut lumen (Gee and Davey, 1986). Each occurrence is counted.

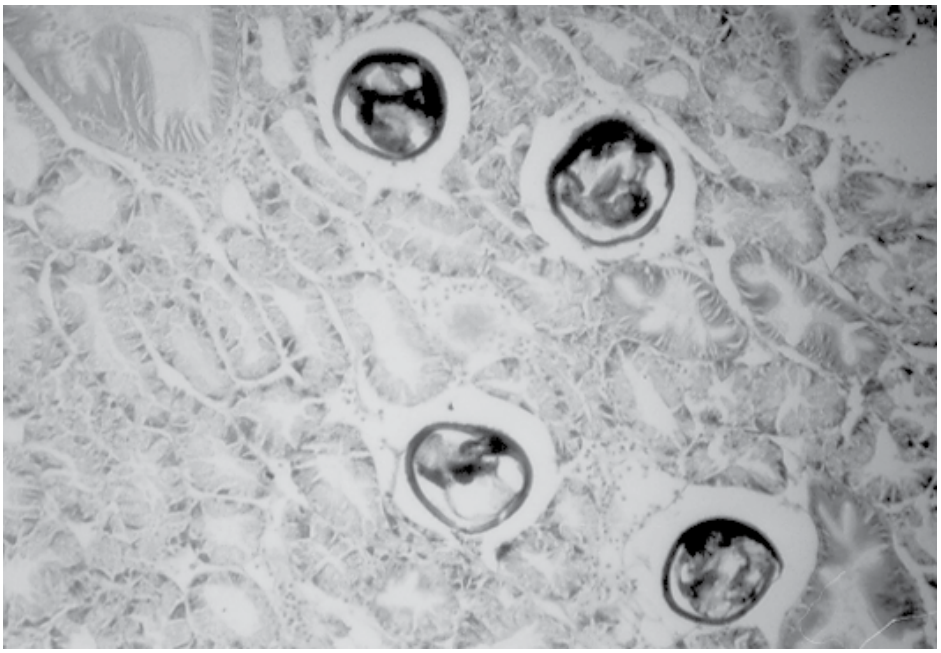


Figure 2-11. Cross-sections of metacercariae encysted in the visceral connective tissue of a mytilid mussel, *M. edulis*. x 63.

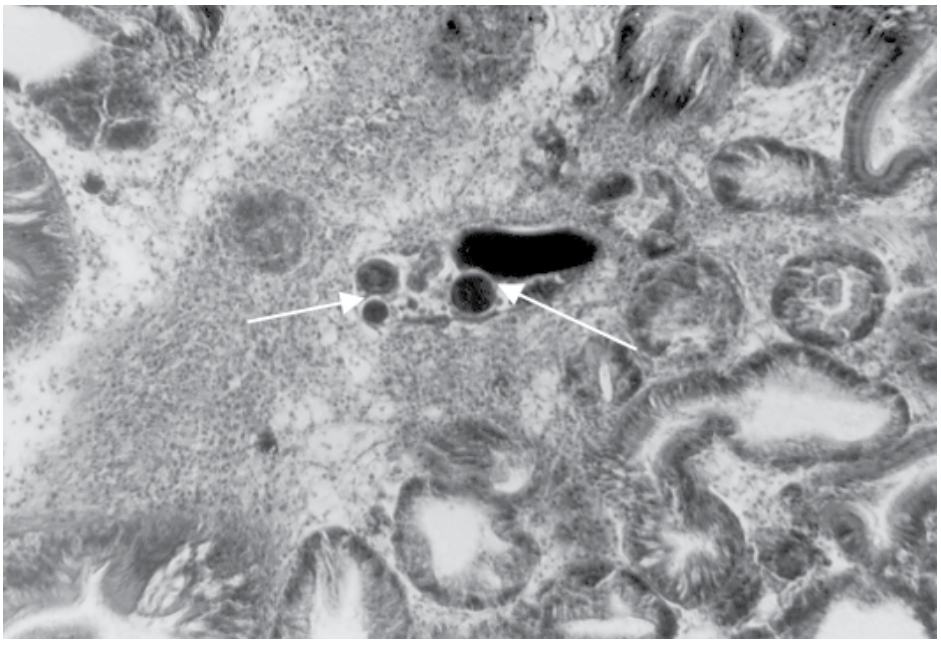


Figure 2-12. Sections of unidentified nematode larvae in the digestive gland connective tissue of an oyster, *C. virginica*. Arrows indicate examples. x 100.

Pinnotherid crabs [Photograph: Stauber (1945), p. 277, Fig. 23; Sandoz and Hopkins (1947), p. 257, Plate III] are occasionally found in the mantle cavity of oysters and mytilid mussels at Mussel Watch sites. Gill damage in infected hosts (Stauber, 1945; Christensen and McDermott, 1958; Haven, 1959) is frequently observed. Pinnotherid crabs also deprive the host of food (Stauber, 1945; Bierbaum and Shumway, 1988). Each occurrence is counted.

Ceroid bodies or brown cells [Photograph: Cheng and Burton (1965), p. 6, Fig. 5; Farley (1968), p. 590 Fig. 16, p. 592 Fig. 23; Murchelano and MacLean (1990), p. 11, Figs. 1-9, 1-12] are distinct brown-yellow aggregates that may occur in large clumps, and appear to be involved in metabolite accumulation and detoxification (Zaroogian and Yevich, 1993). Typically, they occur in greatest abundance in oysters, and in lesser numbers in mytilid mussels and dreissenid mussels. Quantification is obtained by counting each ceroid body. Occasionally, a ceroid body appears fractured or split; in this case only one fragment is counted.

Tumors and neoplasms (Figure 2-13) [Additional photographs: Murchelano and MacLean (1990), p. 19, Figs. 1-27, 1-28; Peters (1988), p. 81, Figs. C, E, F; Sparks (1985), p. 107 Fig. 27, p. 113 Fig. 42; Couch (1985), p. 69 Fig. 10, p. 70 Fig. 11; Figueras *et al*. (1991b), p. 30, Fig. 10; Villalba *et al*. (1997), p. 132, Fig. 16] are occasionally observed. Disseminated sarcomas, probably of hematopoietic origin, are particularly common in mytilid mussels in the Puget Sound region (e.g., Elston *et al*., 1990). The occurrence of neoplasms and tumors in oysters is extremely rare. Examples are described by Farley (1969, 1976), Harshbarger *et al.* (1979) and Ford and Tripp (1996). Neoplasms are occasionally observed in mytilids in Mussel Watch samples. Neoplastic cells with characteristic high nucleus-to-cytoplasm ratios (Ford *et al*., 1997) fill the vesicular connective tissues of the affected mytilids. All observed disseminated sarcomas have been seen in mytilids. For each specimen examined, neoplasms are recorded as either present or absent.

Cases of tissue inflammation (Figures 2-14 – 2-15) [Additional photographs: Farley (1968), p. 590, Fig. 17; Couch (1985), p. 65, Fig. 5; Figueras *et al*. (1991b), p. 28 Fig. 7, p. 29 Fig. 8; Murchelano and MacLean (1990), p. 11, Figs. 1-9, 1-10; Lowe and Moore (1979), p. 138, Figs. 1-4; Villalba *et al*. (1997), p. 132, Fig. 17; Sindermann (1970), p. 110, Fig. 34] characterized by intense infiltration of hemocytes may be focal or diffuse. The type of affected tissue and type of irritation responsible influences the nature of the cellular response (Ford and Tripp, 1996). Diffuse inflammation is differentiated from focal inflammation when the affected area does not appear to have a clear center or focal point of highest hemocyte concentration and hemocytes are abundant and distributed broadly over a large section of tissue. In Mussel Watch samples, most tissue inflammation, characterized by hemocytic infiltration, and most tissue necrosis, characterized by death or decay of cells and tissues, is observed in the visceral connective tissue and is sometimes associated with the presence of parasites. Granulocytomas (Figure 2-15), an inflammatory cellular condition characterized by clusters of hemocytes or the disintegration and sloughing of tissue, occur mainly in the digestive gland of mytilids, as was reported by Villalba *et al*. (1997). These tissue pathologies, focal inflammation, diffuse inflammation and tissue necrosis, are tallied separately. Each affected area is counted.

3.2. SEMI-QUANTITATIVE CATEGORIES

Some conditions are assigned to a semi-quantitative scale related to the intensity or the extent of the affected area (Table 2-1). With one exception, so-called digestive gland atrophy, these are pathologies affecting large tissue areas, diseases characterized by systemic effects, or parasites for which individual counts are not feasible. Semi-quantitative categories include *Haplosporidium nelsoni* (MSX), trematode sporocysts, unusual digestive tubules, gonadal abnormalities (discussed in Kim *et al*., this volume), and

digestive gland atrophy. *Perkinsus marinus*, an oyster parasite that is also assayed semi-quantitatively, is assayed by the more precise thioglycollate method, rather than by histology (Ashton-Alcox *et al*., this volume).

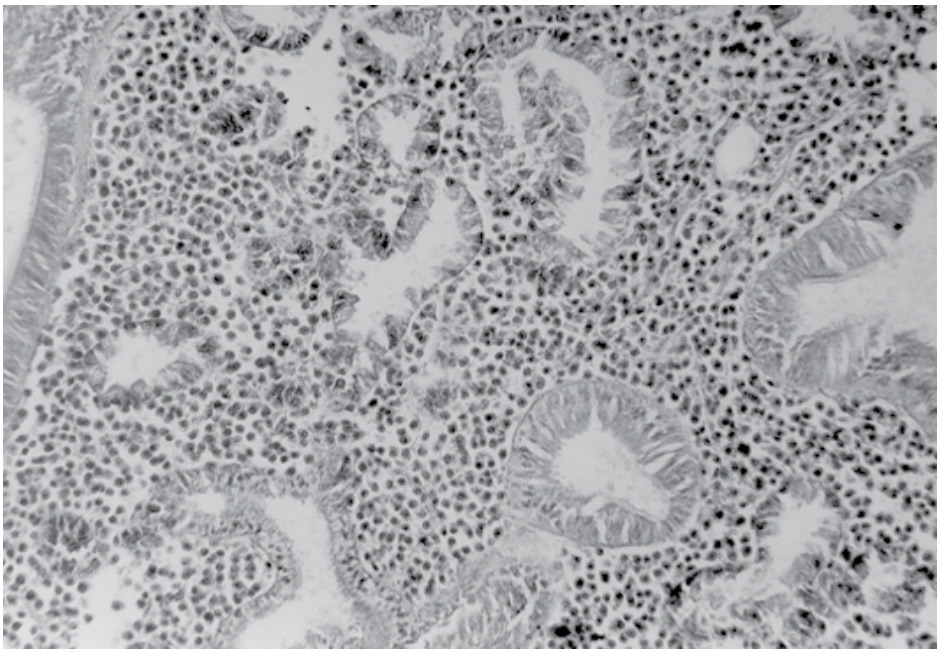


Figure 2-13. Neoplastic cells infiltrating the visceral connective tissue of a mytilid mussel, *M. edulis*. x 100.

Haplosporidium nelsoni (Figure 2-16) [Additional photographs: Farley (1968), p. 590 Fig. 13, p. 592 Fig. 21; Ford (1988), p. 214, Fig. 4; Ford and Tripp (1996), p. 617, Fig. 20], the haplosporidan protozoan responsible for MSX (multinucleated sphere X) disease in eastern oysters, was first reported in Delaware Bay oysters in the late 1950s (Haskin *et al*., 1965). *Haplosporidium nelsoni* was likely introduced from Japan (Burreson *et al*., 2000). It now ranges from Maine to Florida along the entire East coast (Kern, 1988; Ford and Tripp, 1996). Kim *et al*. (1998) observed *Haplosporidium nelsoni* in oysters from Delaware Bay to Georgia at Mussel Watch sites. Multinucleated plasmodia are observed in epithelial cells and the connective tissues of the gills and digestive tract.

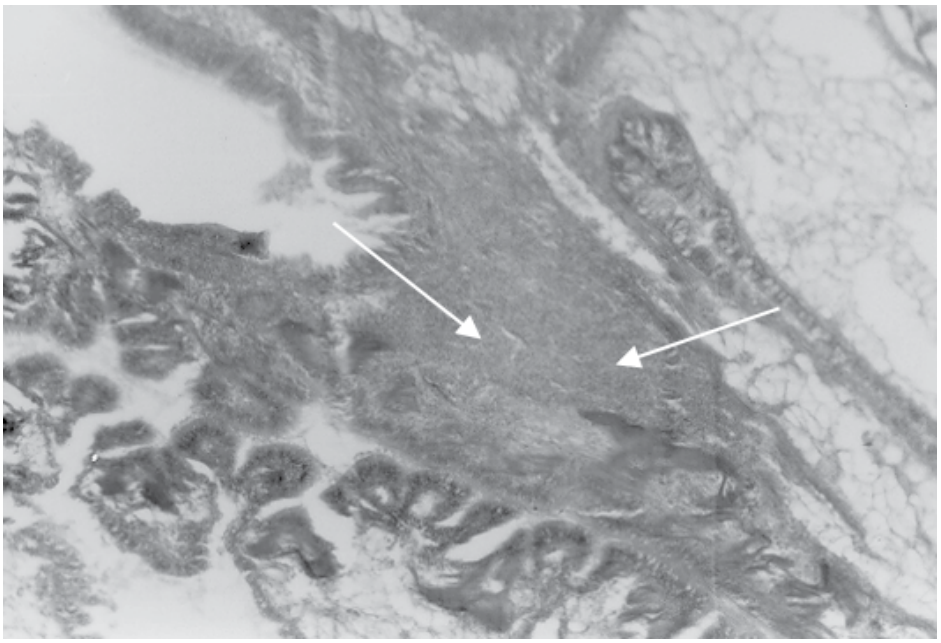


Figure 2-14. Hemocytic infiltration near the gill base of an oyster, *C. virginica*. Arrows indicate examples. x 100.

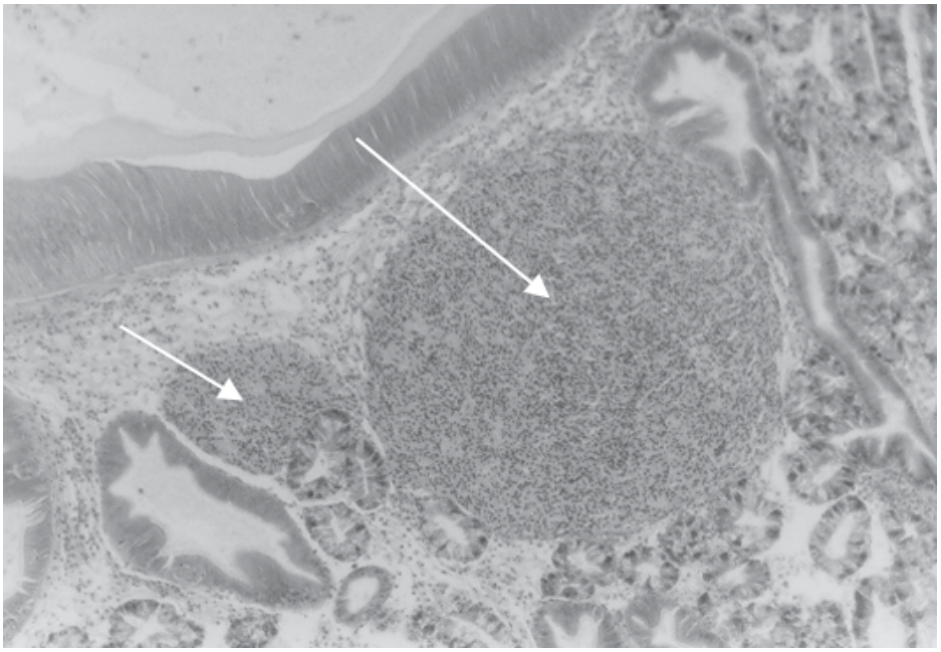


Figure 2-15. Granulocytomas in the digestive gland of a mytilid mussel, *M. edulis*. Arrows indicate examples. x 40.

Haplosporidium nelsoni infections start in the gill epithelium and are limited to this area at light infection levels. As the disease worsens, it becomes systemic and is eventually found throughout the visceral mass. MSX disease, especially heavy infections, is associated with host hemocyte infiltration into the site of infection and tissue necrosis as observed by Farley (1968) and Ford (1985).

Because of the small size of *Haplosporidium nelsoni*, oyster tissues may need to be examined at a

higher power than 10X. MSX infection normally starts in the gill epithelium so the gill tissue must be carefully examined to score early infections accurately. In cases where the disease has become systemic, examination of the visceral mass is necessary to score infection intensity. Grading MSX infection is a two-step process. First, the intensity of the infection is graded according to a semi-quantitative scale (Table 2-2) that records the location, parasite numbers, epithelial or systemic, and extent of infection in the gill tissue or the body tissue separately. In the second step, the two separate ratings are composited into a 0-4 scale (Table 2-3). To get the composite rating, the semi-quantitative rankings for the gill and the body are applied to the matrix in

Table 2-2. Semi-quantitative scale for *Haplosporidium nelsoni* infection modified from Ford (1985, 1986), and Ford and Figueras (1988).

0	Uninfected, no parasites found in the tissue cross-section
1	Parasites confined to gill or digestive tract epithelial tissue, ≤ 10 plasmodia per 100X field of either gill or body tissue
2	Parasites restricted to gill or digestive tract epithelial tissue, Very light infection, 11 ≤ plasmodia ≤ 100 per 100X field of either gill or body tissue
3	Parasites spreading into gill or digestive tract subepithelium, parasites restricted to epithelium and subepithelium area, > 100 plasmodia per 100X field of either gill or body tissue but < 1 per 1000X oil immersion field
4	Parasites more evenly distributed in gill or digestive tract subepithelium and scattered through systemic tissue, > 100 per 100X field of either gill or body tissue but 1 to ≤ 10 per 1000X oil immersion field
5	Moderate systemic infection, averaging 11 to ≤ 20 parasites per 1000X oil immersion field
6	Heavy systemic infection, averaging > 20 parasites per 1000X oil immersion field

Table 2-3. Composite rating matrix for *Haplosporidium nelsoni* infection.

Composite rating scheme

		Body						
		0	1	2	3	4	5	6
	0	0	1	1	2			
	1	1	1	1	2			
	2	1	1	1	2	2		
Gill	3	2	2	2	3	3		
	4	2	2	3	3	3	4	
	5	2	3	3	3	4	4	4
	6				3	4	4	4

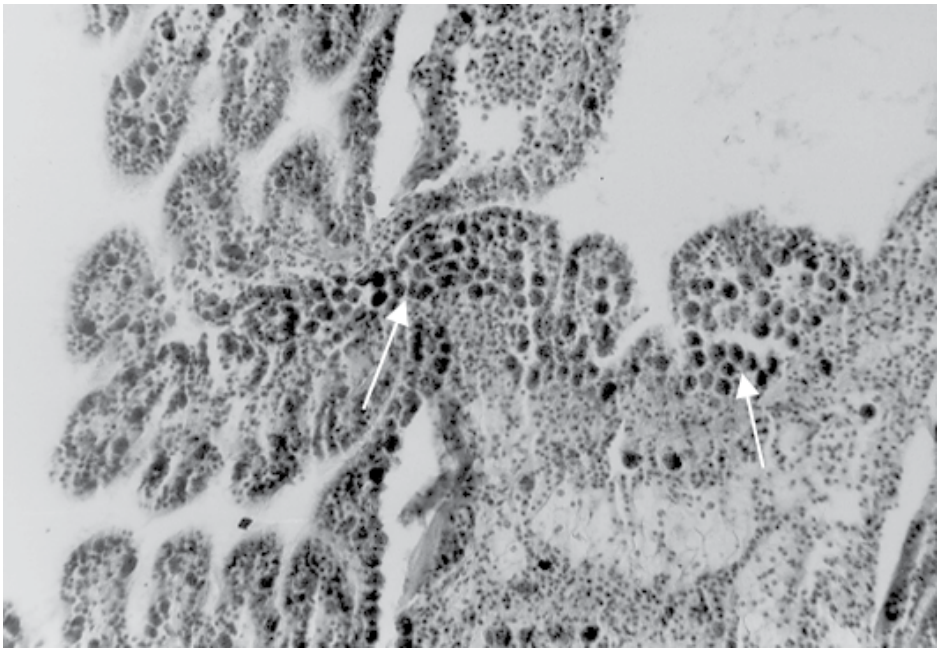


Figure 2-16. Numerous multinucleated plasmodia of *Haplosporidium nelsoni* in the gills of an oyster, *C. virginica*. Arrows point to example parasites. x 100.

Trematode sporocysts of the families Fellodistomidae and Bucephalidae (Figures 2-17 – 2-18) [Additional photographs: Ellis *et al*. (1998), p. 207-208, Figs. 7-9; Cheng and Burton (1965), p. 6 Figs. 1-4, p. 8 Figs. 10-12, p. 10 Figs. 14-17; Tripp and Turner (1978), p. 77, Figs. 1-3; Gauthier *et al.* (1990), p. 112, Fig. 8; Murchelano and MacLean (1990), p. 17, Fig. 1-23; Sindermann (1970), p. 125, Fig. 41; Figueras *et al*. (1991a), p. 92, Fig. 3; Davids and Kraak (1993), p. 751, Fig. 1; Robledo *et al*. (1994), p. 294, Fig. 7; Villalba *et al*. (1997), p. 132, Fig. 14; Powell *et al*. (1999), p. 2061 Fig. 5] occur principally in the gonadal tissue of oysters (Hopkins, 1957) and mytilid mussels. Kim *et al*. (1998) reported trematode sporocyst infections in East and West coast mytilid mussels and in Gulf oysters from Mussel

Watch sites. Carnivorous fish are the final host of bucephalid trematodes (Hopkins, 1954). Fellodistomid trematodes of the genus *Proctoeces* can complete their entire life cycle in a single invertebrate host (e.g., mytilid mussels). Thus, they have a unique life cycle involving molluscs as regular final hosts and bottom fishes as alternative final or as post-cycle hosts (Stunkard and Uzmann, 1959). In Mussel Watch samples, trematodes that have invasive and ramifying sporocysts occur principally in the visceral connective tissues of the digestive gland and the gonadal tissue, destroying gametic tissue and often causing host sterilization. Sindermann (1990) noted that sterilization and tissue destruction are the principal result of the sporocyst invasion. Sterilization is normally observed in infected individuals in Mussel Watch samples (Hillman *et al*., 1988). In extensive and advanced stages of infection, sporocysts infiltrate the gill, mantle and other tissues (see also Cheng and Burton, 1965). Little or no apparent aggregation of host hemocytes around healthy sporocysts and no other evident host reactions have been observed in Mussel Watch samples, which concurs with Cheng and Burton (1965). However, infiltration of hemocytes is occasionally observed around dead or degenerating parasites in Mussel Watch samples, as was reported by Teia dos Santos and Coimbra (1995). The large branching sporocysts are difficult to quantify. Hence, infection intensity is scored on a semi-quantitative scale (Table 2-4). Pictorial examples of the rating scale include Figure 2-17 (scored 1) and Figure 2-18 (scored 2).

Table 2-4. Semi-quantitative scale for trematode sporocyst infection.

Score	Description
0	Uninfected
1	Present in the gonads only (some gametic tissue still present)
2	Completely filling the gonads (no gametic tissue present); may be present in digestive gland or gills in very limited amount
3	Completely filling the gonads; extensive invasion of the digestive gland and/or the gills
4	Completely filling the gonad; substantially filling the digestive gland or gill; individuals appear to be a sac of sporocyst

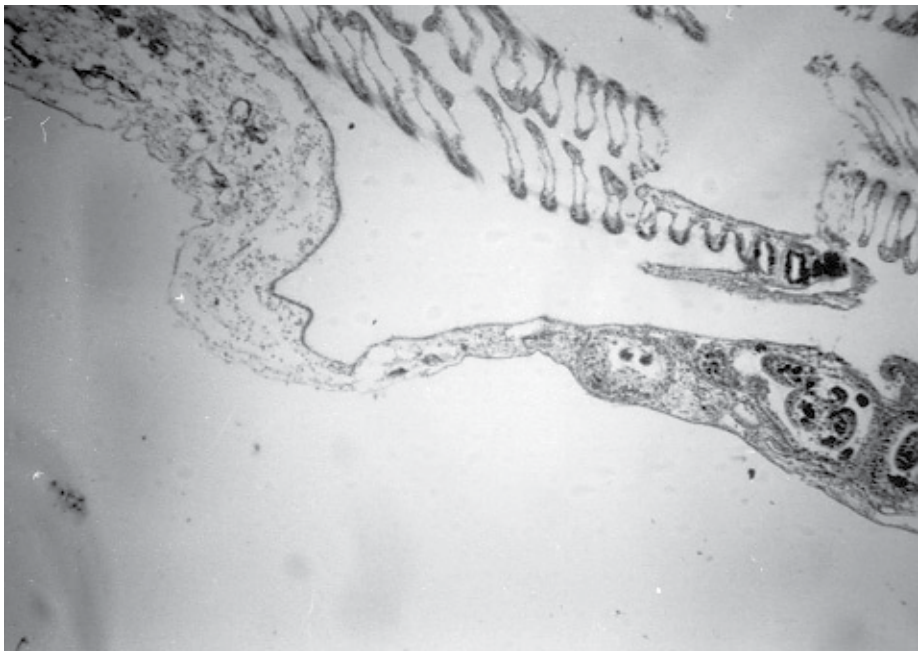


Figure 2-17. This trematode sporocyst infection in *Mytilus edulis* scored a 1 according to Table 2-4. Some gametic tissue is still present.

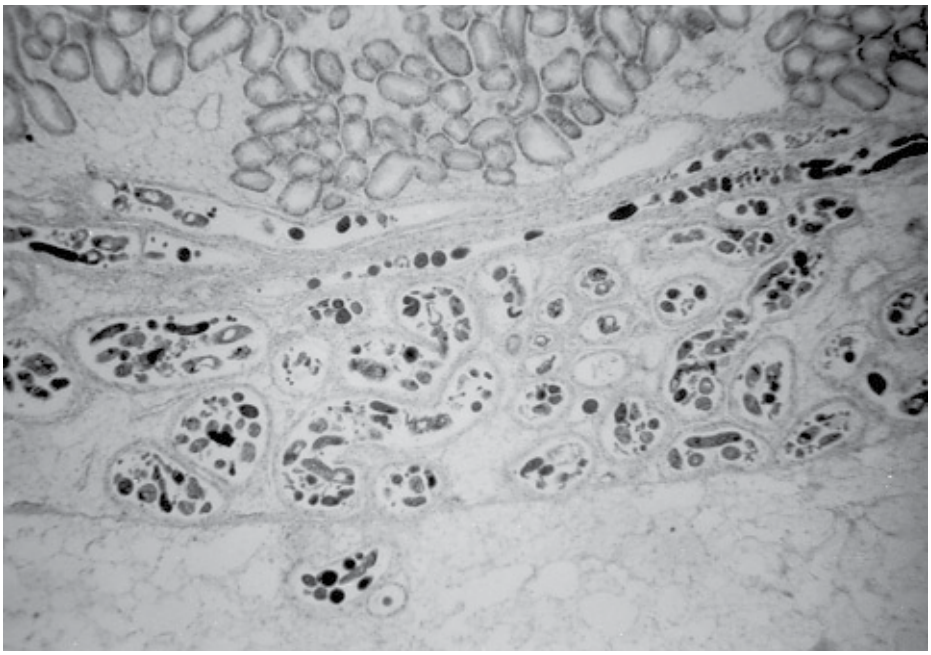


Figure 2-18. This trematode sporocyst infection in *Mytilus edulis* scored a 2 according to Table 2-4. No gametic tissue is present.

Digestive gland atrophy (Figures 2-19 – 2-21 reproduced from Figures 4-6 in Ellis *et al*., 1998) [Additional photographs: Ellis *et al*. (1998), p. 205-206, Figs. 4-6; Couch (1985), p. 66, Fig. 6; Gauthier *et al*. (1990), p. 112, Fig. 10; Winstead (1995), p. 107, Figs. 3-4], a condition characterized by the thinning of the digestive tubule walls, has been described in a number of bivalve species (Bielefeld, 1991; Marigómez *et al.*, 1990; Axiak *et al.*, 1988). Causes of the condition have been ascribed to a variety of stressors including exposure to contaminants and variations in food supply. Winstead (1995) found that poor nutrition was a key element in producing the condition in oysters and that the digestive gland recovered to its normal state relatively rapidly once food supply improved. It is, therefore, not necessarily a pathology. The digestive gland is scanned for tubules showing evidence of epithelial thinning. The average degree of thinning is assigned a numerical rating (Table 2-5). The semi-quantitative assessment permits the reading of many samples in a short time. For increased accuracy, the ratio of tubule diameter to wall diameter (e.g., Winstead, 1995) or a direct measure of wall thickness (Marigómez *et al.*, 1990) can be used.

Table 2-5. Semi-quantitative scale for digestive gland atrophy.

Score	Description
0	Normal wall thickness in most tubules (0% atrophy), lumen nearly occluded, few tubules even slightly atrophied
1	Average wall thickness less than normal, but greater than one-half normal thickness, most tubules showing some atrophy, some tubules still normal
2	Wall thickness averaging about one-half as thick as normal
3	Wall thickness less than one-half of normal, most tubules walls significantly atrophied, some walls extremely thin (fully atrophied)
4	Wall extremely thin (100% atrophied), nearly all tubules affected

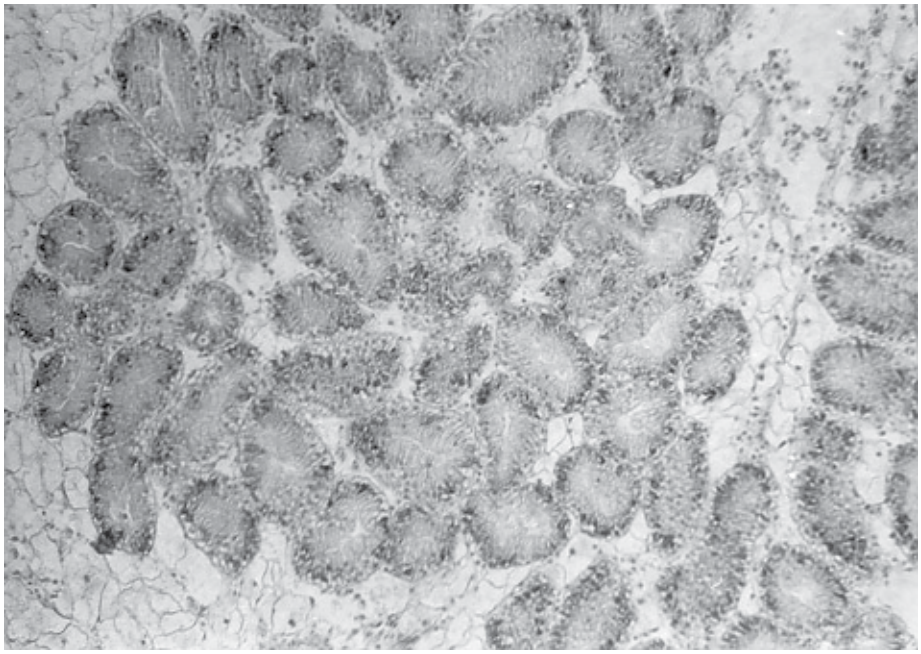


Figure 2-19. *Crassostrea virginica* normal digestive tubule, scored a 0 according to Table 2-5.

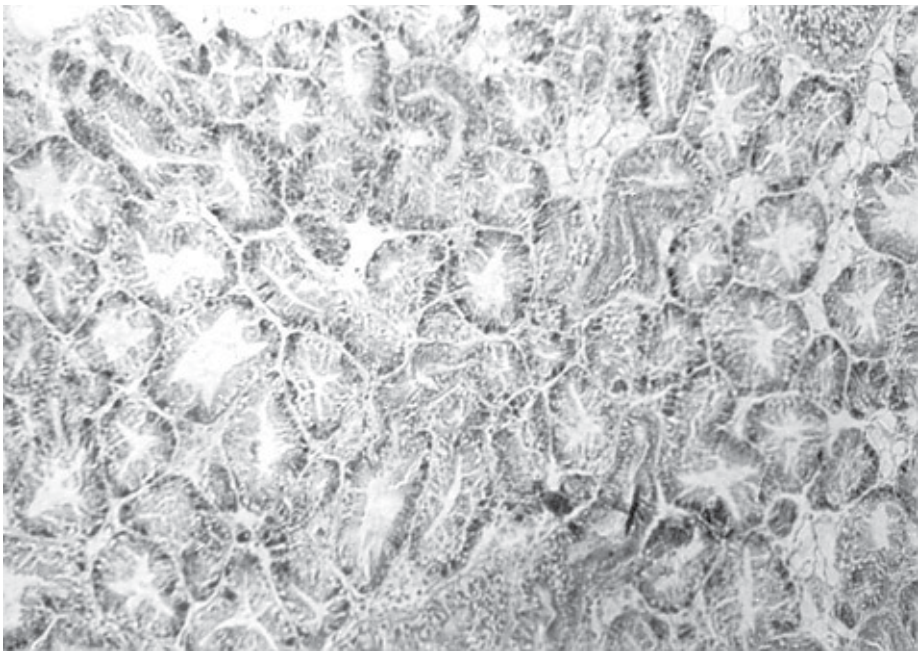


Figure 2-20. Digestive gland atrophy in *Crassostrea virginica* scored a 2 according to Table 2-5.

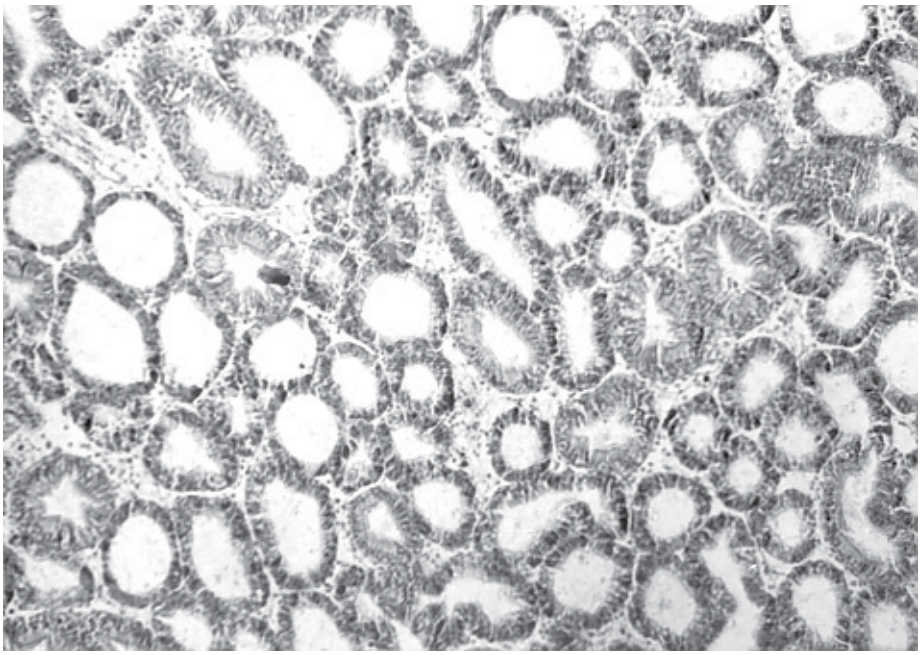


Figure 2-21. Digestive gland atrophy in *Crassostrea virginica* scored a 4 according to Table 2-5.

In Mussel Watch samples, degenerated and/or necrotic digestive glands (Figure 2-22) [Additional photograph: Couch (1985), p. 67, Fig. 7] were frequently observed, particularly in mytilids. This condition is characterized by digestive tubules in unusually poor condition with loss of their normal integrity and structure, and sometimes with vacuolated epithelium. Individual digestive tubules are sometimes not discernible from each other. For each specimen examined, unusual digestive tubules are recorded as either present or absent.

3.3. SUMMARY STATISTICS

Three descriptions of parasite distribution are used: prevalence, infection intensity and weighted prevalence (Ford, 1988). Prevalence describes the proportion of individuals in the population that are infected by a specific parasite or pathology and is calculated as:

$$\text{prevalence} = \frac{\text{number of hosts with parasite or pathology}}{\text{number of hosts analyzed}}$$

Infection intensity is calculated as the average number of occurrences of the parasite or pathology in infected hosts. This is a measure of the intensity of infection in infected individuals.

$$\text{infection intensity} = \frac{\text{total number of occurrences of parasite or pathology}}{\text{number of hosts with parasite or pathology}}$$

Weighted prevalence or mean abundance (Bush *et al*., 1997; Rózsa *et al*., 2000) is the multiple of prevalence and infection intensity, and is a measure of the relative severity of infection within the population. Weighted prevalence is calculated as:

$$\text{weighted prevalence} = \frac{\text{total number of occurrences of parasite or pathology}}{\text{number of hosts analyzed}}$$

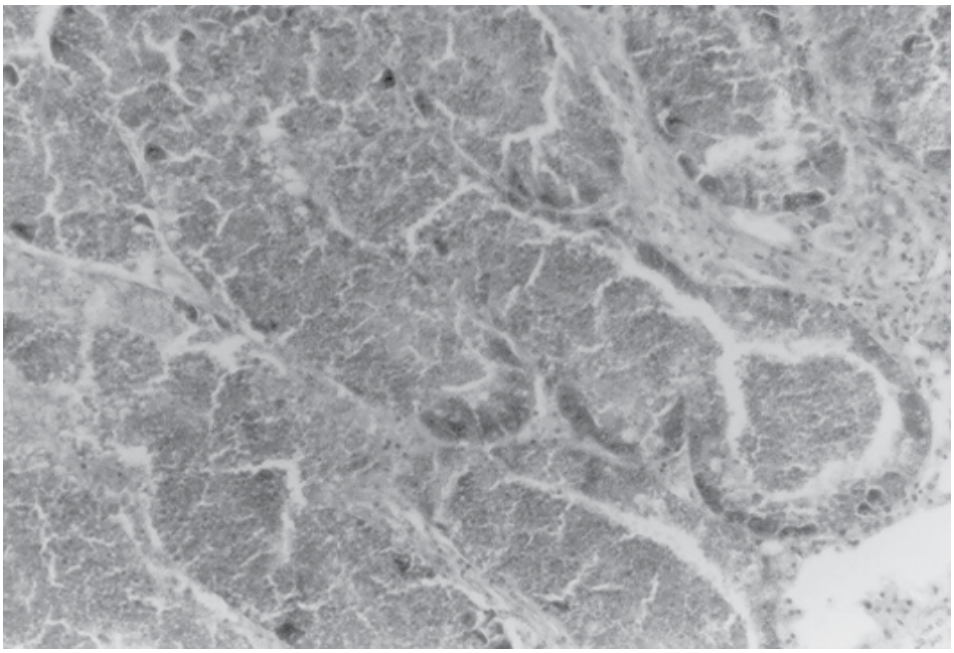


Figure 2-22. Unusual digestive tubules of a mytilid mussel, *M. edulis*. Note the absence of normal tubule structure as shown in Figures 2-20 and 2-21. x 100.

4.0 CONCLUSIONS

The described techniques provide the quantitative and semi-quantitative methods used to determine the prevalence and infection intensity of parasites, pathologies, and diseases affecting oysters, mytilid mussels, and dreissenid mussels in the Mussel Watch Project. The described histopathological method is an approach that targets a wide range of parasites and pathologies. Specific conditions are often better assessed by other methods [e.g., *Perkinsus marinus* infection in oysters (Ashton-Alcox *et al*., this volume)]. The described method emphasizes the quantification of infection intensity. Prevalence rarely provides an adequate description of the population dynamics of disease and, in practice, often yields ambiguous results. Infection intensity as quantified by direct counts or the use of semi-quantitative scales consistently provides a more robust data set for statistical analysis comparing the spatial and temporal distribution of parasites, pathologies, and diseases to contaminant body burden.

5.0 REFERENCES

Abbe, G. R., J. G. Sanders and G. F. Riedel (1994) Silver uptake by the oyster (*Crassostrea virginica*): effect of organism size and storage sites. Estuarine Coastal Shelf Sci., 39:249-60.

Ackerman, E., L. R. Elveback and J. P. Fox (1984) Simulation of Infectious Disease Epidemics. Charles C. Thomas, Springfield, IL. 202 pp.

Anderson, R. S., L. M. Oliver and D. Jacobs (1992) Immunotoxicity of cadmium for the eastern oyster (*Crassostrea virginica* [Gmelin, 1791]): effects of hemocyte chemiluminescence. J. Shellfish Res., 11: 31-5.

Ashton-Alcox, K. A., B. Allam and S. E. Ford (2000) Application of flow cytometry to bivalve pathology. In: Recent Advances in Marine Biotechnology. Vol. 5 Immunobiology and pathology. M. Fingerman and R. Nagabhushanam (eds.). Science Publishers, Inc., New Hampshire. 85-124.

Axiak, V., J. J. George and M. N. Moore (1988) Petroleum hydrocarbons in the marine bivalve *Venus verrucosa*: accumulation and cellular responses. Mar. Biol. (Berl.), 97:225-30.

Barber, B. J. (1996) Gametogenesis of eastern oysters, *Crassostrea virginica* (Gmelin, 1791), and Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) in disease-endemic lower Chesapeake Bay. J. Shellfish Res., 15:285-90.

Berry, G. N. and L. R. G. Cannon (1981) The life history of *Sulcascaris sulcata* (Nematoda: Ascaridoidea), a parasite of marine molluscs and turtles. Intl. J. Parasitol., 11:43-54.

Bielefeld, U. (1991) Histological observation of gonads and digestive gland in starving *Dreissena polymorpha* (Bivalvia). Malacologia, 33:31-42.

Bierbaum, R. and S. E. Shumway (1988) Filtration and oxygen consumption in mussels, *Mytilus edulis*, with and without pea crabs, *Pinnotheres maculatus*. Estuaries, 11:264-71.

Bower, S. M., S. E. McGladdery and I. M. Price (1994) Synopsis of infectious diseases and parasites of commercially exploited shellfish. Ann. Rev. Fish Dis., 4:1-199.

Bowmer, C. T., M. van der Meer and M. C. T. Scholten (1991) A histopathological analysis of wild and transplanted *Dreissena polymorpha* from the Dutch sector of the River Maas. Comp. Biochem. Physiol. C, 100:225-9.

Brun, N. T., A. D. Boghen and J. Allard (1999) Distribution of the turbellarian *Urastoma cyprinae* on the gills of the eastern oyster *Crassostrea virginica*. J. Shellfish Res., 18:175-9.

Burreson, E. M., N. A. Stokes and C. S. Friedman (2000) Increased virulence in an introduced pathogen: *Haplosporidium nelsoni* (MSX) in the eastern oyster *Crassostrea virginica*. J. Aquat. Animal Health, 12:1-8.

Burton, R. W. (1963) Distribution of oyster microparasites in Chesapeake Bay, Maryland, 1959-1960. Proc. Natl. Shellfish. Assoc., 52:65-74.

Bush, A. O., K. D. Lafferty, J. M. Lotz and A. W. Shostak (1997) Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol., 83:575-83.

Cáceres-Martínez, J., R. Vásquez-Yeomans and R. Sluys (1998) The turbellarian *Urastoma cyprinae* from edible mussels *Mytilus galloprovincialis* and *Mytilus californianus* in Baja California, NW Mexico. J. Invertebr. Pathol., 72:214–9.

Cake Jr., E. W. (1977) Larval cestode parasites of edible mollusks of the northeastern Gulf of Mexico. Gulf. Res. Rpt., 6:1-8.

Cake Jr., E. W. and R. W. Menzel (1980) Infections of *Tylocephalum* metacestodes in commercial oysters and three predaceous gastropods of the eastern Gulf of Mexico. Proc. Natl. Shellfish. Assoc., 70:94-104.

Cheng, T. C. (1966a) The coracidium of the cestode *Tylocephalum* and the migration and fate of this parasite in the American oyster, *Crassostrea virginica*. Trans. Amer. Microsc. Soc., 85:246-55.

Cheng, T. C. (1966b) Perivascular leucocytosis and other types of cellular reactions in the oyster *Crassostrea virginica* experimentally infected with the nematode *Angiostrongylus cantonensis*. J. Invertebr. Pathol., 8:52-8.

Cheng, T. C. (1967) Marine molluscs as hosts for symbioses with a review of known parasites of commercially important species. Adv. Mar. Biol., Vol. 5, Academic Press, London. 424 pp.

Cheng, T. C. (1978) Larval nematodes parasitic in shellfish. Mar. Fish. Rev., 40:39-42.

Cheng, T. C. (1988) *In vivo* effects of heavy metals on cellular defense mechanisms of *Crassostrea virginica*: total and differential cell counts. J. Invertebr. Pathol., 51:207-14.

Cheng, T. C. and R. W. Burton (1965) Relationships between *Bucephalus* sp. and *Crassostrea virginica*: histopathology and sites of infection. Chesapeake Sci., 6:3-16.

Christensen, A. M. and J. J. McDermott (1958) Life-history and biology of the oyster crab, *Pinnotheres ostreum* Say. Biol. Bull., (Woods Hole) 114:146-79.

Couch, J. A. (1985) Prospective study of infectious and noninfectious diseases in oysters and fishes in three Gulf of Mexico estuaries. Dis. Aquat. Org., 1:59-82.

Cunningham, P. A. and M. R. Tripp (1975) Accumulation, tissue distribution and elimination of $^{203}HgCl_2$ and $CH_3{}^{203}HgCl$ in the tissues of the American oyster *Crassostrea virginica*. Mar. Ecol., 31:321-34.

Davids, C. and M. H. S. Kraak (1993) Trematode parasites of the zebra mussel (*Dreissena polymorpha*). In: Zebra Mussels: Biology, Impacts, and Control. T. F. Nalepa and D. W. Schloesser (eds.). Lewis Publishers, Boca Raton, FL. 749-60.

Davis, C. V. and B. J. Barber (1994) Size-dependent mortality in hatchery reared populations of oysters, *Crassostrea virginica*, Gmelin 1791, affected by juvenile oyster disease. J. Shellfish Res., 13:137-42.

Dungan, C. F., R. A. Elston and M. H. Schiewe (1989) Evidence for colonization and destruction of hinge ligaments in cultured juvenile Pacific oysters (*Crassostrea gigas*) by cytophaga-like bacteria. Appl. Environ. Microbiol., 55:1128-35.

Ellis, M. S., R. D. Barber, R. E. Hillman, Y. Kim and E. N. Powell (1998) Histopathology analysis. In: Sampling and Analytical Methods of the National Status and Trends Program Mussel Watch Projects: 1993-1996 Update. NOAA Tech. Memo. NOS ORCA 130. NOAA/NOS/ORCA Silver Spring, MD. 198-215.

Ellis, M. S., K-S. Choi, T. L. Wade, E. N. Powell, T. J. Jackson and D. H. Lewis (1993) Sources of local variation in polynuclear aromatic hydrocarbon and pesticide body burden in oysters (*Crassostrea virginica*) from Galveston Bay, Texas. Comp. Biochem. Physiol. C, 106:689-98.

Elston, R. A., A. S. Drum and S. K. Allen, Jr. (1990) Progressive development of circulating polyploid cells in *Mytilus* with hemic neoplasia. Dis. Aquat. Org., 8:51-9.

Farley, C. A. (1968) *Minchinia nelsoni* (Haplosporida) disease syndrome in the American oyster *Crassostrea virginica*. J. Protozool., 15:585-99.

Farley, C. A. (1969) Probable neoplastic disease of the hematopoietic system in oysters, *Crassostrea virginica* and *Crassostrea gigas*. Natl. Cancer Inst. Monogr., 31:541-55.

Farley, C. A. (1976) Proliferative disorders in bivalve mollusks. Mar. Fish. Rev., 38:30-3.

Figueras, A. J., C. F. Jardon and J. R. Caldas (1991a) Diseases and parasites of mussels (*Mytilus edulis*, Linnaeus, 1758) from two sites on the east coast of the United States. J. Shellfish Res., 10:89-94.

Figueras, A. J., C. F. Jardon and J. R. Caldas (1991b) Diseases and parasites of rafted mussels (*Mytilus galloprovincialis* Lmk): preliminary results. Aquaculture, 99:17-33.

Ford, S. E. (1985) Chronic infections of *Haplosporidium nelsoni* (MSX) in the oyster *Crassostrea virginica*. J. Invertebr. Pathol., 45:94-107.

Ford, S. E. (1986) Comparison of hemolymph proteins from resistant and susceptible oysters, *Crassostrea virginica*, exposed to the parasite *Haplosporidium nelsoni* (MSX). J. Invertebr. Pathol., 47:283-94.

Ford, S. E. (1988) Host-parasite interactions in eastern oysters selected for resistance to *Haplosporidium nelsoni* (MSX) disease: survival mechanisms against a natural pathogen. In: Disease Processes in Marine Bivalve Molluscs. W. S. Fisher (ed.). Am. Fish. Soc. Spec. Publ., 18:206-24.

Ford, S. (2002) Testing for molluscan pathogens: an overview. In: Eastern United States Interstate Shellfish Seed Transport Workshop. W. D. Anderson, C. Battey, M. R. DeVoe, J. N. Kraeuter, A. Segars and J. M. Whetstone (eds.). 6-9.

Ford, S. E., R. D. Barber and E. Marks (1997) Disseminated neoplasia in juvenile eastern oysters *Crassostrea virginica*, and its relationship to the reproductive cycle. Dis. Aquat. Org., 28:73-7.

Ford, S. E. and A. J. Figueras (1988) Effects of sublethal infection by the parasite *Haplosporidium nelsoni* (MSX) on gametogenesis, spawning, and sex ratios of oysters in Delaware Bay, USA. Dis. Aquat. Org., 4:121-33.

Ford, S. E. and M. R. Tripp (1996) Diseases and defense mechanisms. In: The Eastern Oyster *Crassostrea virginica*. V. S. Kennedy, R. I. E. Newell and A. F. Eble (eds.). Maryland Sea Grant College,

University of Maryland, College Park, MD. 581-660.

Friedman, C. S., G. R. Gardner, R. P. Hedrick, M. Stephenson, R. J. Cawthorn and S. J. Upton (1995) *Pseudoklossia haliotis* sp.n. (Apicomplexa) from the kidney of California abalone, *Haliotis* spp. (Mollusca). J. Invertebr. Pathol., 66:33-8.

Friedman, C. S., T. McDowell, J. M. Groff, J. T. Hollibaugh, D. Manzer and R. P. Hedrick (1989) Presence of *Bonamia ostreae* among populations of the European flat oyster, *Ostrea edulis* Linnaeus, 1750, in California, USA. J. Shellfish Res., 8:133-7.

Gauthier, J. D., T. M. Soniat and J. S. Rogers (1990) A parasitological survey of oysters along salinity gradients in coastal Louisiana. J. World Aquaculture Soc., 21:105-15.

Gee, J. M. and J. T. Davey (1986) Experimental studies on the infestation of *Mytilus edulis* (L.) by *Mytilicola intestinalis* Steuer (Copepoda, Cyclopoida). J. Cons. Int. Explor. Mer., 42:265-71.

Gulka, G. and P. W. Chang (1984) Host response to rickettsial infection in blue mussel, *Mytilus edulis* L. J. Fish Dis., 8:319-23.

Harshbarger, J. C., S. C. Chang and S. V. Otto (1977) Chlamydiae (with phages), mycoplasmas, and rickettsiae in Chesapeake Bay bivalves. Science, 196:666-8.

Harshbarger, J. C., S. V. Otto and S. C. Chang (1979) Proliferative disorders in *Crassostrea virginica* and *Mya arenaria* from the Chesapeake Bay and intranuclear virus-like inclusions in *Mya arenaria* with germinomas from a Maine oil spill site. Haliotis, 8:243-8.

Haskin, H. H., W. J. Canzonier and J. L. Myhre (1965) The history of MSX on Delaware Bay oyster grounds, 1957-65. Amer. Malacol. Union Bull., 32:20-1.

Haven, D. (1959) Effects of pea crabs *Pinnotheres ostreum* on oysters *Crassostrea virginica*. Proc. Natl. Shellfish. Assoc., 49:77-86.

Heinonen, J., J. V. K. Kukkonen and I. J. Holopainen (1999) The effects of parasites and temperature on the accumulation of xenobiotics in a freshwater clam. Ecol. Appl., 9:475-81.

Hillman, R. E., P. D. Boehm and S. Y. Freitas (1988) A pathology potpourri from the NOAA Mussel Watch Project. J. Shellfish Res., 7:216-7.

Hofmann, E. E., E. N. Powell, J. M. Klinck and G. Saunders (1995) Modeling diseased oyster populations I. Modelling *Perkinsus marinus* infections in oysters. J. Shellfish Res., 14:121-51.

Hopkins, S. H. (1954) The American species of trematode confused with *Bucephalus* (Bucephlopsis) *haimeanus*. Parasitology, 44:353-70.

Hopkins, S. H. (1957) Our present knowledge of the oyster parasite "*Bucephalus*". Proc. Natl. Shellfish. Assoc., 47:58-61.

Johnson, L. L., C. M. Stehr, O. P. Olson, M. S. Myers, S. M. Pierce, B. B. McCain and U. Varanasi (1992) National Benthic Surveillance Project: Northeast Coast. Fish histopathology and relationships between lesions and chemical contaminants (1987-89). NOAA Tech. Memo. NMFS-NWFSC-4. NOAA/

NMFS, Seattle, WA. 95 pp.

Kermack, W. O. and A. G. McKendrick (1991) Contributions to the mathematical theory of epidemics. - I. Bull. Math. Biol., 53:33-55.

Kern, F. G. (1988) Recent changes in the range of "MSX" *Haplosporidium nelsoni*. J. Shellfish Res., 7:543-4.

Kim, Y., E.N. Powell, T.L. Wade, B.J. Presley and J. Sericano (1998) Parasites of sentinel bivalves in the NOAA Status and Trends Program: distribution and relationship to contaminant body burden. Mar. Poll. Bull., 37:45-55.

Ko, Y-T., M. M-Y. Chan, S. E. Ford and D. Fong (1999) A PCR-ELISA method for direct detection of the oyster pathogen *Haplosporidium nelsoni*. Mar. Biotechnol., 1:147-54.

Laird, M. (1961) Microecological factors in oyster epizootics. Can. J. Zool., 39:449-85.

Landau, H. and P. S. Galtsoff (1951) Distribution of *Nematopsis* infection on the oyster grounds of the Chesapeake Bay and in other waters of the Atlantic and Gulf states. Tex. J. Sci., 3:115-30.

Landsberg, J. H. (1996) Neoplasia and biotoxins in bivalves: is there a connection? J. Shellfish Res., 15:203-30.

Laruelle, F., D. P. Molloy, S. I. Fokin and M. A. Ovcharenko (1999) Histological analysis of mantle-cavity ciliates in *Dreissena polymorpha*: their location, symbiotic relationship, and distinguishing morphological characteristics. J. Shellfish Res. 18:251-7.

Lawler, I. F. and J. C. Aldrich (1987) Sublethal effects of bis(tri-N-butyltin)oxide on *Crassostrea gigas* spat. Mar. Pollut. Bull., 6:274-8.

Lee, R. F. (1993) Passage of xenobiotics and their metabolites from hepatopancreas into ovary and oocytes of blue crabs, *Callinectes sapidus*: possible implications for vitellogenesis. Mar. Environ. Res., 35:181-7.

Lee, M., G. T. Taylor, V. M. Bricelj, S. E. Ford and S. Zahn (1996) Evolution of *Vibrio* spp. and microplankton blooms as causative agents of juvenile oyster disease in *Crassostrea virginica* (Gmelin). J. Shellfish Res., 15:319-29.

Lichtenfels, J. R., T. K. Sawyer and G. C. Miller (1980) New hosts for larval *Sulcascaris* sp. (Nematoda, Anisakidae) and prevalence in the calico scallop (*Argopecten gibbus*). Trans. Amer. Micros. Soc., 99:448-51.

Little, J. W., S. H. Hopkins and J. G. Mackin (1969) The viability of an echinostome metacercaria in the American oyster, *Crassostrea virginica*. J. Invertebr. Pathol., 13:455-6.

Lowe, D. M. and M. N. Moore (1979) The cytology and occurrence of granulocytomas in mussels. Mar. Poll. Bull., 10:137-41.

MacKenzie, K., H. H. Williams, B. Williams, A. H. McVicar and R. Siddall (1995) Parasites as indicators of water quality and the potential use of helminth transmission in marine pollution studies.

Adv. Parasitol., 35:85-114.

Marigómez, J. A., V. S. Sáez, M. P. Cajaraville and E. Angulo (1990) A planimetric study of the mean epithelial thickness (MET) of the molluscan digestive gland over the tidal cycle and under environmental stress conditions. Helgol. Meeresunters., 44:81-94.

Millemann, R. E. (1963) Studies on the taxonomy and life history of echinocephalid worms (Nematoda: Spiruroidea) with a complete description of *Echinocephalus pseudouncinatus* Millemann, 1951. J. Parasitol., 49:754-64.

Mo, C. and B. Neilson (1993) Weight and salinity effects on zinc uptake and accumulation for the American oyster (*Crassostrea virginica* Gmelin). Environ. Pollut., 82:191-6.

Morado, J. F., A. K. Sparks and S. K. Reed (1984) A coccidian infection of the kidney of the native littleneck clam *Protothaca staminea*. J. Invertebr. Pathol., 43:207-17.

Moret, K., K. Williams, C. Couturier and J. Parsons (1999) Newfoundland cultured mussel (*Mytilus edulis*) industry 1997 health survey. Bull. Aquacul. Assoc. Canada, 99-3:35-7.

Murchelano, R. A. and S. A. MacLean (1990) Histopathology Atlas of the Registry of Marine Pathology. NOAA/NOS/National Ocean Pollution Program Office, Oxford, Maryland. 77 pp.

Otto, S. V., J. C. Harshbarger and S. C. Chang (1979) Status of selected unicellular eucaryote pathogens, and prevalence and histopathology of inclusions containing obligate procaryote parasites, in commercial bivalve mollusks from Maryland estuaries. Haliotis, 8:285-95.

Peters, E. C. (1988) Recent investigations on the disseminated sarcomas of marine bivalve molluscs**.** In: Disease Processes in Marine Bivalve Molluscs. W. S. Fisher (ed.). Am. Fish. Soc. Spec. Publ., 18:74-92.

Powell, E. N., R. D. Barber, M. C. Kennicutt II and S. E. Ford (1999) Influence of parasitism in controlling the health, reproduction and PAH body burden of petroleum seep mussels. Deep-Sea Res., I 46:2053-78.

Powell, E. N., J. M. Klinck and E. E. Hofmann (1996) Modeling diseased oyster populations. II. Triggering mechanisms for *Perkinsus marinus* epizootics. J. Shellfish Res., 15:141-65.

Prytherch, H. F. (1940) The life cycle and morphology of *Nematopsis ostrearum*, sp. nov., a gregarine parasite of the mud crab and oyster. J. Morph., 66:39-65.

Quick, J. A. and J. G. Mackin (1971) Oyster parasitism by *Labyrinthomyxa marina* in Florida. Fl. Dept. Nat. Resour. Mar. Res. Lab. Prof. Pap, 13:1-55.

Robledo, J. A. F., M. M. Santarém and A. Figueras (1994) Parasite loads of rafted blue mussels (*Mytilus galloprovincialis*) in Spain with special reference to the copepod, *Mytilicola intestinalis*. Aquaculture, 127:287-302.

Rózsa, L., J. Reiczigel and G. Majoros (2000) Quantifying parasites in samples of hosts. J. Parasitol., 86:228-32.

Sandoz, M. and S. H. Hopkins (1947) Early life history of the oyster crab, *Pinnotheres ostreum* (Say).

Biol. Bull. (Woods Hole) 93:250-8.

Sindermann, C. J. (1970) Principal Diseases of Marine Fish and Shellfish. Academic Press, Inc., New York, NY. 369 pp.

Sindermann, C. J. (1990) Principal Diseases of Marine Fish and Shellfish. (Second Edition) Vol. 2: Diseases of Marine Shellfish. Academic Press, Inc., San Diego, CA. 516 pp.

Sparks, A. K. (1985) Synopsis of Invertebrate Pathology Exclusive of Insects. Elsevier Science Publishers, Amsterdam, Netherlands. 423 pp.

Sprague, V. and P. E. Orr (1952) Studies on *Nematopsis*: III. *N. ostrearum* and *N. prytherchi* with special reference to host-parasite relation. Natl. Shellfish. Assoc. Conv. Add., 1952:26-43.

Stauber, L. A. (1945) *Pinnotheres ostreum*, parasitic on the American oyster, *Ostrea (Gryphaea) virginica*. Biol. Bull., 88:269-91.

Stokes, N. A. and E. M. Burreson (2001) Differential diagnosis of mixed *Haplosporidium costale* and *Haplosporidium nelsoni* infections in the eastern oyster, *Crassostrea virginica*, using DNA probes. J. Shellfish Res., 20:207-13.

Stunkard, H. W. and J. R. Uzmann (1959) The life-cycle of the digenetic trematode, *Proctoeces maculatus* (Looss, 1901) Odhner, 1911 [syn. *P. subtenuis* (Linton, 1907) Hanson, 1950], and description of *Cercaria adranocerca* n. sp. Bio. Bull., 116:184-93.

Teia dos Santos, A. M. and J. Coimbra (1995) Growth and production of raft-cultured *Mytilus edulis* L., in Ria de Aveiro: gonad symbiotic infestation. Aquaculture, 132:195-211.

Toews, S., M. Beverley-Burton and T. Lawrimore (1993) Helminth and protist parasites of zebra mussels, *Dreissena polymorpha* (Pallas, 1771), in the Great Lakes region of southwestern Ontario, with comments on associated bacteria. Can. J. Zool. 71:1763-6.

Tripp, M. R. and R. M. Turner (1978) Effects of the trematode *Proctoeces maculatus* on the mussel *Mytilus edulis*. In: Comparative Pathobiology. Vol. 4 Invertebrate Models for Biomedical Research. L. A. Bulla Jr. and T. C. Cheng (eds.). Plenum Press, New York, NY. 73-84.

Villalba, A., S. G. Mourelle, M. J. Carballal and C. Lopez. (1997) Symbionts and diseases of farmed mussels *Mytilus galloprovincialis* throughout the culture process in the Ras of Galicia (NW Spain). Dis. Aquat. Org., 31:127-39.

Warburton, F. E. (1958) Control of the boring sponge on oyster beds. Fish. Res. Board Can. Prog. Rpt. Atl. Coast Stns., 69:7-11.

Weis, P., J. S. Weis, J. Couch, C. Daniels and T. Chen (1995) Pathological and genotoxicological observations in oysters (*Crassostrea virginica*) living on chromated copper arsenate (CCA)-treated wood. Mar. Environ. Res., 39:275-8.

White, M. E., C. L. Kitting and E. N. Powell (1985) Aspects of reproduction, larval development and morphometrics in the pyramidellid *Boonea* (=*Odostomia*) *impressa* (Gastropoda: Opisthobranchia). Veliger, 28:37-51.

White, M. E., E. N. Powell, E. A. Wilson and S. M. Ray (1989) The spatial distribution of *Perkinsus marinus*, a protozoan parasite, in relation to its oyster host (*Crassostrea virginica*) and an ectoparasitic gastropod (*Boonea impressa*). J. Mar. Biol. Assoc. UK, 69:703-17.

Wilson-Ormond, E.A., M.S. Ellis, E.N. Powell, Y. Kim and S. Li (2000) Effects of gas-producing platforms on continental shelf megafauna in the northwest Gulf of Mexico: reproductive status and health. Internat. Rev. Hydrobiol., 85:293-323.

Winstead, J. T. (1995) Digestive tubule atrophy in eastern oysters, *Crassostrea virginica* (Gmelin, 1791), exposed to salinity and starvation stress. J. Shellfish Res., 14:105-11.

Winstead, J. T. and J. A. Couch (1981) *Proctoeces* sp. (Trematoda: Digenea) in the American oyster, *Crassostrea virginica*. Trans. Am. Micros. Soc., 100:296-305.

Winstead, J. T. and J. A. Couch (1988) Enhancement of protozoan pathogen *Perkinsus marinus* infections in American oysters *Crassostrea virginica* exposed to the chemical carcinogen *n-nitrosodiethylamine* (DENA). Dis. Aquat. Org., 5:205-13.

Winstead, J. T., R. M. Overstreet and L. A. Courtney (1998) Novel gonadal parasites in the eastern oyster *Crassostrea virginica* from two Gulf of Mexico bays. J. Shellfish Res., 17:341-342.

Wolf, P. H., J. T. Winstead and J. A. Couch (1987) *Proctoeces* sp. (Trematoda: Digenea) in Australian oysters, *Saccostrea commercialis* and *Crassostrea amasa*. Trans. Am. Micros. Soc., 106:379-80.

Yoo, M. S. and T. Kajihara (1985) Effect of the pea crab (*Pinnotheres pholadis*) on the reproductive capacity of the blue mussel (*Mytilus edulis galloprovincialis*). Bull. Korean Fish. Soc., 18:581-5.

Zander, C. D. (1998) Ecology of host parasite relationships in the Baltic Sea. Naturwiss., 85:426-36.

Zaroogian, G., P. Yevich and S. Anderson (1993) Effect of selected inhibitors on cadmium, nickel, and benzo[*a*]pyrene uptake into brown cells of *Mercenaria mercenaria*. Mar. Environ. Res., 35:41-5.

MICROTOX® BASIC TEST (PHENOL STANDARD)

Katy W. Chung, Peter B. Key and Michael H. Fulton

1.0 OBJECTIVE

This method tests a standard toxicant, whose test results are well documented, and also checks the performance of the complete Microtox system. The advantage of using phenol is that its toxicity is extremely well characterized and produces a clear effect rapidly.

2.0 HEALTH AND SAFETY

Personnel should wear lab coats, lab aprons, safety goggles, and chemical resistant gloves when preparing chemical stocks, and when dosing with test chemicals or effluents.

3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

This method should be restricted to use by or under the supervision of professionals experienced in toxicity testing.

4.0 REQUIRED AND RECOMMENDED MATERIALS

Microtox Test Reagent 1-L Volumetric Flask

Microtox Reconstitution Solution Phenol Standard

2.5% NaCl Diluent 22% NaCl Diluent

Test Cuvettes Lab coat

Gloves Repeat Pipettor

0.6ml Syringe Pipet Tips

Sodium Chloride 1000-ml Pipettor

500-ml Pipettor 250-ml Pipettor

100-ml Glass Beaker De-ionized Water (DI H2O)

SDI (Strategic Diagnostics Inc.) Model 500 Analyzer

5.0 PROCEDURE

5.1 Preparation

5.1.1 Analyzer and Incubator Preparation Plug in the SDI M500 Toxicity Analyzer. Place a cuvette in the REAGENT well.

5.1.2 Making Phenol Standard

Weigh out 0.025g of Phenol (located in Rm. 238) into a 100-ml glass beaker. Add 100 ml of DI water. Pour into a 250-ml volumetric flask and mix well. Pour into clean amber bottle. Date and label the bottle.

5.1.3 Making 2.5% NaCl Diluent

Weigh out 25g of Microbiology grade Sodium Chloride (located in Rm. 230) into a 50-ml glass beaker. Pour the NaCl into a 1-L volumetric flask. Add DI water to the mark on the neck of the flask. Cap with a stopper and invert to mix. Pour into a pre-clean 1000-ml Pyrex Culture Bottle. Date and label the bottle

5.1.5 Making 22% NaCl Diluent

Weigh out 220g of Microbiology Grade Sodium Chloride (located in Rm. 230) into a 100-ml glass beaker. Pour the NaCl into a 1-L volumetric flask. Add DI water to the mark on the neck of the flask. Cap with a stopper and invert to mix. Pour into a pre-clean 1000-ml Pyrex Culture Bottle. Date and label the bottle

5.2 Phenol Standard Test

Phenol Standard Test should be conducted at least once a day when running samples. Add 2-ml of Reconstitution Solution (water) to a cuvette and place it in REAGENT well. Take out Phenol Standard, 2% and 22% NaCl diluents from the Beige Refrigerator. Place cuvettes in Rows A & B of the SDI M500 Analyzer.

Pipette 500µl of 2% NaCl diluent into cuvettes in Row B for 5 mins. Pipette 1ml of 2% NaCl solution into cuvettes 1-4 in Row A. Pipette 250µl of 22% NaCl in to A5. Pipette 2.5ml of Phenol Standard in to A5. Mix 3-4 times with pipette. Use the 500µl-pipettor first to add 0.5 ml standard, then use the 1ml-pipettor (broken one) to add 2ml of standard. Transfer 1ml from A5 to A4. Mix three times. Transfer 1ml from A4 to A3. Mix three times. Transfer 1ml from A3 to A2. Mix three times. Discard 1ml from A2 into a beaker appropriately labeled “Spent Waste”. Using the 250µl-pipettor, discard 750µl from A5. When timer reaches 5 minutes, reconstitute 2 vials of reagent (stored in the freezer). Remove the foil from the vials and add the 2-ml of Reconstitution Solution from the REAGENT cuvette to them. Cap the vials and invert several times to dissolve the reagent. Pour the solution back into the cuvette and place it back in the REAGENT well. Try to get everything out of the vials by using the repeat pipettor. Attach a 0.6ml syringe to the pipettor and get all of the reagent out of the vials. Mix reagent ~20 times with a 1ml-pipettor. Using the repeat pipettor with a 0.6ml syringe, add 10µl of reagent to each of the cuvettes in Row B. Mix by shaking it by hand. Set timer for 15 minutes. After everything is set up for this test, use the remainder of the 15 mins to start setting up the next sample. Set up computer for a BASIC TEST:

1 CONTROL

4 DILUTIONS

45 = INITIAL CONC.

2 = DILUTION FACTOR

MG/L = UNITS

5 MINS. TEST TIME (Delete the 15 min.)

Zero time readings should be (□)ed After 15 mins, place cuvette B1 in READ well and press the SET button. When the green light comes back on, touch the space bar and take initial readings, as prompted by the computer. Transfer 500µl from A1 to B1, A2 to B2, A3 to B3, A4 to B4 and A5 to B5. Pipet tip does not need to be changed between transfers. Touch space bar and wait for the remainder of the 5 mins. to expire. After 5 mins. expires, take readings as prompted by the computer. After taking the readings, check results: EC50 = 13-26 mg/L, Coefficient of Determination > 0.95, Confidence Factor should be between 1 and 2. If the results are not within these parameters, another phenol test needs to be conducted again.

5.3 End of the Test

The cuvette contents are disposed of in the sink and the cuvettes are thrown away in the BROKEN GLASS DISPOSAL (SOP 00-009) by the door.

6.0 QUALITY ASSURANCE/QUALITY CONTROL

After taking the readings, check results: EC50 = 13-26 mg/L, Coefficient of Determination > 0.95, Confidence Factor should be between 1 and 2. If the results are not within these parameters, another phenol test needs to be conducted again. The Phenol Standard will need to be properly disposed of after 90 days. Assurances will be made to confirm that the Reconstitution Solution has not exceeded the expiration date.

Personnel should follow good laboratory practices during Microtox testing.

7.0 REFERENCES

Microtox Manual. Microbics Corporation. 1992. Carlsbad, CA. 476 pp.

Taxonomy Analysis
Thomas Nelepa
NOAA/GLERL (retired)

Benthic Taxonomy and Sorting: Measurement/Data Acquisition

1.Sampling Process Designs

1.1. Picking and Sorting

In the laboratory, sample residue will be placed in a white enamel pan and all macroinvertebrates will be picked and sorted with the aid of a low power lamp-magnifier. The red-stained organisms are more effectively and efficiently picked when placed against a white background.

1.2. Species Identification

If possible, all macroinvertebrates in a given sample will be identified to the lowest practical taxonomic level using standard keys. In samples where numbers of a particular group are high, the number will be proportionately reduced with a Folsom Plankton Splitter and between 75 to 100 individuals of the groups will be identified. Identification of oligochaetes and chironomids will be subcontracted.

2. Sampling Methods Requirements

2.1. Labeling Laboratory Sample Vials

Before a particular sample is processed in the laboratory, glass vials with seal-tight caps are labeled with the station identifier, replicate number (triplicate samples per station are identified as A, B, or C), and the date the sample was collected. Enough vials are labeled to separately contain each of the major benthic groups (amphipods, oligochaetes, sphaeriids, chironomids, Dreissena , and “others”). A dilute preservative (5 % buffered formalin) is placed in each of the vials.

2.2. Sample Preparation

The sample is washed into a brass screen with 500-um openings (U. S. Standard No. 35 Sieve) and gently rinsed to remove preservative. The sample is then washed into a shallow, white enamel pan. The white background of the pan allows the red-stained organisms to be seen more effectively. The proportion of the total sample placed into the pan will depend upon the amount of sediment debris. If the sample contains a large amount of debris, the sample is processed (see below) in portions until the entire sample is completed.

2.3. Sample Processing: Picking and Sorting

Under a magnifier lamp (2x), a technician picks, sorts, and places all macroinvertebrates into the

appropriate vials. Accurate counts of the number placed in each vial is kept (laboratory counter) and later recorded on a bench sheet after all organisms have been removed from the sample. Since the focus is on macroinvertebrates, organisms such as copepods and nematodes will not be sorted and counted. Uncommon taxonomic groups such as Mysis, leeches, and planarians, etc. are placed in the vial labeled "others". Once the technician has picked all the macroinvertebrates from the sample, the next procedure will vary depending on the technician's experience. For a new technician, the first 10 samples that are picked will be checked by senior personnel to make sure all the organisms have been removed. If the number left (not removed) is less than 5 % of the total number picked and counted for each group, then the technician can proceed to pick and sort samples without senior personnel checking each sample. If the number left is greater than 5 %, checking by senior personnel will continue until this figure is achieved. All original counts and re-counts (i. e., number found by senior personnel when checking the sample) will be placed on a laboratory bench sheet. Even for experienced technicians, the same technician will not sort all replicates within a given site and 10% of all samples will be re-checked by a different person (technician or senior personnel) and any missed individuals will be recorded.

In some samples, the number of dreissenid mussels can be great. In such cases, the sample is randomly split into quarters and all individuals (dreissenids or otherwise) in one randomly-selected quarter are picked and counted. If the number of dreissenids exceeds 200, then all individuals but dreissenids in the rest of the sample are picked and counted. If the number of dreissenids does not exceed 200 in the first quarter, then all individuals in other random quarters are picked and counted until the number exceeds 200.

2.4. Identification and Count Verification

Amphipoda - Amphipods will be removed from the vial, placed in a petri dish, and genus determined under a stereoscope. The number of amphipods in every tenth sample will be recounted (only intact specimens with heads are counted). If the value is not within 10 % of the original count as given on the bench sheet, the number of individuals in the sample will be recounted. Once this is completed, all individuals will be returned to the original vial for storage.

Oligochaeta - Oligochaetes need to be cleared and mounted prior to identification. The procedure is time consuming, therefore no more than 130 individuals are identified per sample. Prior to mounting, the number of oligochaetes in every tenth sample will be recounted. If the value is not within 10 % of the original count, the number of individuals in the sample will be recounted. If there are less than 130 individuals in a sample, then all are identified. If there are more than 130 individuals, then the sample is proportionally split with a folsom plankton splitter as many times as needed to obtain less than 130 individuals. The number of splits is recorded on the laboratory bench sheet. Oligochaetes are cleared in lactophenol and then mounted in glycerol on a microscope slide. A coverslip is then placed over the organisms and care taken to avoid air bubbles. When mounting, individuals should not be overlapping and individuals of the same general size (thickness) should be placed under the same coverslip. The number of individuals mounted is recorded on the bench sheet. All slides are labeled with the date, station, and replicate. The slides are then placed in a microscope slide tray and given to Freshwater Benthic Services, Inc. for identification to the lowest possible taxonomic level. Only individuals with heads are identified. The fraction mounted relative to the total number originally counted is determined, and this fraction is used as the multiplier to obtain the total number of each species in the sample.

Sphaeriidae - Sphaeriids will be removed from the vials, placed in a petri dish, and genus determined under a stereoscope (most all sphaeriids are of the genus Pisidium and can be easily identified). The

number of sphaeriids in every tenth sample will be recounted. If the value is not within 10 % of the original count, the number of individuals in the sample will be recounted. Once this is completed, all individuals will be returned to the original vial for storage.

Chironomidae – Chironomids need to be mounted and examined under high power magnification to be identified to the genus/species level. The headcapsule is separated from the body and then mounted in glycerol on a microscope slide with the mentum facing up. The corresponding body is mounted alongside. After a coverslip is placed over the top, the slide is labeled (date, station, replicate), placed in a slide tray, and given to Freshwater Benthic Services, Inc. for identification to the lowest possible taxonomic level. All chironomids in the sample will be identified in this manner.

Dreissena – Dreissena in each of the vials will be placed in a petri dish and examined under a stereoscope. The number of Dreissena in every tenth sample will be recounted. If the value is not within 10 % of the original count, the number of individuals in the sample will be recounted. Once this is completed, all individuals will be returned to the original vial for storage.

"Others" – All individuals in the vial labeled "others" will be identified to the lowest practical taxonomic level.

3. Sample Handling and Custody Requirements

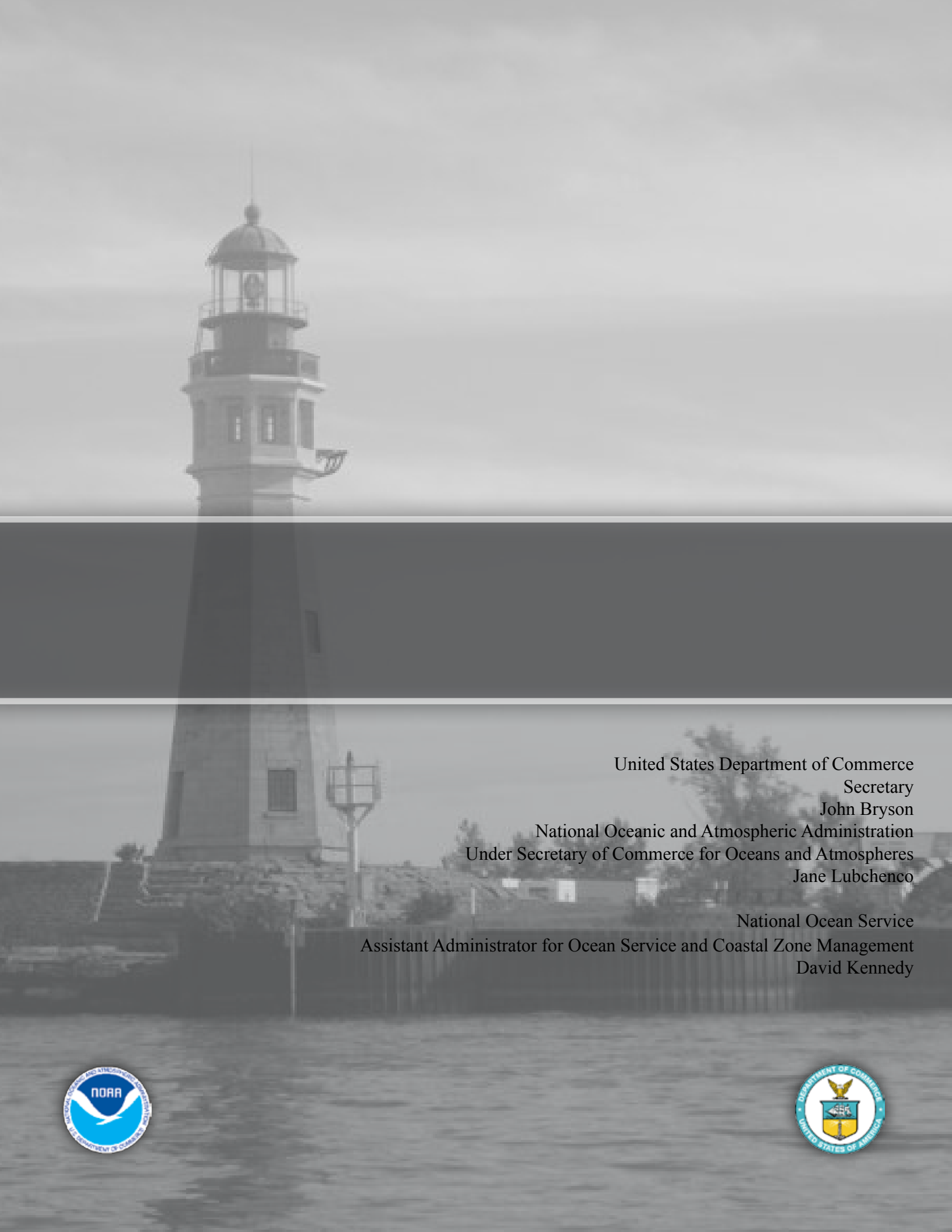
After collection, all samples will be stored at GLERL and in the custody of T. Nalepa. Samples will be processed, and vials containing the organisms will be stored at the same facility. Slide trays containing oligochaetes and chironomids for identification will be picked up by M. Winnell of Freshwater Benthic Services, Inc. and identified at his facility. A list will be made of the slides provided to Mr. Winnell and the date the transfer occurred. The date in which the identifications were provided by Mr. Winnell will also be recorded.

4. Data Management

All laboratory bench sheets will be kept at GLERL and in the custody of T. Nalepa. Once all individuals are identified to the lowest practical taxonomic level, including the data provided by Freshwater Benthic Services, all data will be inputted into a spreadsheet by T. Nalepa. Accuracy of data input will be checked independently.

5. Data Review, Validation, and Verification Requirements

Criteria for accuracy and completeness of sample processing (picking and counting) have been established. Species identification of oligochaetes and chironomids will be made by Michael Winnell of Freshwater Benthic Services, Inc. M. Winnell has over 20 years experience in identifying Great Lakes macroinvertebrates. T. Nalepa will identify individuals in the other macroinvertebrate groups. Individuals that cannot be identified with certainty will be sent to specialists for the particular taxonomic group in question.



United States Department of Commerce
Secretary
John Bryson
National Oceanic and Atmospheric Administration
Under Secretary of Commerce for Oceans and Atmospheres
Jane Lubchenco

National Ocean Service
Assistant Administrator for Ocean Service and Coastal Zone Management
David Kennedy