NATIONAL CENTERS FOR COASTAL OCEAN SCIENCE

MUSSELWATCHPROGRAM

Great Lakes Monitoring Project Plan under GLRI Action Plan II

Mussel Watch Program: Great Lakes Monitoring Project Plan under GLRI Action Plan II

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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 federal, 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 for the period FY2015-FY2019 under Great Lakes Restoration Initiative - Action Plan II. 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 I summarized the actions of federal agencies implemented during the period 2010 through 2014. Action Plan II covers the period FY2015 through FY2019 and federal agencies will continue to use GLRI resources to strategically target the biggest threats to the Great Lakes ecosystem and to accelerate progress toward long term goals. The specific Action Plan II goals addressed by Great Lakes Mussel Watch is cleaning up Areas of Concern, with a special emphisis on characterizing chemicals of emerging concern and their effects on fish and wildlife. The U.S.-Canada Great Lakes Water Quality Agreement (GLWQA, 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." The most recent amendment, *Great Lakes Water Quality Protocol of 2012*, addresses current impacts on Great Lakes water quality in ten annexes. Annex three specifically addresses chemicals of mutual concern, the first list includes several emerging chemcials of concern.

This document includes descriptions of sampling, analyses, reporting, and associated quality assurance and quality control methods used by the Mussel Watch Program during Action Plan II. 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

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Table of Contents

Table 1. QAPP distribution list

Name	Organization
Steve Thur	NOAA/National Centers for Coastal Ocean Science (NCCOS)
Sherri Fields	NOAA/NCCOS
Marie Delorenzo	NOAA/NCCOS
John Hayes	NOAA/NCCOS
W. Edward Johnson	NOAA/NCCOS/Mussel Watch Program
Kimani Kimbrough	NOAA/NCCOS/Mussel Watch Program
Annie Jacob	NOAA/NCCOS/Mussel Watch Program
Annie John Gibbs	NOAA/Office of Response and Restoration (ORR)
Deborah Lee	NOAA/Great Lakes Environmental Research Laboratory (GLERL)
Rebecca Held Knoche	NOAA/GLERL
Sean Campbell	SGS AXYS
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Juan Ramirez	TDI-Brooks, International, Inc.
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Table 2. Project task organization

Name	Affiliation	Responsibility
NOAA - National Centers for Coastal Ocean Science		
Steve Thur	National Centers for Coastal Ocean Science	Director
Sherri Fields	Stressor Detection & Impacts Division	Deputy Division Cheif
Marie Delorenzo	Monitoring & Assessment Branch	MAB Chief (Acting)
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Kimani Kimbrough	Mussel Watch Program	Quality Assurance Manager
W. Edward Johnson	Mussel Watch Program	Project Field Coordinator
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Annie Jacob	Mussel Watch Program	Project Data Manager
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Rebecca Held Knoche	Great Lakes Environmental Research Laboratory	GLRI Coordinator
Deborah Lee	Great Lakes Environmental Research Laboratory	GLERL Director and NOAA's GLRI Director
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Contractors		
Juan Ramirez	TDI-Brooks International, Inc.	Chemistry

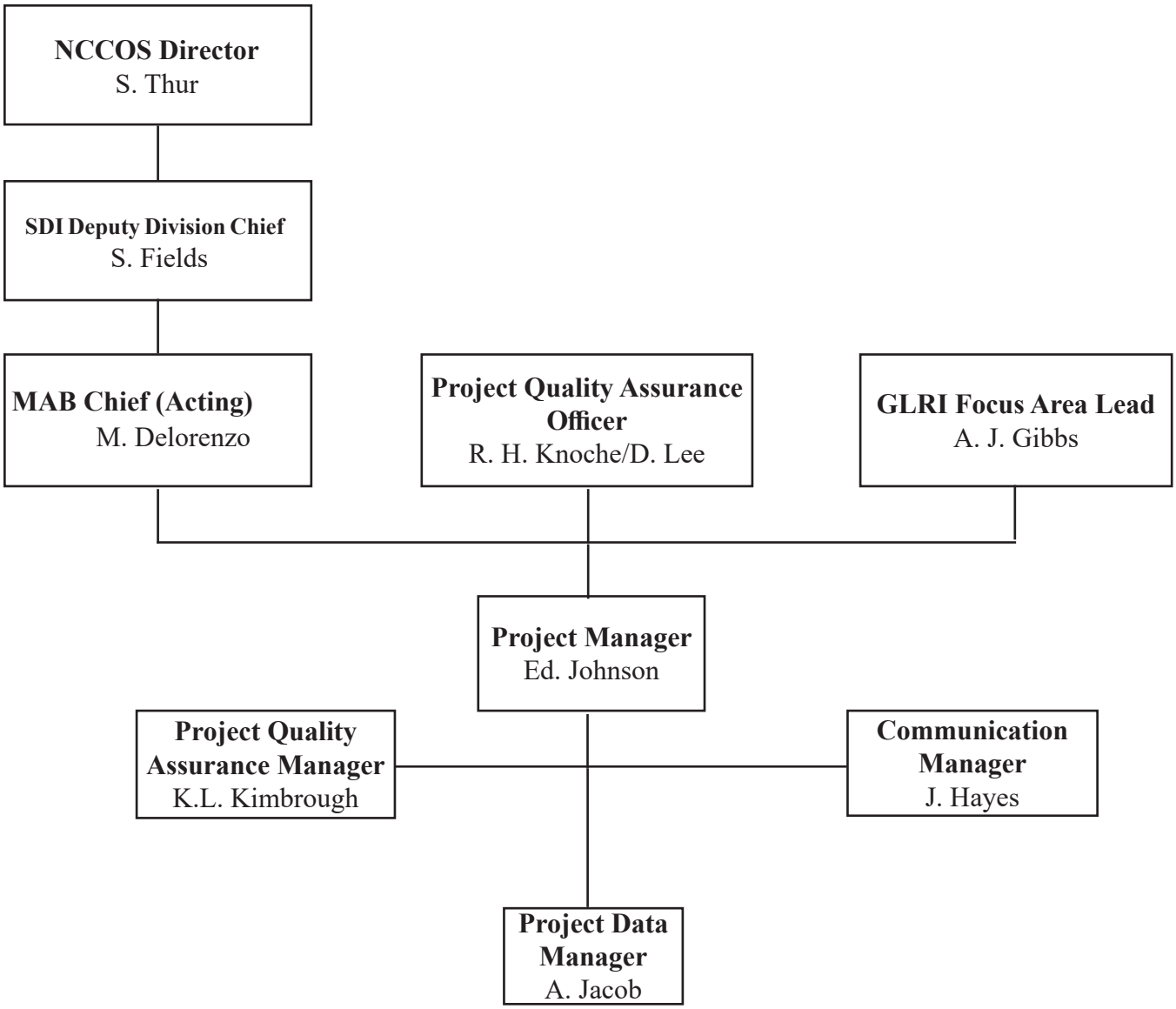

Figure 1. Organizational chart that shows lines of authority and reporting responsibilities.

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 and the Great Lakes. 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. 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 (https://products.coastalscience.noaa.gov/collections/ltmonitoring/nsandt/). 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) offshore and river-harbors. The program will be enhanced with additional sites and indicators including mussel tissue, mussel health metrics and passive samplers in support of:

1. Characterizing emerging chemicals of concern
2. Assessing the potential for impacts on fish and wildlife
3. Leverage MWP national data to provide a national perspecitve to Great Lakes contamination;
4. Policy, remediation, regulatory and legislative decisions

Great Lakes

The Mussel Watch Program began collecting mussels from sites in the Great Lakes in 1992 after the invasion and proliferation of Ponto-Caspian mussels in the region. 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).

Dreissenid mussels are widely used as bioindicators in Europe and their native Ponto-Caspian region, because of their high filtering rates, high lipid content and the ability to bioaccumulate contaminants from water, algae and suspended sediments (de Kock and Bowmer 1993;Bervoets et al., 2005; Berny et al., 2003; Gossiaux et al., 1998; Reeders and bij de Vaate 1992). Other favorable characteristics of dressenids as biomonitors include ease of collection, abundance, sessile life cycle, limited ability to metabolize lipophilic contaminants, and utility in effects-based monitoring. 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). In addition, the recognition of dressenid mussels as keystone species of the Great Lakes ecosystem (Vanderploeg et al., 2002), reinforces its critical value in the assessment of the overall health and ecological forecasting of the Great Lakes.

MWP expanded its monitoring activities in the Great Lakes under the Great Lakes Restoration Initiative (GLRI) in 2010, based on Action Plan I (2010-2014), Focus Area "Toxic Substances and Areas of Concern". MWP added sites in all the U.S. Areas of Concern (AOC) and data from the basin-wide assessment conducted in 2009-2010 is summarized in Kimbrough et al., 2014. During the first five

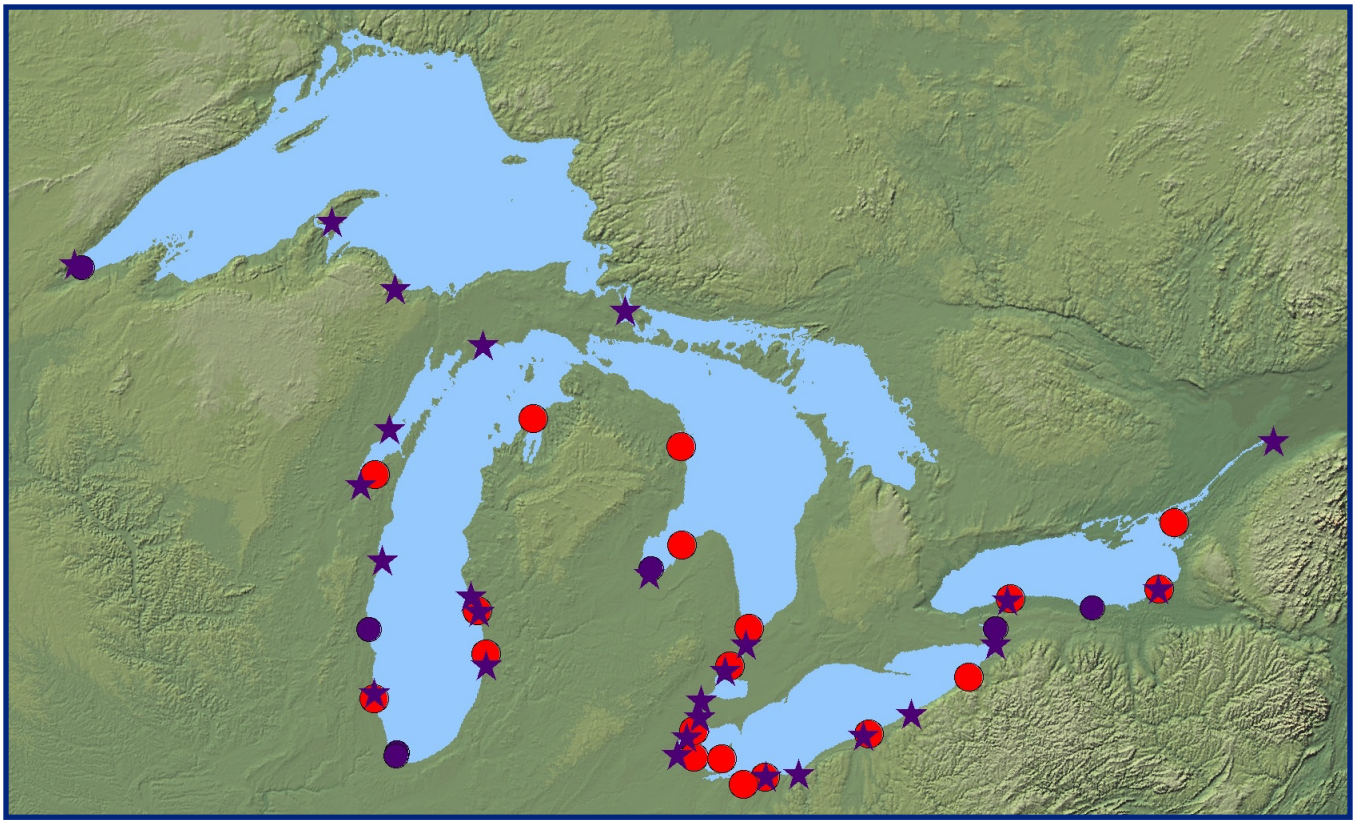

Figure 3. Great Lakes Mussel Watch Program sites. (●Mussel Watch sites, ●Mussel Watch/AOC sites, ★AOC sites).

NOAA characterized presence and distribution of a suite of legacy and selected CECs in biota and sediment. Intensive characterizations of selected priority AOCs and the addition of mussel sites in the lake nearshore and offshore zones were conducted. Concurent with this effort was the application of mussel health indicators including cellular biomarkers, transcripomics gene experssion assays, and NMR metabolomics.

The GLRI Action Plan II (2015-2019) Focus Area "Toxic substances and Areas of Concern" calls for federal partners to "identify emerging contaminants and assess impacts on Great Lakes fish and wildlife." The multi-agency team uses collaborative research, monitoring and surveillance program to achieve the goals outlined in the Action Plan II to effectively address the issue of CECs in the Great Lakes. MWP particpates in Action Plan II activities by providing mussel tissue concentrations of contaminants of emerging concern and supplemental bivalve health data and passive sampler data. MWP uses caged mussels to conduct monitoring in areas where natural Caged mussels are typically deployed for approximately four weeks and strategically located in areas with known or suspected pollution.

Data and documents of the Mussel Watch Program including some of this work are available online (https://coastalscience.noaa.gov/) as static data files and through a data query tool that allows custom data searches. Reports, methods documents and other publications related to this project are available. Field logs, chain-of-custody logs, are retained on file for a minimum of ten years at NCCOS headquarters in Silver Spring, MD 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.The project work schedule is shown in Figure 4.

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*
Bivalve chemistry	Critical
BivalveHealth- Metabolomics, Gene expression, DNA damage and Cellular biomarkers	Critical
POCIS	Critical
SPMD	Critical
Physico-chemical variables- Temperature and Dissolved oxygen	Critical

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

Compound class	Compound
Trace elements	Mercury, Methyl Mercury
Chlordane	Alpha-Chlordane, Cis-Nonachlor, Gamma-Chlordane, Heptachlor, Heptachlor-Epoxide, Oxychlordane, Trans-Nonachlor
Dichlorodiphenyltrichloroethane and metabolites (DDT)	2,4'-DDD, 4,4'-DDD, 2,4'-DDE, 4,4'-DDE, 2,4'-DDT, 4,4'-DDT, DDMU
Dieldrin	Aldrin, Dieldrin, Endrin
Endosulfan	Endosulfan I, Endosulfan II, Endosulfan Sulfate
Hexachlorocyclohexane	Alpha-Hexachlorocyclohexane, Beta-Hexachlorocyclohexane, Delta-Hexachlorocyclohexane, Gamma-Hexachlorocyclohexane
Polychlorinated biphenyls (PCB)	PCB1, PCB101_90, PCB105, PCB107, PCB110_77, PCB114_131_122, PCB118, PCB128, PCB129_126, PCB136, PCB138_160, PCB141_179, PCB146, PCB149_123, PCB15, PCB151, PCB153_132_168, PCB156_171_202, PCB158, PCB16_32, PCB166,PCB167, PCB169, PCB170_190, PCB172, PCB174, PCB176_137, PCB177, PCB178, PCB18, PCB180, PCB183, PCB185, PCB187, PCB189, PCB191, PCB194, PCB195_208, PCB196_203, PCB199, PCB200, PCB201_173_157, PCB205, PCB206, PCB209, PCB22_51, PCB24_27, PCB25, PCB26, PCB28, PCB29, PCB31, PCB33_53_20, PCB40, PCB41_64, PCB42_59_37, PCB43, PCB44, PCB45, PCB46, PCB47_48_75, PCB49,PCB52, PCB56_60, PCB66, PCB7_9, PCB70, PCB74_61, PCB8_5, PCB81, PCB82, PCB83, PCB84, PCB85, PCB86, PCB87_115, PCB88,PCB92, PCB95, PCB97, PCB99
Other Organochlorines	Mirex, Chlorpyrifos
Polybrominated diphenyl ethers (PBDE)	BDE1, BDE10, BDE100, BDE11, BDE116, BDE118, BDE119, BDE12, BDE126, BDE13, BDE138, BDE15, BDE153, BDE154, BDE155, BDE166, BDE17, BDE181, BDE183, BDE190, BDE194, BDE195, BDE196, BDE197, BDE198, BDE2, BDE201, BDE202, BDE204, BDE205, BDE206, BDE207, BDE208, BDE209, BDE25, BDE28, BDE3, BDE30, BDE32, BDE33, BDE35, BDE37, BDE47, BDE49_71, BDE66, BDE7, BDE75, BDE77, BDE8, BDE85, BDE99

Polycyclic aromatic hydrocarbons (PAH) & select individual isomers	Acenaphthene, Acenaphthylene, Anthracene, Benz[a]anthracene, Benzo[a]pyrene, Benzo[e]pyrene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[g,h,i]perylene, Benzothiophene, C1-Benzothiophene, C1-Chrysenes, C1-Dibenzothiophenes , C1-Fluorenes, C1-Fluoranthenes_Pyrenes, C1-Naphthobenzothiophene, C1-Naphthalenes, C1-Phenanthrenes_Anthracenes, C2-Dibenzothiophenes, C2-Fluorenes, C2-Fluoranthenes_Pyrenes, C2-Naphthobenzothiophene, C2-Naphthalenes, C2-Phenanthrenes_Anthracenes, C3-Benzothiophene, C3-Chrysenes, C3-Dibenzothiophenes, C3-Fluorenes, C3-Fluoranthenes_Pyrenes, C3-Naphthobenzothiophene, C3-Naphthalenes, C4-Chrysenes, C4-Naphthalenes, C4-Phenanthrenes_Anthracenes, Chrysene, Dibenzo[a,h]anthracene, Dibenzofuran, Dibenzothiophene, Fluoranthene, Fluorene, Indeno[1,2,3-c,d]pyrene, Naphthobenzothiophene, Naphthalene, Phenanthrene, Pyrene
Hexabromocyclododecane (HBCDD)	alpha-HBCDD, gamma-HBCDD and beta-HBCDD
Dioxins and furans	1,2,3,4,6,7,8-HPCDD,1,2,3,4,6,7,8-HPCDF, 1,2,3,4,7,8,9-HPCDF, 1,2,3,4,7,8-HXCDD, 1,2,3,4,7,8-HXCDF, 1,2,3,4,7,8-HXCDF, 1,2,3,6,7,8-HXCDF, 1,2,3,7,8,9-HXCDD, 1,2,3,7,8-PECDD, 1,2,3,7,8-PECDF, 2,3,4,6,7,8-HXCDF, 2,3,4,7,8-PECDF, 2,3,7,8-TCDD, 2,3,7,8-TCDF, OCDD, OCDF, 1,2,3,7,8,9-HXCDF
Alkylphenols	4N-OP, NP, NP1EO, NP2EO

Table 4 Cont'd. List of contaminants (Pharmaceuticals and personal care products) analyzed as part of the Expanded Great Lakes.

1,7-Dimethylxanthine	Citalopram	Gemfibrozil	Prednisone
10-Hydroxy-amitriptyline	Clarithromycin	Glipizide	Progesterone
17 alpha-Dihydroequilin	Clinafloxacin	Glyburide	Promethazine
17 alpha-Estradiol	Clonidine	Hydrochlorothiazide	Propoxyphene
17 alpha-Ethinyl-Estradiol	Clotrimazole	Hydrocodone	Propranolol
17 beta-Estradiol	Cloxacillin	Hydrocortisone	Ranitidine
2-Hydroxy-ibuprofen	Cocaine	Ibuprofen	Rosuvastatin
4-Epianhydrochlortetracycline [EACTC]	Codeine	Iopamidol	Roxithromycin
4-Epianhydrotetracycline [EATC]	Colchicine	Isochlortetracycline [ICTC]	Sarafloxacin
4-Epichlortetracycline [ECTC]	COMPOUND	Lincomycin	Sertraline
4-Epioxytetracycline [EOTC]	Cotinine	Lomefloxacin	Simvastatin
4-Epitetracycline [ETC]	Cyclophosphamide	Medroxyprogesterone Acetate	Sulfachloropyridazine
Acetaminophen	Daunorubicin	Melphalan	Sulfadiazine
Albuterol	DEET	Meprobamate	Sulfadimethoxine
Allyl Trenbolone	Dehydronifedipine	Mestranol	Sulfamerazine
Alprazolam	Demeclocycline	Metformin	Sulfamethazine
Amitriptyline	Desmethyldiltia-zem	Methylprednisolone	Sulfamethizole
Amlodipine	Desogestrel	Metoprolol	Sulfamethoxazole
Amphetamine	Diatrizoic acid	Metronidazole	Sulfanilamide
Amsacrine	Diazepam	Miconazole	Sulfathiazole
Androstenedione	Digoxigenin	Minocycline	Tamoxifen
Androsterone	Digoxin	Moxifloxacin	Teniposide
Anhydrochlortetracycline [ACTC]	Diltiazem	Naproxen	Testosterone
Anhydrotetracycline [ATC]	Diphenhydramine	Norethindrone	Tetracycline [TC]
Atenolol	Doxorubicin	Norfloxacin	Theophylline
Atorvastatin	Doxycycline	Norfluoxetine	Thiabendazole
Azathioprine	Drospirenone	Norgestimate	Trenbolone
Azithromycin	Enalapril	Norgestrel	Trenbolone acetate
Benzoylecgonine	Enrofloxacin	Norverapamil	Triamterene
Benztropine	Equilenin	Ofloxacin	Triclocarban
Betamethasone	Equilin	Ormetoprim	Triclosan
Bisphenol A	Erythromycin-H2O	Oxacillin	Trimethoprim
Busulfan	Estriol	Oxazepam	Tylosin
Caffeine	Estrone	Oxolinic Acid	Valsartan
Carbadox	Etoposide	Oxycodone	Venlafaxine
Carbamazepine	Flumequine	Oxytetracycline [OTC]	Verapamil
Cefotaxime	Fluocinonide	Paroxetine	Virginiamycin M1
Chlortetracycline [CTC]	Fluoxetine	Penicillin G	Warfarin
Cimetidine	Fluticasone propio-nate	Penicillin V	Zidovudine
Ciprofloxacin	Furosemide	Prednisolone	

Table 4 Cont'd. List of contaminants (pesticides_ analyzed as part of the Expanded Great Lakes.

2,4'-DDD	Chlorpyriphos-Methyl	Fenitrothion	Nonachlor, trans-
2,4'-DDE	Chlorpyriphos-Oxon	Flufenacet	Octachlorostyrene
2,4'-DDT	Cyanazine	Flutriafol	Parathion-Ethyl
4,4'-DDD	Cypermethrin-A	Fonofos	Parathion-Methyl
4,4'-DDE	Cypermethrin-B	HCH, alpha	Pendimethalin
4,4'-DDT	Cypermethrin-C	HCH, beta	Permethrin, cis-
Alachlor	Dacthal	HCH, delta	Permethrin, trans-
Aldrin	Desethylatrazine	HCH, gamma	Perthane
alpha-Endosulfan	Diazinon	Heptachlor	Phorate
Ametryn	Diazinon-Oxon	Heptachlor Epoxide	Phosmet
Atrazine	Dieldrin	Hexachlorobenzene	Pirimiphos-Methyl
Azinphos-Methyl	Dimethenamid	Hexazinone	Quintozene
beta-Endosulfan	Dimethoate	Linuron	Simazine
Butralin	Disulfoton	Malathion	Tebuconazol
Butylate	Disulfoton Sulfone	Methoprene	Tecnazene
Chlordane, alpha (cis)	Endosulfan Sulfate	Methoxychlor	Terbufos
Chlordane, gamma (trans)	Endrin	Metolachlor	Triallate
Chlordane, oxy-	Endrin Ketone	Metribuzin	Trifluralin
Chlorothalonil	Ethalfluralin	Mirex	
Chlorpyriphos	Ethion	Nonachlor, cis-	

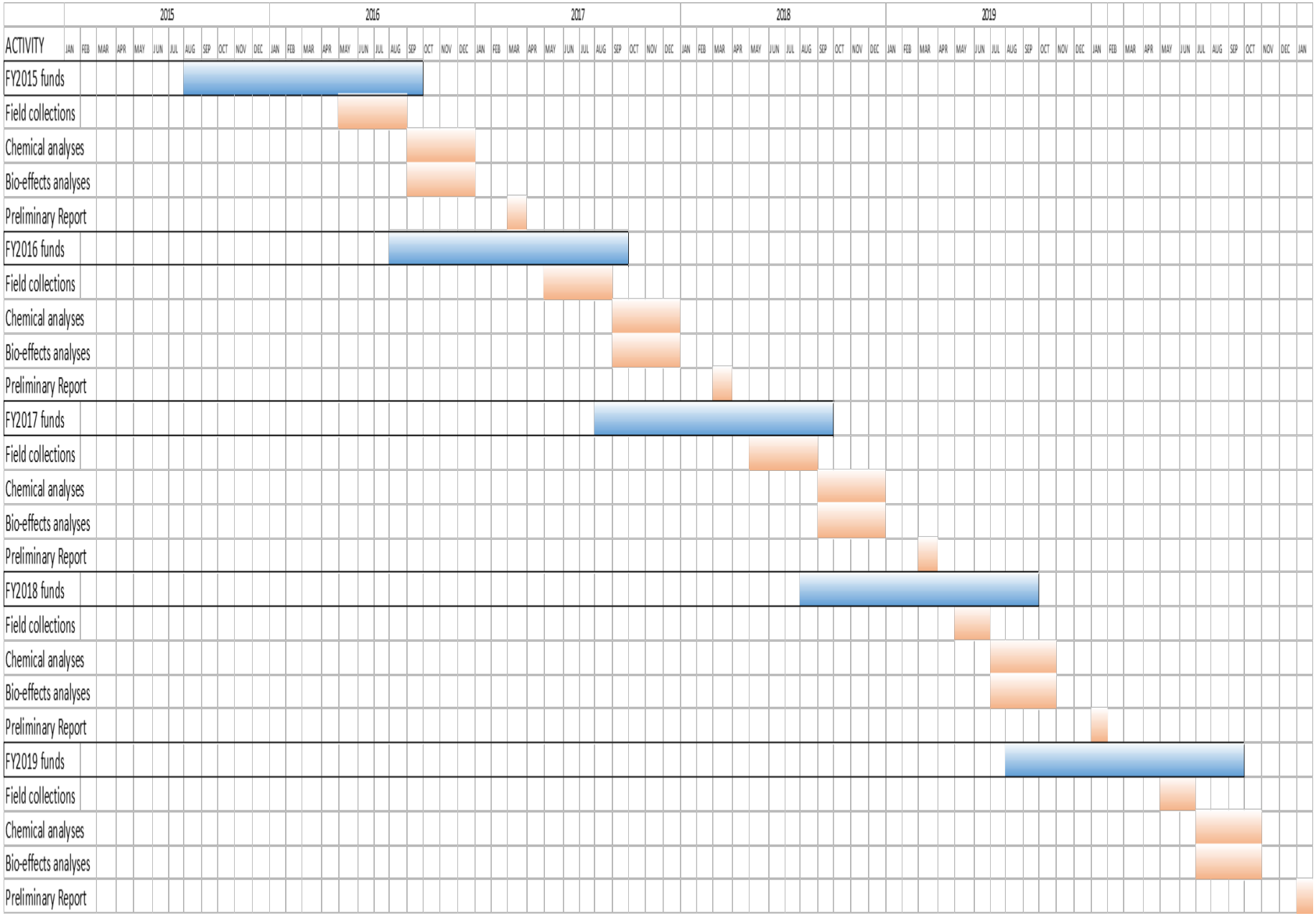

Figure 4. Expanded Mussel Watch Great Lakes Project work schedule.

Data Quality Objectives

Under Action Plan II (2015-2019) Great Lakes Mussel Watch aligned with a group of federal partners focused on characterizing CECs and their potenital effects on fish and wildlife. The specific goals of this new alignment are :

- To characterize and evaluate the extent to which chemicals of emerging concern (CECs) threaten fish and wildlife populations relative to other stressors present in the Great Lakes, and,
- To pilot and develop a short and long term state-of-the-art bioeffects surveillance program for the Great Lakes basin.

Project Objectives

- Characterize chemical body burdens in mussel tissue ;
- Characterize contaminant exposure of mussels using passive samplers (POCIS, SPMDs);
- Assess bivalve health metrics- metabolomics, DNA damage, cellular biomarkers as appropriate and determine whether these results could be correlated with tissue chemistry
- Determine whether tissue chemistry and mussel health results could differentiate between impacted sites and reference sites.

Measurements will support the weight of evidence approach to characterize the potential effects of CEC on fish and wildlife. Action levels and criteria do not exist for bivalve tissue measurements. As a result, measurements will be compared to CEC data obtained by MWP from monitoring efforts in other regions to gain perspective on concentration.

The data obtained from the contract labortaories are verified by the quality assurance lead and data project manager for quality control. 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.

NOAA scuba divers involved in the collection of samples are certified through NOAA's Diving Program. Dive Plans and Diving Emergency Assistance Plans are submitted and approve prior to all NOAA diving activities. Annual recertifications and training are integral to the program.

Section 3: Site Selection and Sample Collection

Site Selection

MWP is part of a federal team working collaboratively to acheive the goals of Action Plan II. MWP works at the sites chosen by the CEC team and the exact locations are finalized by MWP depending on the availability of in situ mussels and the feasibility of deploying caged dreissend mussels in the upper reaches of the river chosen for a particular year. Additional sites are chosen by MWP based on existing

data gaps and the requirements of a specific project.
MWP ensures the work carried out is in full environmnetal compliance standards set by National Ocean Service, NOAA. All required permits to collect dreissenid mussels are obtained prior to the start of the field mission.

Criteria for bivalve site selection

- Bivalves sites are located in river harbors, near shore and offshore zones.
- Sites that are chosen for in situ monitoring should have sufficient bivalves to obtain approximately one-half gallon of mussels. Sites that are used to harvest mussels for caged studies should have abundant population for cage deployments.
- Substrates are limited to rock or concrete (including rip-rap and jetties), and sand or mud. Structures such as wooden pilings and metallic substraits such as sheet pile or anchor chains on navigation aids are avoided in order to eliminate potential contamination.
- 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 May- August. Once established new sites must be documented with Global Positioning Satellite System (GPS).
- For monitoring contaminants of emerging concerm, caged bivalves should be strategically located along a suspected pollution gradient around outfalls and at sites that integrate contaminant accumulation from nearby or surrounding areas. The reference sites should be outside effluent discharge zone.
- 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.

Bivalves

Bivalve sample attainment is dependant on the presence of abundant resident population. In situ bivalves are collected by diving. Samples are a combination of *in situ* bivalves collected from outer harbor stone breakwaters and caged bivalves deployed in rivers. Bivalve samples are collected for the analysis of organic and inorganic contaminants and health measurements. All samples are accompanied by chain of custody forms which included the date and time of sample collection and the site name.

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. Samples which are not harmed by freezing (e.g., tissue for health measurements) and once frozen can be maintained frozen for the duration of the mission may be held and shipped at the end of the mission. Samples for chemistry that are kept in cooler with water ice should be shipped within three days of collection

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. 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

Table 5. 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 6. 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

and provided tracking numbers when samples are shipped. Chain of custody forms are included with each sample as described above (Figure 6).

CAGED MUSSEL STUDIES

MWP has incorprated the use of caged dreissenid mussels as a tool to assess bioavailability at locations where either natural mussels are scarce or are hard to collect to complement the existing biomonitroing efforts in AOCs. Collection of mussels from a "clean" site and redeployment in cages at locations where data gaps exist is a valuable approach that has been widely used worldwide. NOAA's Great Lakes Mussel Watch Program has successfully established new sites and multiple collecting stations within AOCs due to the well-established dreissenid populations on rock breakwaters in the outer harbor areas and the use of scuba divers. Collection of mussels from inner harbor and lower river reach areas present greater challenges due to increased boat traffic, unseen fouling or entanglement hazards for divers in low visibility conditions, and greater risk of diver exposure to water borne pathogens especially following rainfall/runoff events. Biomonitoring of mussels in the inner harbors and lower river reach of AOCs can provide more accurate and precise information on the spatial and temporal pattern of chemical contamination. The use of caged dreissenid mussels suspended above the bottom sediment provides a good alternative that can be readily compared to the dreissenid mussel results obtained in the outer harbor and nearshore areas. The use of native mussels in this region is a less desirable option for several reasons, many species are a scarce resource (threaten or endangered), these native mussels live in the sediment, which in some AOCs is uninhabitable due to anoxic conditions, high ammonia, or habitat disturbance from dredging or sediment capping activities.

Caged mussel deployments can be targeted to known outfalls or hot spots, and/or follow a transect line from outer to inner harbor or lower river reach placements. Alternatively, caged mussels can be

Table 7. 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*
Bivalves- Metals/Organics	cooler filled with ice chips	water ice/refrigerate	Within 3 days with water ice
Bivalves- Metabolomics	teflon bags in cryoshipper	-80°C	At the end of the mission
Bivalves- DNA damage	teflon bags in cryoshipper	-80°C	At the end of the mission
Bivalves- Cellular biomarkers	teflon bags in cryoshipper	-80°C	At the end of the mission
POCIS	cooler filled with blue ice	-80°C	Within 3 days with water ice
SPMDs	cooler filled with blue ice	-80°C	Within 3 days with water ice

randomly deployed at a series of stations using a probabilistic sampling design such as the Generalized Random-tessellation Stratified (GRTS), developed by Don Stevens, Tony Olsen and colleagues of EPA (Stevens, 1997; Stevens and Olsen, 1999, 2000, 2004; EPA 2013). This probabilistic sampling framework results in data that can be used to make unbiased statistical estimates of spatial extent and magnitude of contaminant condition.

Results from cage mussel deployments can thus be designed to provide greater spatial resolution of the biological availably of contamination with improved accuracy and precision of the assessment. These enhancements in data/results will, in general, help mangers more precisely target resource and management actions to areas within AOCs to assess remediation effectiveness and identify areas that may require further management action.

In 2012 (phase 1) the MWP used cage dreissenid mussels in the Manistique River AOC to help in tracking bioavailable sources of PCBs after sediment dredging for remediation. Deployments followed a 1.5 km long transect from the outer harbor to a suspected source upstream with additional targeted deployments. The spacing of caged mussels varied depending on location within the 1.5 km transect and ranged from about 25m to 200m between cages. Mussels used in the cage deployments were first harvested from established populations collected from stone breakwater of the outer harbor. A subsample was sent to laboratories for determination of pre-deployment of chemical burdens, gonald development, lipid content, and mussel health indicators (biomarkers, and gene expression).

This study was further enhanced through a multi-agency collaboration (NOAA, EPA, USGS) that provided multiple biotic and abiotic indicators including sediment chemistry, mussel chemistry and health (biomarkers and gene expression/omics assays), Polyethelene Devices (PEDs), and Hester Denys (benthic colonizing substrate). These indicators were further enhanced by land based indicators, spiders and birds. The “weight-of-evidence” approach from multiple indicators suggest that our preliminary caged mussel data in this AOC provided a the spatial resolution of 25 to 50 meters for contaminant burdens

In 2013 (phase 2) the MWP caged study was repeated in the Manistique River in the same location but with a larger geographic scope and density of sites. PCB analyses of mussel tissues included 84 congeners rather than 39 congeners usually reported. In 2015 and 2016, under GLRI Action Plan II, the MWP conducted a caged bivalve studies in the Maumee River AOC (dreissenids, and corbicula), and the Rouge River AOC (dreissenids only). In 2017, and 2018, the MWP will conduct a caged mussel study in the Milwaukee Estuary AOC. Beginning in 2015, the MWP began deploying passive samplers called polar organic chemical integrative samplers (POCIS) along side the polyethylence deveices (PEDs) that were depolyed by EPA. In 2017 MWP anticipates deploying both POCIS and SPMDs (SemiPermeable Membrane Devises). Both of these devices are obtained from Environmental Sampling Technologies, Inc. of St. Joesph, Mouisri and analyzed by SGS AXYS Analytical Services, in Sidney, British Columbia, Canada.

Section 4: Analytical Methods and Quality Control Requirements

Analytical methods for trace elements and legacy 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

Recorder initials:_____ ***SITE VISIT LOGSHEET*** (v4) **DATE: _**

SITE-STATION:_____ TIME (arrival on station):_____
Lat / lon:_____

GPS unit # / waypoint #(s): _____

TOPSIDE SITE DESCRIPTION (location, distance/bearing to shore, depth):

DIVE SITE: YES / NO; **SITE DESCRIPTION from diver perspective:**
(water temp, depth, visibility; bivalve abundance, size, appearance, substrate; bottom type; fish presence; etc.)

WHAT DID YOU DO (check all that apply)**:**

☐ **1ST mooring deployment** (☐-bivalves, ☐-SPMD, ☐-POCIS, ☐-logger, ☐-Other _____.

☐ **Mooring recovery** (☐-bivalves, ☐-SPMD, ☐-POCIS, ☐-logger, ☐-Other _____.

☐ **Mooring redeployment** (☐-bivalves, ☐-SPMD, ☐-POCIS, ☐-logger, ☐-Other _____.

☐ **Logger** (☐ -in sample cooler, ☐ -downloaded, ☐ -redeploy) SN: _____

☐ **Samples** (☐-caged bivalve,☐-in situ bivalve,☐-POCIS,☐-SPMD,☐- logger,☐-fish,☐-Other___

☐ **Photos** (for species ID and condition include 3x5 card with site-station # in the photos)

☐ **Fish** (☐-in situ round goby, ☐-other species: _____

☐ **Other** _____

TELL US ABOUT THE MOORING: **tied-off:** YES / NO / NA

Where tied-off:_____

Line lay: (distance & bearing):_____

Mooring location by intersecting bearings (from mooring-set get 2 bearings to shore objects)**:**

Object-1 (obj., distance, & bearing):_____

Object-2 (obj., distance, & bearing):_____

TIME (departure from station):_____

Figure 4. Site visit field sheet

CHAIN OF CUSTODY RECORD

Client:_____

Project ID:_____

B&B Contact:_____

Sampler Signature:_____

Sample ID	Sample Date	Sample Time	Sample Matrix	Preservative	Containers Type	Containers No.	Analyses	Other Instructions / Comments
				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
S=Soil/Sediment
R=Rinseate
P=Product
G=Gas
Ws=Waste
HW=Hazardous Waste
W=Water

Sample Container: Vol/material

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

Figure 5. Chain of Custody Form

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.

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., 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 that are part of standard MWP suite 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. Contaminants of emerging concern including pharmaceuticals and personal Care Products (PPCPs), hormones, pesticides, alkylphenols, hexabromocyclododecane are analyzed using strict QA/QC 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 relational data strucuture. The relational data is reviewed and compared 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 https://www.diver.orr.noaa.gov

Section 6: References

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Appendix 1: Tissue Chemistry Methods

TRACE ELEMENT QUANTIFICATION TECHNIQUES

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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 HNO3 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.

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

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.

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

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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.

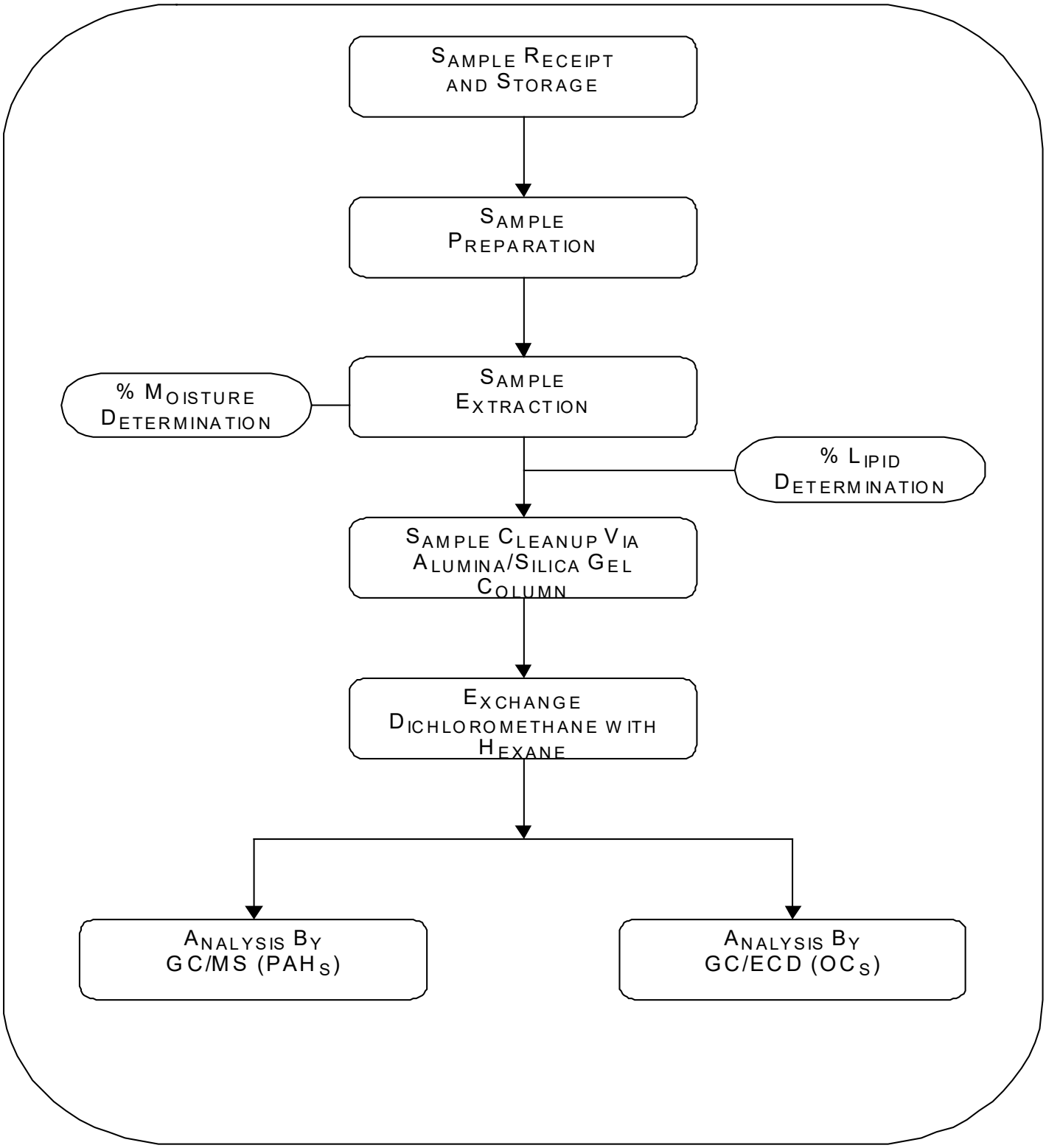

Figure 3-1. Methodology for extraction, isolation, and quantification of tissue samples for polycyclic aromatic hydrocarbons (PAHs) and organic contaminants (OC).

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

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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:

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

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:

$$\%\ \text{Recovery} = \frac{C_{ESU}}{C_{SU}} x100$$

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:

$$C_{Corrected} \frac{C}{\%\ \text{Recovery}} x\ 100$$

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

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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 standa

- 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:

$$M_A = \frac{(A_A M_S)}{(A_S \overline{RRF_i})}$$

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:

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

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:

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

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.

QUANTITATIVE DETERMINATION OF POLYBROMINATED DIPHENYL ETHERS USING SELECTED ION MONITORING GAS CHROMATOGRAPHY/MASS SPECTROMETRY 1999 – 2006

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ABSTRACT

Selected polybrominated diphenyl ethers (PBDEs) are detected using a gas chromatograph/mass spectrometer in selected ion monitoring mode. This method is capable of detecting ppb concentrations of PBDEs 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 polybrominated diphenyl ethers in tissues and sediments at ppb. Samples are injected into a temperature-programmed GC/MS, operated in splitless mode. The capillary column is a DB-XLB (30 m x 0.25 mm ID and 0.1 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. The data acquisition system continuously acquires and stores all data for quantitation.

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 DB-XLB (30 m x 0.25 mm ID and 0.10 µm film thickness)
- Micropipetters, calibrated, 1% accuracy, disposable tips

2.2 REAGENTS

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

2.3 STANDARDS

2.3.1 SURROGATE SPIKING SOLUTION

Surrogate spiking solution is prepared from aliquots of pure compounds (Wellington Laboratories) that are diluted with dichloromethane to a final concentration of 1.0 µg/mL. The surrogate spiking solution includes 2,4,4'-TriBDE (13C12) and 2,2'3,4,4',6-HexaBDE (13C12). The surrogate spiking 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.

2.3.2 INTERNAL STANDARD SOLUTION

The internal standard solution is made from a solution purchased from a commercial vendor (Wellington Laboratories, Guelph, Ontario, Canada) and diluted with dichloromethane to a final concentration of 1.0 µg/mL. The internal standard solution includes 2,2'4,4'-TetraBDE (13C12). 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 instrument analysis. Internal standards are used to calculate relative response factors and specific analyte concentrations based on retention time.

2.3.3 MATRIX SPIKING SOLUTION

A certified solution containing tri to deca PBDE compounds is purchased from a commercial vendor (Cambridge Isotope Laboratories, Inc. Andover, MA) and diluted with dichloromethane to prepare the matrix spiking solution (Table 1). The matrix spiking solution is diluted to approximately 10 times the method detection limit (MDL) and is added to all matrix spike samples.

2.3.4 CALIBRATION SOLUTION

Calibration solutions are prepared at 5 concentrations ranging from approximately 0.05 to 1 µg/mL (Table 2) by diluting a commercially available certified solution (Cambridge Isotope Laboratories, Inc.) containing the analytes of interest.

Table 1. Polybrominated Diphenyl Ethers Contained in the Matrix-Spiking Solution.

Analyte	CAS	Spiking Solution Concentration (µg/mL)
2,2'4-TriBDE (BDE-17)	NA	1.00
2,4,4'-TriBDE (BDE-28)	41318-75-6	1.00
2,2',4,4'-TetraBDE (BDE-47)	5436-43-1	1.00
2,3'4,4'-TetraBDE (BDE-66)	NA	1.00
2,3',4',6-TetraBDE (BDE-71)	NA	1.00
2,2'3,4,4'-PentaBDE (BDE 85)	182346-21-0	1.00
2,2'4,4',5-PentaBDE (BDE-99)	60348-60-9	1.00
2,2'4,4',6-PentaBDE (BDE-100)	189084-64-8	1.00
2,2'3,4,4'5'-HexaBDE (BDE-138)	NA	1.00
2,2'4,4'5,5'-HexaBDE (BDE-153)	68631-49-2	1.00
2,2'4,4'5,6'-HexaBDE (BDE-154)	NA	1.00
2,2'3,4,4'5',6-HeptaBDE (BDE-183)	NA	1.00

2,3,3'4,4'5,6-HeptaBDE (BDE-190)	68928-80-3		1.00			

Table 2. Polybrominated Diphenyl Ethers 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						
2,2'4,4'-TetraBDE (13C12)	NA	0.1	0.1	0.1	0.1	0.1
Surrogates						
2,4,4'-TriBDE (13C12)	NA	0.05	0.10	0.25	0.50	1.0
2,2'3,4,4',6-HexaBDE (13C12)	NA	0.05	0.10	0.25	0.50	1.0
Analytes						
2,2'4-TriBDE (BDE-17)	NA	0.05	0.10	0.25	0.50	1.0
2,4,4'-TriBDE (BDE-28)	41318-75-6	0.05	0.10	0.25	0.50	1.0
2,2',4,4'-TetraBDE (BDE-47)	5436-43-1	0.05	0.10	0.25	0.50	1.0
2,3'4,4'-TetraBDE (BDE-66)	NA	0.05	0.10	0.25	0.50	1.0
2,3',4',6-TetraBDE (BDE-71)	NA	0.05	0.10	0.25	0.50	1.0
2,2'3,4,4'-PentaBDE (BDE 85)	182346-21-0	0.05	0.10	0.25	0.50	1.0
2,2'4,4',5-PentaBDE (BDE-99)	60348-60-9	0.05	0.10	0.25	0.50	1.0
2,2'4,4',6-PentaBDE (BDE-100)	189084-64-8	0.05	0.10	0.25	0.50	1.0
2,2'3,4,4'5'-HexaBDE (BDE-138)	NA	0.05	0.10	0.25	0.50	1.0
2,2'4,4'5,5'-HexaBDE (BDE-153)	68631-49-2	0.05	0.10	0.25	0.50	1.0
2,2'4,4'5,6'-HexaBDE (BDE-154)	NA	0.05	0.10	0.25	0.50	1.0
2,2'3,4,4'5'6-HeptaBDE (BDE-183)	207122-16-5	0.05	0.10	0.25	0.50	1.0
2,3,3'4,4'5,6-HeptaBDE (BDE-190)	68928-80-3	0.05	0.10	0.25	0.50	1.0

3.0 QUANTITATIVE DETERMINATION OF PBDES BY GC/MS-SIM

3.1 MASS SPECTROMETER TUNING

Prior to calibration, the MS is autotuned to perfluorotributylamine (PFTBA) using 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. A RRF is determined, for each analyte, for each calibration level using the following equation:

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 (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.

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

Where:

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \overline{X})^2}{(n-1)}}$$

x_i = each RRF value used to calculate the mean RRF

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.

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

Where:

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

$\overline{RRF_c}$ = relative response factor from the continuing calibration standard

3.4 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, blank spikes, and matrix spikes. 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/ splitless
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 masses for the PBDEs listed in Table 3.

3.5 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 determined by the analysis of the daily calibration check or PBDE Reference solution.
- 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 3). The reference mass spectrum is obtained from the continuing calibration solution. 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 3.
- Data not meeting the criteria established in this section are appropriately qualified or re-analyzed.

Table 3. Target Analyte Parameters.

Analyte	CAS	Reference to	Ion
Internal Standard and Surrogate			
2,2'4,4'-TetraBDE (13C12) (I-1)	NA	I-1	338.0
2,4,4'-TriBDE (13C12) (S-1)	NA	S-1	418.0

Analyte	CAS	Reference to	**Ion**
BDE 2 (3-MonoBDE)	6876-00-2	I-1, S-1	248.0
BDE 3 (4-MonoBDE)	101-55-3	I-1, S-1	248.0
BDE 4 (2,2'-DiBDE)	NA	I-1, S-1	248.0
BDE 7 (2,4-DiBDE)	NA	I-1, S-1	327.9
BDE 8 (2,4'-DiBDE)	147217-71-8	I-1, S-1	327.9
BDE 10 (2,6-DiBDE)	NA	I-1, S-1	327.9
BDE 11 (3,3'-DiBDE)	6903-63-5	I-1, S-1	327.9
BDE 12 (3,4-DiBDE)	NA	I-1, S-1	327.9
BDE 13 (3,4'-DiBDE)	83694-71-7	I-1, S-1	327.9
BDE 15 (4,4'-DiBDE)	2050-47-7	I-1, S-1	327.9
BDE 17 (2,2',4-TriBDE)	NA	I-1, S-1	405.8
BDE 25 (2,3',4-TriBDE)	NA	I-1, S-1	405.8
BDE 28 (2,4,4'-TriBDE)	41318-75-6	I-1, S-1	405.8
BDE 30 (2,4,6-TriBDE)	NA	I-1, S-1	405.8
BDE 32 (2,4',6-TriBDE)	NA	I-1, S-1	405.8
BDE 33 (2',3,4-TriBDE)	NA	I-1, S-1	405.8
BDE 35 (3,3',4-TriBDE)	NA	I-1, S-1	405.8
BDE 37 (3,4,4'-TriBDE)	NA	I-1, S-1	405.8
Analyte	CAS	Reference to	Ion
BDE 47 (2,2',4,4'-TetraBDE)	5436-43-1	I-1, S-1	485.7
BDE 49/71 (2,2',4,5'-TetraBDE/2,3',4',6-TetraBDE)	NA/NA	I-1, S-1	485.7
BDE 66 (2,3',4,4'-TetraBDE)	NA	I-1, S-1	485.7
BDE 75 (2,4,4',6-TetraBDE)	NA	I-1, S-1	485.7
BDE 77 (3,3',4,4'-TetraBDE)	93703-48-1	I-1, S-1	485.7
BDE 85 (2,2',3,4,4'-PentaBDE)	182346-21-0	I-1, S-1	563.6
BDE 99 (2,2',4,4',5-PentaBDE)	60348-60-9	I-1, S-1	563.6
BDE 100 (2,2',4,4',6-PentaBDE)	189084-64-8	I-1, S-1	563.6
BDE 116 (2,3,4,5,6-PentaBDE)	NA	I-1, S-1	563.6
BDE 118 (2,3',4,4',5-PentaBDE)	NA	I-1, S-1	563.6
BDE 119 (2,3',4,4',6-PentaBDE)	NA	I-1, S-1	563.6
2,2'3,4,4',6-HexaBDE (13C12) (S-2)	NA	S-2	496.0
BDE 126 (3,3',4,4',5-PentaBDE)	NA	I-1, S-2	563.6
BDE 138 (2,2',3,4,4',5'-HexaBDE)	NA	I-1, S-2	643.5
BDE 153 (2,2',4,4',5,5'-HexaBDE)	68631-49-2	I-1, S-2	643.5
BDE 154 (2,2',4,4',5,6'-HexaBDE)	NA	I-1, S-2	643.5
BDE 155 (2,2',4,4',6,6'-HexaBDE)	NA	I-1, S-2	643.5

BDE 166 (2,3,4,4',5,6-HexaBDE)	NA	I-1, S-2	643.5
BDE 181 (2,2',3,4,4',5,6-HeptaBDE)	NA	I-1, S-2	563.6
BDE 183 (2,2',3,4,4',5',6-HeptaBDE)	207122-16-5	I-1, S-2	563.6
BDE 190 (2,3,3',4,4',5,6-HeptaBDE)	68928-80-3	I-1, S-2	563.6

(I-#) = Internal 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 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 target analytes not contained in the initial calibration solution are presumed equal to the response factor of a respective similar PBDE compound.

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

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

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

The concentration 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

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

W = the sample weight (g)

Analyte concentrations are reported as corrected for individual surrogate recoveries. The corrections

for each compound are based on the surrogates referenced in Table 3. Percent surrogate recoveries (SURecovery) for each surrogate are calculated using the following equation:

$$SU_{Recovery} = \frac{C_{ESU}}{C_{SU}} \times 100$$

Where:
CESU = calculated surrogate concentration in the extract
CSU = known concentration of surrogate added to extract

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

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

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, and matrix spike duplicate. 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 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 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%.
- 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) 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.

6) Method Detection Limit
- The method detection limit (MDL) is determined following the procedures outlined in Federal Register (1984), Vol. 49, No. 209: 98-199.

DETERMINATION OF METHYLMERCURY IN TISSUES AND SEDIMENTS

1.0 INTRODUCTION

Determination of the methylmercury (MeHg) concentration in sediments and tissues has been developed in order to quantify the most bioavailable and toxic forms of this element. In addition to its toxic behavior, MeHg is important because it occurs naturally in the environment and is displays a pattern of bioaccumulation up the food chain.
The method described here is based on procedures that extract MeHg from biological tissue and sediment and then determine its concentration by an instrumental method involving purge-and-trap isolation, isothermal gas chromatographic separation of species, and then atomic fluorescence detection.

2.0 APPARATUS AND LABWARE

2.1 Apparatus

2.1.1 Methylmercury analyzer

Methylmercury is analyzed by atomic fluorescence following extraction, trapping, and peak separation using a Brooks-Rand "MERX" Automated Methylmercury Analytical System.

2.2 Labware

The following labware is needed to perform the analytical procedure:
Culture tubes: 15mL, disposable borosilicate glass, combusted at 450°C.
Centrifuge tube racks
Pipets and tips: Eppendorf and Finnpettes in following volumes- 0.1, 1, 5 ml
Glass vials: 42 mL amber VOA vials, certified clean, with Teflon-lined septa caps
Analytical balance: 0.0001 g; for particularly small samples, a 0.00001 g balance is used
Repipette dispenser: Borosilicate glass, filled with reagent water

3.0 Reagents

The procedure requires the following:
Reagent Water: Reagent water contains no analytes above the method detection limit. Reagent water is produced by passing deionized water through a MilliQ water system.
Calibration standard: MeHg Standard, 1 ppm in 0.5% HOAc, 0.2% HCl, SKU 06610, Brooks Rand Instruments, Seattle, WA
Spiking solution: MeHgCl(s) dissolved in reagent water with 0.5% HOAc and 0.2% HCl, diluted to approximately 0.5 ppm MeHg as Hg.
Nitric acid: concentrated, trace metal grade
Antifoam/pH bubber solution: Acetate buffer with antifoam to eliminate or reduce sample foaming, SKU 06120, Brooks Rand Instruments, Seattle, WA
Sodium tetraethylborate (NaTEB): Ethylation reagent kit, SKU 06110, Brooks Rand Instruments, Seattle, WA
Argon: Liquid argon is used as the carrier gas in analyzing MeHg. It is passed through a gold bead trap at the MERX instrument to remove trace Hg.
Nitrogen: High purity nitrogen (compressed) is used as the sample transfer and purge-and-trap gas. It is passed through a gold bead trap at the MERX instrument to remove trace Hg.

4.0 PROCEDURES

4.1 Sample collection, preservation and storage

4.1.1 Samples must be collected in such a manner that speciation changes and cross-

contamination are minimized. Methylmercury is generally not a contaminant in field sampling equipment, but care should be taken that tissues likely to have high concentrations (e.g. fish muscle) do not contaminate those having lower concentrations (e.g. plant tissue).

4.1.2 Samples can be stored in either glass or plastic containers after collection. If glass is used, it should be precleaned or combusted to prevent contamination. One advantage of plastic containers (including heavy duty, zip lock bags) is reduced breakage during shipping.

4.1.3 Samples should be stored frozen at minus 10-20°C in order to eliminate the possibility of speciation changes.

4.2 Extraction of methylmercury from tissue

4.2.1 Samples may be analyzed either wet or after freeze drying. Because freeze drying generally allows for more effective grinding and improved sample homogeneity, the following instruction assumes that samples have been freeze dried and ground to a fine powder.

4.2.2 Label combusted culture tubes with Lab#'s for samples and QA. Obtain tube tare weights.

4.2.3 Weigh approximately 0.03xx g of tissue or 1.0 g of sediment into the culture tubes, recording weights to the nearest 0.0001 g. If sample sizes are such that less than 0.1 g is available, a more sensitive balance should be used in order to obtain at least three significant figures.

4.2.4 Add 0.02 ml (gravimetrically) of a ~0.5 ppm MeHg spike solution (prepared from higher concentration stock daily) to matrix spikes and the laboratory control sample. This results in an approximate spike level of 0.3 ppm for a nominal sample weight of 0.03 g; if tissues are known to be substantially higher or lower in concentration, then a different spike volume should be added that is still consistent with the goal of adding more methylmercury than was initially present in the sample.

4.2.5 Add 4.9 mL reagent water with a Repipet and then 2.1 mL concentrated nitric acid with an adjustable Finnpette; cap and mix immediately; place into centrifuge tube rack with other samples and then into an ice bag.

4.2.6 Place into a forced air oven at 60°C for 8 hours; mix samples after each hour.

4.2.7 Remove from oven and allow samples to cool before analysis. If samples are not to be analyzed immediately, store at 4°C in refrigerator.

4.3 Analysis of methylmercury

4.3.1 Sample and standard preparation

4.3.1.1 Label sufficient 42 mL amber vials for samples, calibration standards, and quality control standards.

4.3.1.2 Fill vials approximately half-way with reagent water

4.3.1.3 Add 0.3 mL sodium acetate buffer/antifoam solution, swirl to mix.

4.3.1.4 Add (gravimetrically) approximately 0.025 mL of solution from "MeHg extract" culture tubes to sample vials

4.3.1.5 Add sufficient 1 ppm MeHg calibration solution to calibration vials and QC checks to provide for calibration and periodic evaluation (one QC check after every 10 samples)

4.3.1.6 Add reagent water to amber vials until they are almost full. Thaw frozen NaTEB and add 0.025 mL to each vial.

4.3.1.7 Immediately fill to "convex" surface each vial with reagent water; cap and analyze within 24 hours.

4.3.2 Operation of MERX analyzer

Follow manufacturer's instructions after ensuring that argon and nitrogen gasses are available and that regulators are adjusted to proper settings.

4.4 Calculation of methylmercury concentration

4.4.1 Methylmercury concentrations are determined from the MERX results, the amount of extract solution added to the amber vial, the volume of extract solution (5 mL), and the amount of

sample extracted (

4.4.4 Methylmercury in tissue is calculated as

MeHg (ppm dry wt, as Hg) = HgMERX ÷ Volextract xf'd x Volextract ÷ Wtspl

5.0 QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

Quality control samples are processed in a manner identical to actual samples, and include reagent blanks, spiked blanks, duplicates, and spiked samples.

5.1 Reagent Blanks: At least one reagent blank is analyzed with each batch of samples. Blank levels should be no more than 2x method detection limit (MDL). If blank levels for any analyte are above the 2x MDL, samples analyzed in that sample set should be reprocessed after the source of contamination is isolated.

5.2 Spiked blanks: At least one reagent blank is analyzed with each batch of samples. Percent recovery of the spike is calculated and used as a measure of accuracy.

5.3 Matrix Spikes: Matrix spikes are used to investigate possible interferences that may result in either signal enhancement or suppression. Samples are spiked with methylmercury at levels higher than expected. Matrix spikes consist of at least 5% of the number of samples analyzed.

An inorganic mercury spike may be included to demonstrate that inorganic mercury species are not extracted along with the methylmercury fraction.

5.4 Duplicate Analysis: Duplicate samples are run with every 20 samples or with every sample set. Duplicates should be ±20% for low-level samples, and ±10% for higher samples. Heterogeneous samples may result in greater variability between duplicates.

5.5 Certified Reference Materials: Certified reference materials (CRM's) samples are run with every 20 samples or with every sample set. Percent recovery of the certified value is calculated and used as a measure of accuracy.

6.0 REPORTING AND PERFORMANCE

6.1 Normal reporting units for methylmercury are ppm (µg/g, dry weight). If requested, and if moisture values are also determined, samples can be reported on a wet weight basis.

6.2 Minimum method performance criteria

Method performance standards are based on reagent blanks, spiked blanks, and spiked samples. Reagent blanks must be less than 2x the MDL. Spiked blanks are considered acceptable when recovery is between 80-120%. Spiked samples are evaluated similarly, but will in general show less precision because they will be susceptible to matrix effects.

6.3 Significant figures

Results are reported to a number of significant figures that matches the number of such figures in the instrument or gravimetric readings.

6.4 Duplicate Analyses

All duplicate analyses are reported. Duplicate analyses are run at least every 20 samples.

7.0 REFERENCES

U.S. EPA. January 2001. Method 1630: Methyl Mercury in Water by Distillation, Aqueous Ethylation, Purge and Trap, and CVAFS. EPA 821-R-01-020.

Hammerschmidt,C.R.; Fitzgerald, W.F. Methylmercury in Mosquitoes Related to Atmospheric Mercury Deposition and Contamination. Environ. Sci. Technol. 2005, 39, 3034-303

METHOD DETECTION LIMITS

Table A-1. Reporting units for organic contaminants 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 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-3. 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-3. 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

METHODS FOR CONTAMINANTS OF EMERGING CONCERN

AXYS Analytical Services Ltd. provided the analytical services for determining CECs in tissue and POCIS. The methods are proprietary and confidential. Hence, in this document, we will refer to the name of the method and provide the point of contact at AXYS to whom all questions can be directed to.

1. MLA-075 R06.01 for Pharmaceuticals and Personal Care Products
2. MLA-035 R07.02 for Multi Residue Pesticides
3. MLA-080 R02.04 for Alkylphenols in tissues
4. MLA-070 R02.03 for Hexabromocyclododecane
5. MLA-037 R05 for Acid Extractable Herbicides

The point of contact at AXYS:

Richard Grace
AXYS Analytical Services Ltd, 2045 Mills Road W.,
Sidney, BC, Canada, V8L 5X2.
Tel. (250) 655-5800, fax (250) 655-5811

Appendix 2: Bivalve Health Methods

CELLULAR BIOMARKERS

LIPID PEROXIDATION ASSAY

FOR ULTROSPEC 4300pro SPECTROPHOTOMETER

Jennifer Hoguet, Peter B. Key and Michael H. Fulton

1.0 OBJECTIVE

To perform the lipid peroxidation assay.

2.0 HEALTH AND SAFETY

Personnel should wear a lab coat and chemical resistant gloves. Personnel should be aware that trichloroacetic acid, thiobarbituric acid, and butylated hydroxytoluene are used in this assay and due caution should be taken.

3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

Any employee who routinely works in the laboratory should be capable of performing this task. Training of new staff should be carried out under supervision of an experienced technical employee familiar with the SOP before the employee can work unsupervised.

4.0 REQUIRED AND RECOMMENDED MATERIALS

This section lists the required supplies and equipment:

spectrophotometer	microcentrifuge tubes
water bath	cuvettes
centrifuge	pipetors
lab coat	ice and ice bucket
chemical resistant gloves	tissue grinder
5-place balance	assay reagents and buffers

5.0 PROCEDURE

5.1 SOLUTIONS NEEDED

1 N Hydrochloric Acid (HCl)
Add 8.2 ml of 12.1 N HCl to 91.8 ml distilled H_2O.
Store at room temperature.

50 mM Potassium Phosphate (K_2PO_4) Buffer
Dissolve 1.7011 g monobasic K_2PO_4 and 2.177 g dibasic K_2PO_4 in 495 ml distilled H_2O.
Adjust pH to 7.0 with 1 N HCl.
Adjust final volume to 500 ml with distilled H_2O.
Filter through a 0.22 µm screen.
Store at 2 to 8° C for up to 2 weeks.

10 mM Malondialdehyde Tetraethylacetal (MDA) (Primary stock)
Combine 24 µl 1,1,3,3-tetraethoxypropane (TEP), 1 ml 1 N HCl, and 90 ml distilled H_2O in a volumetric flask.
Bring volume up to 100 ml with distilled H_2O, and mix well.
Cap and seal with parafilm.
Heat in a 50° C water bath for 60 minutes.
Cool to room temperature.
Make fresh daily.

0.375% Thiobarbituric Acid (TBA) Instructions for 20 samples plus standards
Dissolve 6.0 g of 15% trichloroacetic acid (TCA) and 0.15 g thiobarbituric acid (TBA) in 40 ml 0.25 N HCl (10 ml 1 N HCl to 30 ml distilled H_2O).
Make fresh daily.
Keep at 2 to 8° C.

2% Butylated Hydroxytoluene (BHT) Instructions for 20 samples plus standards
Dissolve 0.04 g BHT in 2ml absolute alcohol.
Seal solution tightly to avoid evaporation before use.
Make fresh daily.
Keep at room temperature.

5.2 SAMPLE PREPARATION

1. Label 2 sets of microcentrifuge tubes for each sample.

2. Weigh tissues in tared plastic cups on 5-place balance and prepare at 250 mg/ml in K_2PO_4 (e.g., if tissue weighs 0.25 g, add 1.0 ml buffer).

3. Homogenize tissues on ice, for two minutes, using a Pro Scientific model Pro 200 motor with a 20 mm x 150 mm stainless steel generator. Transfer homogenates to a glass tube/glass pestle homogenizer and homogenize for an additional minute.

4. While homogenates are still well mixed, transfer up to 1 ml to microcentrifuge tubes. Keep samples cold.

5. Centrifuge samples cold (4° C) at 13,000 x g for 5 minutes.

6. Transfer 100 µl of supernatants to new set of microcentrifuge tubes for assay.

1.3 STANDARD PREPARATION

1. Prepare MDA standards by first making a secondary stock as follows:
Secondary Stock (3200 µM): add 192 µl of Primary Stock (see page 2) to 408 µl K_2PO_4

2. Prepare serial dilutions by adding 300µl of the secondary stock (3200 µM) to 300µl of K_2PO_4 to yield a 1600 µM MDA solution. Mix well, and repeat serial dilution until reaching a 50 µM concentration.

3. Transfer 100 µl of each standard to a set of microcentrifuge tubes for assay.

5.4 LIPID PEROXIDATION ASSAY

1. Turn on the spectrophotometer and allow to warm-up, for at least 15 minutes, while continuing with assay. Set up software protocol according to Appendix 1.

2. Mix the following solutions with 100 µl of each sample or standard (including a blank of 100µl of K_2PO_4) in microcentrifuge tubes:

1400 µl (1.4 ml) TBA
14 µl BHT

3. Vortex, then heat samples and standards in a 100^{o} C water bath for 15 minutes.
Make holes at the top of each microcentrifuge tube with a hypodermic needle to allow for pressure-release while the samples are being heated. If you do not do this, the pressure may cause the tops to pop off the tubes; thereby spilling the samples.

4. Centrifuge samples and standards at 13,000 x g for 5 minutes at room temperature.

5. Transfer supernatant to cuvettes, place cuvettes in spectrophotometer, and read absorbance at 532 nm.

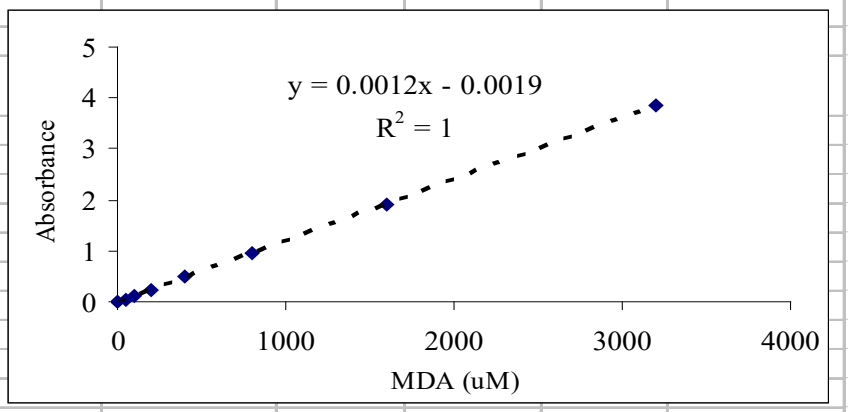
5.5 DATA ACQUISITION

1. Below is an example of a possible spreadsheet format. Data types are denoted by the letters at the top of each column: (A) date samples were collected, (B) sample numbers assigned to each sample, (C) sample type (e.g., organism, tissue type, or standard concentration), (D) absorbance readings for standards and samples, (E) MDA concentrations in samples based on the standard curve, (F) wet weights of samples prior to homogenization, (G) volumes of buffer added to samples, and (H) adjusted MDA concentrations based on data in columns E, F, and G.

A	B	C	D	E	F	G	H
Collected	Sample #	Sample	Abs@532nm	MDA (uM)	Wet Wt. (g)	Volume (ml)	MDA (nmol/g)
n/a	n/a	0	0.0000	0	n/a	n/a	n/a
n/a	n/a	50	0.0500	50	n/a	n/a	n/a
n/a	n/a	100	0.1200	100	n/a	n/a	n/a
n/a	n/a	200	0.2400	200	n/a	n/a	n/a
n/a	n/a	400	0.4800	400	n/a	n/a	n/a
n/a	n/a	800	0.9600	800	n/a	n/a	n/a
n/a	n/a	1600	1.9200	1600	n/a	n/a	n/a
n/a	n/a	3200	3.8400	3200	n/a	n/a	n/a
1/6/2005	1	shrimp	2.9000	2418.25	0.05230	0.209	9663.75
1/6/2005	2	shrimp	3.3325	2778.67	0.06942	0.2780	11127.48
1/6/2005	3	shrimp	3.1259	2606.50	0.07156	0.2860	10417.26

Standard Curve:

MDA (uM)	Abs @ 532nm
0	0.0000
50	0.0500
100	0.1200
200	0.2400
400	0.4800
800	0.9600
1600	1.9200
3200	3.8400



2. Determine the MDA concentrations (both µM and nmol/g wet weight) of samples using the standard curve and the following equations:

MDA (µM): (D – y-intercept of standard curve)/slope of standard curve = E
Example (sample #1): (2.9000 + 0.0019)/0.0012 = 2418.25
Note: in this example, the y-intercept is negative (- 0.0019); therefore, 0.0019 is added to the absorbance reading (2.9000).

MDA (nmol/g wet weight): (((E/1000)*G)/F)*1000 = H
Example (sample #1): (((2418.25/1000)*0.209)/0.05230)*1000 = 9663.75
Note: this equation involves dividing and multiplying by 1000. This is to demonstrate the conversion from µM to nM and from ml to liter (nM = nmol/liter). The calculation can be reduced to ((E*G)/F) = H for simplicity.

5.6 ASSAY CLEANUP

1. After assay is complete, homogenate mixture in the cuvettes and excess reagents should be flushed down the drain with plenty of water.

2. Place cuvettes in the trash.

3. Wash glassware according to its SOP.

6.0 REFERENCES

Ringwood, A.H., J. Hoguet, C.J. Keppler, M.L. Gielazyn, B.P. Ward, and A.R. Rourk. 2003. Cellular biomarkers (lysosomal destabilization, glutathione, lipid peroxidation) in three common estuarine species: A methods handbook. Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET), 49 pp.

USEPA. 1979. Good laboratory practice standards for health effects, Part 772 – Standards for development of test data. Fed. Reg., 44: 27362 – 27378.

APPENDIX 1: Assay Protocol for Ultrospec 4300pro

1. After spectrophotometer has warmed up, click on Programs
   Swift II
   Quantification
   Files of type – Methods
   Open "LPx Assay"

2. Click on parameter icon and verify:
   Parameters
   Wavelength - 532 nm
   Method - Standard Curve
   Filename - (whatever you wish)
   Sample # - 8

   Standards
   Standards (in left column) - 50, 100, 200, 400, 800, 1600, and 3200
   Units - µmol/liter (same as µM)
   Replicates - 1

   Run Options
   Save and Save Spreadsheet

3. Click OK.

4. A blank Graph will pop up.

5. Load Cell Changer window appears. Load Blank and Standards as follows:
   Cell 1 (light blue) - Blank cuvette (reference)
   Cell 2 - 50 µM standard
   Cell 3 - 100 µM standard
   Cell 4 - 200 µM standard
   Cell 5 - 400 µM standard

Cell 6 - 800 µM standard
Cell 7 - 1600 µM standard
Cell 8 - 3200 µM standard

6. Run Standards – Click on Guy with Flag.

7. Run Samples – Click on Guy Running (without flag). Box will appear in which samples are defined. Remove all sample names in left column and click "Define Samples." This will prompt you to specify number of samples (not including blank).

8. Next Load Samples as follows (making sure to use all 8 cells):
Cell1 is the Blank.
Cells 2 - 8 are samples 1 - 7.
*For more than 8 samples:
Cell 1 is sample 8
Cells 2 - 8 are samples 9 - 15.
*For more than 15 samples:
Cells 1 - 8 are samples 16 - 23.

GLUTATHIONE ASSAY

FOR ULTROSPEC 4300pro SPECTROPHOTOMETER

1.0 OBJECTIVE

To perform the glutathione (GSH) assay.

2.0 HEALTH AND SAFETY

Personnel should wear a lab coat and chemical resistant gloves.

3.0 PERSONNEL/TRAINING/RESPONSIBILITIES

Any employee who routinely works in the laboratory should be capable of performing this task. Training of new staff should be carried out under supervision of an experienced technical employee familiar with the SOP before the employee can work unsupervised.

4.0 REQUIRED AND RECOMMENDED MATERIALS

This section lists the required supplies and equipment:

spectrophotometer
centrifuge
lab coat
chemical resistant gloves
5-place balance
cuvettes
microcentrifuge tubes
pipetors
ice and ice bucket
tissue grinder
assay reagents and buffers

5.0 PROCEDURE

5.1 SOLUTIONS NEEDED

5% Sulfosalicylic Acid (SSA)
Dissolve 12.5 g sulfosalicylic acid in 250 ml distilled H_2O.
Store at 2 to 8° C for up to 2 weeks.

143 mM Sodium Phosphate Buffer
Dissolve 4.29 g monobasic sodium phosphate and 0.5988 g tetrasodium EDTA in
250 ml distilled H_2O. Dissolve 5.0765 g dibasic sodium phosphate and 0.5988 g tetrasodium EDTA in 250 ml distilled H_2O.
Mix 248 ml monobasic solution and 248 ml dibasic solution together.
Adjust pH to 7.5.
Adjust final volume to 500 ml with distilled H_2O if necessary.
Store at 2 to 8° C for up to 2 weeks.

10 mM 5,5'-Dithiobis(2-Nitrobenzoic acid) (DTNB)
Dissolve 0.03963 g DTNB in 10 ml sodium phosphate buffer.
Make fresh daily.

0.238 mg/ml NADPH Buffer
Dissolve NADPH in sodium phosphate buffer.
Make fresh daily.
*Determine the volume (ml) of NADPH buffer needed to run samples and divide 0.238 mg by that volume.

50 U/ml Glutathione (GSSG) Reductase
The specific activity and concentration of GSSG reductase may vary between suppliers and batches, so the volumes and dilution factors may also vary. Purified GSSG reductase may be diluted into a working solution of 500 U/ml, to be further diluted when needed, or it may be left undiluted. Either way, store both undiluted and diluted stocks at 2 to 8° C. Working stocks (50 U/ml) should be made fresh daily.

5.2 SAMPLE PREPARATION

1. Weigh tissues in tared plastic cups on 5-place balance and prepare at 100 mg/ml in 5% SSA (e.g., if sample weighs 0.1 g, add 1.0 ml 5% SSA).

2. Homogenize tissues on ice, for two minutes, using a Pro Scientific model Pro 200 motor with a 20 mm x 150 mm stainless steel generator. Transfer homogenates to a glass tube/glass pestle homogenizer and homogenize for an additional minute.

3. While homogenates are still well mixed, transfer up to 1 ml to microcentrifuge tubes. Keep samples cold.

4. Centrifuge samples cold (4° C) at 13,000 x g for 5 minutes.

5. Combine 100 µl supernatants with 100 µl 5% SSA, in microcentrifuge tubes, and store at 2 to 8° C until used. Samples can then be stored for up to 24 hours at 4° C prior to running assay.

5.3 STANDARD PREPARATION

1. Prepare GSH standards (6.25 – 200 µM) by first making a primary stock as follows:
   Primary Stock (1 mM): dissolve 3.073 mg GSH in 10 ml 5% SSA

2. Prepare secondary stock to be used for serial dilutions as follows:
   Secondary Stock (200 µM): add 60 µl of Primary GSH Stock to 240 µl 5% SSA

3. Prepare serial dilutions by adding 150µl of the secondary stock (200 µM) to 150µl of 5% SSA to yield a 100 µM GSH solution. Mix well, and repeat serial dilution until reaching a 6.25 µM concentration.

5.4 GLUTATHIONE ASSAY

1. Turn on the spectrophotometer and allow to warm-up, for at least 15 minutes, while continuing with assay. Set up software protocol according to Appendix 1.
2. Mix the following solutions with samples, standards, and blanks (in microcentrifuge tubes) in the following relative proportions:

700 µl NADPH Buffer
100 µl DTNB
175 µl distilled H_2O
25 µl sample, standard, or 5% SSA for blanks*

Note: A bulk "cocktail" solution of the above reagents can be made and added as a single volume to each sample or standard. Use the estimated number of samples to be run (including the standards and blanks) to determine the volumes of each reagent needed (i.e., NADPH, DTNB, and distilled H_2O). Mix reagents together accordingly, and add 975 µl of the resultant "cocktail" to each sample, standard or blank.

*Two blanks must be made: one to be used to zero the spectrophotometer before readings, and another to be treated as a 0 µM standard (i.e., add GSSG reductase). The standards and sample rates are adjusted from the 0 µM standard rate.

3. Vortex.

4. Transfer 900 µl of cocktail/sample mixtures to 1.5 ml cuvettes.

5. Quickly add 15 µl GSSG reductase to cuvettes, shake, and read absorbance at 405 nm every 15 seconds for at least 90 seconds. In order to determine GSH concentrations, rates of change in GSH activity are needed. If using a spectrophotometer with kinetic software, rates should be provided. If not, slopes for each sample/standard can be manually generated using raw data from the15 second time intervals.

6. Zero spectrophotometer between samples with blank.

5.5 DATA ACQUISITION

1. Below is an example of a possible spreadsheet format. Data types are denoted by the letters at the top of each column: (A) date samples were collected, (B) sample number assigned to each sample, (C) sample type (e.g., organism, tissue type, or standard concentration), (D) absorbance rates "rate 1" (from spectrophotometer) for standards and samples, (E) 0 µM – adjusted rates "rate 2" for standards and samples, (F) GSH concentrations in samples based on the standard curve, (G) wet weights of samples prior to homogenization, (H) volume of buffer added to samples, and (I) adjusted GSH concentrations based on data in columns F, G, and H.

2. Determine the 0 µM – adjusted absorbance rates ("rate 2") for samples and standards by subtracting

"rate 1" of 0 µM standard from "rate 1" of each sample and standard. This must be done before generating a standard curve.

3. Determine the GSH concentrations (both µM and nmol/g wet weight) of samples using the standard curve and the following equations:

GSH (µM): ((E – y-intercept of standard curve)/slope of standard curve)*2 = F
Example (sample #1): ((0.0548 - 0.002)/0.0037)*2 = 28.541
*Note: multiplying by 2 adjusts the 1:1 dilution of sample to 5% SSA during sample preparation. See 5.2 Sample Preparation, step 5.

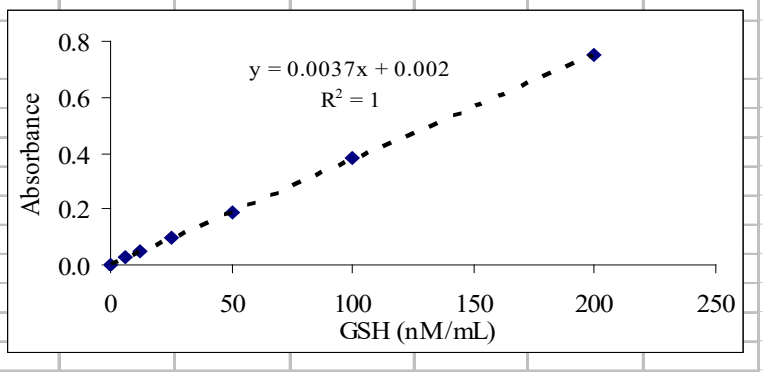
GSH (nmol/g wet weight): (((F/1000)*H)/G)*1000 = I
Example (sample #1): (((28.541/1000)*6.550)/0.6550)*1000 = 285.405

Note: this equation involves dividing and multiplying by 1000. This is to demonstrate the conversion from µM to nM and from ml to liter (nM = nmol/liter). The calculation can be reduced to ((F*H)/G) = I for simplicity.

A	B	C	D	E	F	G	H	I
Collected	Sample #	Sample	Rate 1 @405nm	Rate 2 @405nm	GSH (µM)	Wet Wt. (g)	Volume (ml)	GSH (nmol/g)
n/a	n/a	0	0.0173	0.0000	0	n/a	n/a	n/a
n/a	n/a	6.25	0.0423	0.0250	6.25	n/a	n/a	n/a
n/a	n/a	12.5	0.0673	0.0500	12.5	n/a	n/a	n/a
n/a	n/a	25	0.1123	0.0950	25	n/a	n/a	n/a
n/a	n/a	50	0.2073	0.1900	50	n/a	n/a	n/a
n/a	n/a	100	0.3973	0.3800	100	n/a	n/a	n/a
n/a	n/a	200	0.7673	0.7500	200	n/a	n/a	n/a
1/7/2005	1	shrimp	0.0721	0.0548	28.541	0.6550	6.550	285.405
	2	shrimp	0.0810	0.0637	33.351	0.6290	6.290	333.514
	3	shrimp	0.0800	0.0627	32.811	0.5700	5.700	328.108

Standard Curve:

GSH (µM)	Abs@ 405nm
0	0.0000
6.25	0.0250
12.5	0.0500
25	0.0950
50	0.1900
100	0.3800
200	0.7500



5.6 ASSAY CLEANUP

1. After assay is complete, homogenate mixture in the cuvettes and excess reagents should be flushed down the drain with plenty of water.

2. Place cuvettes in the trash.

3. Wash glassware according to its SOP.

6.0 REFERENCES

Ringwood, A.H., J. Hoguet, C.J. Keppler, M.L. Gielazyn, B.P. Ward, and A.R. Rourk. 2003. Cellular biomarkers (lysosomal destabilization, glutathione, lipid peroxidation) in three common estuarine species: A methods handbook. Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET), 49 pp.

USEPA. 1979. Good laboratory practice standards for health effects, Part 772 – Standards for development of test data. Fed. Reg., 44: 27362 – 27378.

APPENDIX 1: Assay Protocol for Ultrospec 4300pro

1. After spectrophotometer has warmed up, click on Programs
   - Swift II
   - Reaction Kinetics
   - Files of type – Methods
   - Open "GSH Assay"
2. Click on parameter icon and verify:
   - Parameters
     - Wavelength - 405 nm
     - Method - Serial Mode
     - Filename - (whatever you wish)
     - No. of Assays - Number of Samples (default is 20)
     - Set Reference - Check
   - Run Options
     - Post Run
       - Save
       - Save Spreadsheet
     - View
       - Individual Graphs
       - Overlay Graphs
     - Function
       - Auto Scale
       - Auto Slope
3. Click OK.
4. Run Samples - Click on Running Guy.
5. A blank Graph will pop up.

6. “Load Cell Changer” window appears. Load Blank (reference) in Cell 1 (light blue) and run. When finished, run standards and samples (one at a time). This program does not generate a standard curve. Use spreadsheet, such as Excel, to do so after completing assay.

Acetylcholinesterase Assay

Solutions Needed:

20mM Sodium Phosphate Buffer

- Dissolve 1.4196 g Dibasic Sodium Phosphate in 500mL Ultra-Pure deionized (DI) H_2O
- Dissolve 0.240 g Monobasic Sodium Phosphate in 100 mL Ultra-Pure DI H_2O
- Mix 400 mL of Dibasic with 50 mL of Monobasic and adjust pH to 7.6; use the phosphate buffers to adjust pH (dibasic to increase pH, monobasic to reduce pH)
- Split buffer: 250 ml in one bottle, and label one as Buffer #1; put remaining buffer in another bottle and label as Buffer #2
- Add 250 ul of Triton X-100 to Buffer #1
- Store both at 4°C for up to two weeks

PMSF (Phenylmethylsulfonylfluoride)

- Dissolve 0.00174g PMSF (protease inhibitor) in 100 uL absolute ethanol per 10 mLs of Buffer #1(with Triton X-100) needed for the day just before use (estimate 500ul per sample).
  Note: the PMSF must be dissolved in ethanol before adding to buffer; do not add PMSF directly to buffer.

5mM DTNB (5,5'-Dithiobis 2-Nitrobenzoic acid)

- Add 19.8175mg DTNB to 10mL of Buffer #2

25mM ACTC (Acetylcholine Iodide – 99% pure)

- Add 36.1475 mg of ACTC to 5mL of D.I. immediately before use

Sample Preparation:

1. Weigh out tissue samples and homogenize in 2x volume Buffer #1 ($NaPO_4$ buffer with Triton X and PMSF (e.g. if the sample weighs 0.2g, add 0.4mL Buffer #1). Transfer homogenate to micro centrifuge tubes and keep cold (the centrifuge set to 4°C is a good place to store while homogenizing). Note that a minimum volume of 160uL is needed for the assay, so samples should weigh no less than 0.1g, or will still be brought up to 160 ul.
2. Cold centrifuge samples at 10,000g for 20 minutes in the 4°C centrifuge
3. Transfer 40ul of supernatant to second set of micro centrifuge tubes and freeze at -20°C for future protein analysis

AChE Assay

UV/Vis Spectrophotometer / Platereader, 412 nm, 30°C

Step 1: To a 90-well plate, add the following per well:

-180ul 20mM Sodium Phosphate Buffer - from Buffer #2
-40ul Sample or Blank (Buffer #2)

Use 3 replicates per Sample and Blanks

Step 2: Just before placing plate in the spectrophotometer / platereader, add to each well-

-30ul 5mM DTNB

Insert plate and begin read. The protocol is setup to have a 3 minute incubation after the plate reaches the target temperature of 30°C. (Note the total incubation time).

Step 3: After the incubation time, the plate will eject. Add the following to each well:

-30ul 25mM ACTC

Read immediately.

*The assay is a kinetic assay at 412nm. Read interval is 30 seconds for 5 – 7 minutes.

Data Processing for AChE:
Compute the slope of the reaction (time vs absorbance reading) for each subsample, and confirm that the r^2 is > 0.9; use the averaged value of the 3 subsamples for the blank and sample values, and compute a blank corrected value for each.

Bio-Rad Protein Assay

UV Vis Spectrophotometer, 595 nm

Bio-Rad Dye Reagent

- Mix 100ml Bio-Rad dye reagent with 400ml DI H_2O ($1/5^{th}$ dilution)
- Filter through a 0.22um filter
- Store remaining dye for up to 2 weeks at 4°C

Standards

- Prepare standards by making a primary BSA stock solution (A) of 1mg/ml from 10mg/ml frozen BSA stock.
- Serially dilute 1:1 for the following concentrations:
  B) 0.5 mg/ml
  C) 0.25 mg/ml
  D) 0.125 mg/ml
  E) 0.0625 mg/ml
  F) 0.03125 mg/ml

Sample Preparation

1. Using frozen supernatant, dilute 1:1 with Buffer #2 (no Triton)
2. Dilute samples $1/20^{th}$ by adding 380ul of Buffer #2 to 20ul of supernatant from step 1
3. Pipette 20ul of each sample/standard into separate wells in triplicate, then add 180ul of diluted dye reagent to wells using a multi-pipettor. Mix well and incubate at room temperature for a minimum of 5 minutes before reading on the plate reader.
4. Measure absorbance at 595nm within 1 hour of loading plate.

Data Processing for Protein Concentration

Generate a standard curve for the protein standards, concentration vs blank-corrected readings; r^2 should be > 0.9. Determine sample protein concentrations based on blank-corrected values. Note that based on the dilutions above, multiple the concentrations by 40 as a dilution factor (or appropriate dilution factor if different proportions are used).
The results are reported as protein concentration (mg) / ml.

Final Calculations for AChE Activity

$$\text{AChE activity (umol ACTC /min / mg protein)} = \frac{\Delta A_{412} \times Vol_T \times 1000}{1.36 \times 10^4 \times \text{lightpath} \times Vol_S \times [\text{protein}]}$$

ΔA_{412} = change in absorbance (OD) per min, corrected for spontaneous hydrolysis

Vol_T = total assay volume (ml)

1.36×10^4 = extinction coefficient of TNB (M/ cm)

Lightpath = microplate well depth (1 cm)

Vol_S = sample volume (in ml)

[protein] = concentration of protein in the enzymatic extract (mg / ml).

Note: to convert to nmol/ min/ mg protein, multiply by 1000.

Calculation of Expected AChE based on Temperature

Ricciardi et al. reported that baseline levels of AChE in zebra mussels are affected by field or laboratory exposure temperatures (°C). Therefore to calculate the expected AChE levels of zebra mussels based on different temperature regimes, use the following parabolic equation.

$$\text{Expected Baseline AChE activity (nmol / min / mg protein)} = 0.0117T^2 + 0.3946T - 0.5747$$

Francesco Ricciardi, Andrea Binelli, Alfredo Provini. 2006. Use of two biomarkers (CYP450 and acetylcholinesterase) in zebra mussel for the biomonitoring of Lake Maggiore (northern Italy) Ecotoxicology and Environmental Safety 63: 406–412.

DNA DAMAGE ASSAYS

EQUIPMENT and MATERIALS

Instruments and Accessories

GC/MS System

• Autosampler, Agilent 7693. Agilent Technologies, Wilmington, DE
• MassHunter Workstation Software, Version B 03.01 Agilent Technologies, Wilmington, DE
• Gas chromatograph: 7890A GC System. Agilent Technologies, Wilmington, DE
• Mass spectrometer: 7000 GC/MS TripleQuad. Agilent Technologies, Wilmington, DE

GC Gas and Accessories

• Helium, ultra-high purity grade, 99.999%
• Nitrogen, ultra-high purity grade, 99.999%
• Big Universal Trap A Superior Helium Purifier BMSH-2. Agilent Technologies, Wilmington, DE
• Big Universal Trap A Superior Nitrogen Purifier RMSN-2. Agilent Technologies, Wilmington,DE
• Helium regulator, two-stage, Praxair gas connector, model 4143301-000
• Nitrogen regulator, 2 stage nitrogen, inert gas, w/ 2 diaphragms - 2nd stage approx. 3-1/8". dia., internal filtering system.

GC Column and Accessories

• GC column, fused-silica, Agilent Technologies HP-Ultra 2, 12 m x 0.2 mm, coated with cross-linked 5 % phenylmethylsilicone gum phase (film thickness, 0.33 µm), Agilent Technologies, Wilmington, DE
• Inlet Septa, Advanced Green Septa, Agilent Technologies, Wilmington, DE
• Liner, split w/cap, Agilent Technologies, Wilmington, DE filled w/DMCS treated glass wool, Alltech Associates, Inc., Deerfield, IL.
• Column nut, GC inlet, 5181-8830. Agilent Technologies, Wilmington, DE
• Column nut, MS interface, 05988-20066. Agilent Technologies, Wilmington, DE
• Ferrule for GC end of GC column, PN 5080-8853, Agilent Technologies, Wilmington, DE
• Ferrule for MS end of GC column, PN 5062-3508, Agilent Technologies, Wilmington, DE
• Target clear vials, 12 mm x 32 mm, Agilent Technologies, Wilmington, DE
• Glass inserts, 250 µL, deactivated, Agilent Technologies, Wilmington, DE
• Aluminum crimp seal, 11 mm, teflon/silicone/Teflon, Agilent Technologies, Wilmington, DE
• Hand crimper, for use with 11mm crimp seals, Agilent Technologies, Wilmington, DE

Equipment and accessories

• Bullet Blender Storm 24 high-throughput bead-mill homogenizer (Next Advance, Averill Park, NY)
• 1.5 mL Rhino Screw cap tubes, Next Advance, Averill Park, NY,
• 2 mm zirconium oxide beads, Next Advance, Averill Park, NY
• 1,5 mL and 2 mL Eppendorf tubes, DNase free, Eppendorf, Hamburg, Germany.
• Pipettors, 0.5 µL to 10 µL, 2 µL to 20 µL, 20 µL to 200 µL and 100 µL to 1000 µL, pipette tips 10, 200, 1000 µL.
• Centrifuge, Eppendorf size tubes, refrigerated.
• SpeedVac
• Freeze Dryer
• Analytical balance

• UV/Vis Spectrometer
• Tube vortex

Solvents and Reagents

• E.Z.N.A. Mollusc DNA Kit, Omega Bio-tek, Norcross, GA
• Ethanol, 200 proof for molecular biology, Sigma-Aldrich, St. Louis, MO
• Chloroform:Isoamyl alcohol 24:1, Sigma-Aldrich, St. Louis, MO
• 2-Propanol, bioreagent for molecular biology, Sigma-Aldrich, St. Louis, MO
• Acetonitrile CHROMASOLV™ LC-MS Ultra, tested for UHPLC-MS, Morris Plains, NJ
• Water CHROMASOLV™ LC-MS, Morris Plains, NJ
• Ethylenediaminetetraacetic acid (EDTA) solution, pH 8, 0.5 mol/L, BioUltra, for molecular biology, Sigma-Aldrich, St. Louis, MO
• UltrPure 1 mol/L Tris-HCl pH 7.5, Invitrogen, Waltham, MA
• N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane, BSTFA + TMCS, for GC derivatization, contains 1 % TMCS, Sigma-Aldrich, St. Louis, MO
• Sodium acetate, powder, BioReagent, ≥99 %, Sigma-Aldrich, St. Louis, MO
• Sodium phosphate monobasic, BioXtra, ≥99.0 %, Sigma-Aldrich, St. Louis, MO
• Sodium phosphate dibasic, BioXtra, ≥99.0 %, Sigma-Aldrich, St. Louis, MO
• DL-Dithiothreitol, BioUltra, for molecular biology, ≥99.5 %, Sigma-Aldrich, St. Louis, MO
• Potassium chloride, for molecular biology, ≥99.0%, Sigma-Aldrich, St. Louis, MO
• E. coli formamidopyrimidine-DNA glycosylase, Fpg, M0240L, New England BioLabs, Ipswich, MA
• E. coli endonuclease III, Nth, Prospec-Tany Technogene Ltd., Ness-Ziona7414003 Israel
• Trizma® hydrochloride, BioUltra, for molecular biology, ≥99.0 %, Sigma-Aldrich, St. Louis, MO
• Zinc chloride, BioReagent, for molecular biology, Sigma-Aldrich, St. Louis, MO
• Nuclease P1 from Penicillium citrinum, US Biological, Salem, MA
• Phosphodiesterase I from Crotalus adamanteus venom, vial of ≥0.40 units, Purified, Sigma-Aldrich, St. Louis, MO
• Phosphatase, alkaline from calf intestine, Roche Diagnostics, Indianapolis, IN

Modified bases and nucleosides internal standards, labeled with stable isotopes for GC-MS/MS analysis

• SRM 2396 - Oxidative DNA Damage Mass Spectrometry Standards, NIST, Gaithersburg (https://www-s.nist.gov/srmors/view_detail.cfm?srm=2396). Attached pdf of the Certificate
• Standards for modified 2'-deoxynucleosides, i.e., (5′S)-8,5′-cyclo-2′-deoxyguanosine-15N5 (S-cdG-15N5), (5′R)-8,5′-cyclo-2′-deoxyguanosine-15N5 (R-cdG-15N5), (5′S)-8,5′-cyclo-2′-deoxyadenosine-15N5 (S-cdA-15N5) and (5′R)-8,5′-cyclo-2′-deoxyadenosine-15N5 (S-cdA-15N5) were synthesized and isolated as described (Birincioglu M, et al. DNA base damage by the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine). J Am Chem Soc.2003;125(38):11607–11615 and Jaruga P, et al. Mass spectrometric assays for the tandem lesion 8,5′-cyclo-2′-deoxyguanosine in mammalian DNA. Biochemistry. 002;41(11):3703–3711).

DNA PREPARATION and GC-MS/MS ANALYSIS

DNA isolation

Isolated DNA samples are customarily analyzed in batches of 6 to 8 samples. After removal from the Dewar container, mussels (selected ≈ 2 cm long) were thawed on ice, then washed with ice-cold deionized water. Mussel tissues (gills, foot, ≈ 100 mg) separated from shells with a scalpel were

processed according to the product manual of E.Z.N.A. Mollusc DNA Kit* with modification involving homogenization of wet tissue with Bullet Blender Storm 24 high-throughput bead-mill homogenizer. For the final DNA elution, two portions of 100 µL of sterile high-performance liquid chromatography grade water warmed to 70 oC were used. The UV absorbance spectrum of each DNA sample was recorded by absorption spectrophotometry between the wavelengths of 200 nm and 350 nm to ascertain the quality of DNA and to measure the DNA concentration at 260 nm (absorbance value of 1 corresponds to 50 µg/mL). Aliquots (50 µg) of DNA samples were dried in 1.5 mL deoxyribonuclease-free Eppendorf tubes labeled with sample numbers in a SpeedVac under vacuum and then kept at –80 oC for further analysis.

DNA digestion for oxidatively modified bases analysis

After the internal standards from the SRM 2396 set (1 µL each, 10x diluted) are added, the samples are dried in SpeedVac, and then dissolved in 50 µL of an incubation buffer consisting of 50 mmol/L phosphate buffer (pH 7.4), 100 mmol/L KCl, 1 mmol/L EDTA, and 0.1 mmol/L dithiothreitol. Subsequently, they are incubated with 1 µg of E. coli formamidopyrimidine-DNA glycosylase (Fpg) and 1 µg of E. coli endonuclease III (Nth), at 37 °C for 1 h to release the modified bases from DNA. An aliquot of 125 µL of pure ethanol was added to precipitate DNA and to stop the reaction for 1 h in -20 °C. After centrifugation, 14000 g, 30 min. the supernatant fractions are separated to 2 mL target glass vials. After removal of ethanol in the SpeedVac and addition of 200 µL HPLC grade water, samples are frozen in liquid nitrogen and undergo overnight lyophilization in a FreezeDryer.

Derivatization

An aliquot of 60 µl of a mixture of nitrogen-bubbled bis(trimethylsilyl)trifluoroacetic acid [containing trimethylchlorosilane (1%; v/v)] (BSTFA) and pyridine (1:1, v/v) is added to lyophilized supernatant fractions from hydrolyzed DNA. Samples are vortexed and purged individually with ultrahigh-purity nitrogen, tightly sealed under nitrogen with Teflon-coated septa, and then heated at 120°C for 30 min. After cooling, the clear supernatant fractions are removed and placed in target vials with 250 µL glass inserts used for injection of samples onto the GC column. Vials are purged with ultrahigh-purity nitrogen and tightly sealed with Teflon-coated septa.

GC-MS/MS analysis of oxidatively modified bases

For GC-MS/MS measurements, a fused silica capillary column (12.5 m, 0.2 mm i.d.) coated with cross-linked 5 % phenylmethylsilicone gum phase (film thickness, 0.33 µm) is used. Ultrahigh purity helium is used as the carrier gas. The injection port and the GC/MS interface are kept at 250 °C and 280 °C, respectively, with a column head pressure at 65 kPa. The ion source temperature of the mass spectrometer is kept at 230°C. Aliquots of 4 µl of the derivatized samples are injected onto the GC column by means of an automatic sampler, with split mode of injection and split ratio of 10 to 1. The split ratio is adjusted electronically. Analyses by the mass spectrometer are performed in the EI-mode at 70 eV. The oven temperature of the gas chromatograph is programmed from 130 to 280°C at a rate of 8°C/min after 2 min at 130 °C. For identification and quantification, samples were analyzed by GC-MS/MS using multiple reaction monitoring (MRM). The mass transitions used for this purpose were: m/z 331→m/z 316 and m/z 334→m/z 319 for 5-OH-5-MeHyd and 5-OH-5-MeHyd-13C,15N2, respectively; m/z 432→ m/z 417 and 435→ m/z 420 for 5,6-diOH-Ura and 5,6-diOH-Ura-13C,15N2, respectively; m/z 448→ m/z 259 and m/z 452→ m/z 262 for ThyGly and ThyGly-d4, respectively; m/z 369→ m/z 368 and m/z 372→ m/z 371 for FapyAde and FapyAde-13C,15N2, respectively; m/z 457 → m/z 368 and m/z 460 → m/z 371 for FapyGua and FapyGua-13C,15N2, respectively; m/z 455 → m/z 440 and m/z 460 → m/z 445 for 8-OH-Gua and 8-OH-Gua-15N5, respectively. These transitions are based on the known mass spectra of the trimethylsilyl derivatives of modified DNA bases and their

fragmentation patterns.

GC-MS/MS analysis of 8,5′-cyclopurine-2′-deoxynucleosides

Another set of DNA samples (50 µg each) is used for the measurement of 8,5′-cyclopurine-2′-deoxynucleosides, i.e., (5'S)-8,5′-cyclo-2′-deoxyadenosine (S-cdA), (5'R)-8,5′-cyclo-2′-deoxyguanosine (R-cdG) and (5'S)-cyclo-2′-deoxyguanosine (S-cdG). DNA samples are supplemented with the aliquots of the stable isotope-labeled analogs of these compounds. The stable isotope-labeled internal standards for modified 2'-deoxynucleosides, i.e., (5′S)-8,5′-cyclo-2′-deoxyguanosine-15N5 (S-cdG-15N5), (5′R)-8,5′-cyclo-2′-deoxyguanosine-15N5 (R-cdG-15N5), (5′S)-8,5′-cyclo-2′-deoxyadenosine-15N5 (S-cdA-15N5) and (5′R)-8,5′-cyclo-2′-deoxyadenosine-15N5 (S-cdA-15N5) were synthesized and purified at DNA Damage and Repair Laboratory, NIST, Gaithersburg, MD. The samples are dried in SpeedVac and then dissolved in 50 µL of 10 mmol/L Tris-HCl solution (pH 7.5) containing 45 mmol/L ZnCl2, supplemented with 2.5 µL of 1 mol/L sodium acetate (final pH 6.0). Aliquots of nuclease P1 (2 U), snake venom phosphodiesterase (0.004 U) and alkaline phosphatase (16 U) are added and the samples are incubated at 37 °C for 24 h. After hydrolysis, the samples are filtered using ultrafiltration membranes with a molecular mass cutoff of 3 kDa by centrifugation at 12000 g for 30 min. Filtered samples are lyophilized and then trimethylsilylated as described abowe. MRM scans were performed with mass transitions m/z 465 → m/z 309 for S-cdA, m/z 470 → m/z 314 for S-dA-15N5, m/z 553 → m/z 397 for R-cdG and S-cdG, and m/z 558 → m/z 402 for R-cdG-15N5 and S-cdG-15N5. These transitions are based on the known mass spectra of the trimethylsilyl derivatives of 8,5′-cyclopurine-2′-deoxynucleosides and their fragmentation patterns The optimal (maximum) collision energies of the trimethylsilyl derivatives of S-cdA, R-cdG and S-cdG were determined by varying the collision energy between 5 V and 35 V with 5 V increments. The maximum collision energy for each of these compounds was found to be 15 V, and this was used for the measurements.

*Certain commercial equipment or materials are identified here in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

METABOLOMICS- NUCLEAR MAGNETIC RESONANCE

Overview of Project

Metabolomics has been shown to be a unique approach to identify the physiological status of an organism at a given time in its natural environment. Metabolomic assessment is a technique sensitive to many external variables and stressors, including stressors that are incidental to (and possibly unrecognized in) the study being performed. Thus it is very important to harvest organisms with zero added stress and with as many factors under control as possible. This is often difficult in practice, so the development of this project will lay a foundation for optimal data quality and data interpretability.

Goals

1. Develop a robust field collection, preparation, preservation and transport protocol.
2. Develop robust laboratory protocols for sample extraction and NMR-based metabolomics analysis.
3. Explore the spatial and temporal variability of the metabolomic signature of the mussels to understand the optimum sampling scheme for using mussel metabolomics as an *in situ* assessment and monitoring tool.

Assumptions

1. Individual mussels can be collected and analyzed.
2. Whole body metabolome analysis is informative.
3. Whole body wet tissue weights are more than 100 mg.
4. Suitable control material can be developed.
5. A 'sample site' is considered to be a very small area (probably less than 25 cm diameter) where mussels are collected. There may be several 'sample sites' at one 'diving site'.

Preliminary findings

A bag of zebra mussels from the Manistique deployment site was delivered to the laboratory on ice on August 31, 2012. Over the next few days, various forms of sample processing were explored. The delivered samples were apparently rather impacted, so one impression was that there was not very much tissue in most shells. The net wet tissue weight from a sampling of the shells was a little over 100 mg, which seems like enough for extraction of metabolites. The shells were thin and crushable with nominal finger pressure, and a scalpel could be used to open the shells. The shells were washed with gentle rubbing of a gloved hand in a stream of DI-water (removing sand, byssal threads by pulling, some algae coating) and frozen with various techniques (dropping into a -80 °C freezer, dropping into liquid nitrogen, dropping onto a layer of synthetic ice). We found that the intact frozen shells could be easily opened and a whole-body tissue-ice sample excised intact, forgoing the effective stress impact that may be identified from live dissections in the field.

Two of the individuals were extracted using our modified Bligh-Dyer two-phase extraction technique and NMR spectra were collected. The resultant amount of extracted metabolites seemed somewhat low, resulting in noisy spectra. The extraction and data collection parameters can be optimized, eventually, so that the results are satisfactory.

Continual analysis of the NMR spectra for 4 days showed the extracts were stable to chemical change.

Questions:

1. For diving collectors:
    a. How are mussels removed from the substrate? Can the byssal threads be gently cut to release the mussel?
    b. Can you 'mark' a 'sample site' so that it can be revisited (within a few cm) at a later date?
    c. On a given 'dive', how big of an area is sampled?
    d. Is it possible to use a small pump for a stream of site-water to rinse the shells? (This would be to avoid using water at a significantly different temperature or oxygenation level than present at the point of collection.)
2. For a mussel ecologist/biologist:
    a. Is there large mussel biological/phenotypical variability within a dive area? How are the phenotypes distinguished? How big of a dive area has constant phenotypic character based on phenotypical observations?
    b. Are the dive sites characterized by standard indexes, like gonadal index, viability, etc.?
    c. Is it possible to identify zebra mussels from quagga mussels so that we could collect these individually from the same site and look for metabolic biomarkers that might distinguish the species?
    d. Is it known whether cutting or trimming the byssal threads causes any stress on the organisms?

Standard Operating Procedure 001
Collection of Freshwater Mussels Quality Control Material

1.0 OBJECTIVE

To collect a large number of mussels from one dive site to produce a material for quality control purposes. These samples will be processed at the laboratory to produce a composited, homogeneous sample of at least 10 grams tissue wet weight.

2.0 REQUIRED MATERIALS

- No materials required other than appropriate data log sheets.

3.0 PROCEDURE

3.1 Identification of a suitable site

1. The site should have a large number of mussels available that can be collected in one dive period.
2. Samples can be collected from a larger area to facilitate the number of mussels needed, but the extent of the collection area should not exceed 10 m in diameter.
3. Ideally, the site should be considered a non-impacted site so that the samples could represent a 'baseline sample'.
4. If possible, mark the site for potential return in subsequent trips or photo-document the site with appropriate landmarks.

3.2 Collection of Samples

1. Following the sample collection procedure (Standard Operating Procedure 003), place individual groups of mussels into large bags. Place no more than 20 in each bag to facilitate rapid freezing.
2. Collect enough mussels to generate at least 10 grams tissue wet weight.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

- Personnel should follow good laboratory practices in order to ensure that all materials are contaminant free and ready to use.
- Please make note of any deviations during the collection no matter how trivial they seem.

Standard Operating Procedure 002

Collection of Freshwater Mussels for Time Variation Analysis

1.0 OBJECTIVE

To collect mussels from one sampling site during one field trip to gauge the temporal variability of the metabolome at a site.

2.0 REQUIRED MATERIALS

- No materials required other than appropriate data log sheets.

3.0 PROCEDURE

3.1 Identification of a suitable site

1. The site should have a large number of mussels available that can be collected at different times and not exceed 25 cm in diameter.
2. The site should fit into the logistical float plan so that it can be visited at least twice (but more is preferable) during the field trip.
3. Mark the sample site for accurate return to the sample site in subsequent trips or photo-document the site with appropriate landmarks.
4. This site could be identical to the site identified in SOP-001.

3.2 Collection of Samples

1. Following the sample collection procedure (Standard Operating Procedure 003), place individual mussels into small bags. Place only one mussel in each bag (with no added water) to avoid cross contamination or exchange of body fluids.
2. Collect a minimum of 10 individuals, but up to 15 individuals per visit to the site.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

- Personnel should follow good laboratory practices in order to ensure that all materials are contaminant free and ready to use.
- Please make note of any deviations during the collection no matter how trivial they seem.

Standard Operating Procedure 003
Collection of Freshwater Mussels for NMR-based Metabolomic Analysis

1.0 OBJECTIVE

To obtain frozen, intact mussels for metabolomics analysis.

2.0 REQUIRED MATERIALS

- Bucket and/or flowing stream of fresh site water
- Small and large Teflon bags
- Zip ties for closing Teflon bags
- Pre-labeled ID tags
- Heavy-duty Scissors or sharp scalpel
- Cold, charged dry shipper*
- Appropriate data sheets

*NIST will provide a charged dry shipper for flash freezing and storage of metabolomic samples. The shipper will maintain a charge for up to 4 days if stored out of direct sunlight/ heat and open/closed quickly and minimally.

3.0 PROCEDURE

3.1 Collection of Mussels During Dive

1. Identify the group of mussels that represents a 'sample site'.
    a. If this is a 'control site', mark or photo-document the sample site appropriately.
2. Question: Can any 'processing' be done at the sample site by the diver? Such as separating the mussels to individuals? Or must a 'clump' be brought to the surface for processing? Can the byssal be cut under water? How will 'sampling site' collections be distinguished and labeled if all samples are collected during one dive? Separate collection bags?
3. Retrieve the sample(s) and bring to the surface for further processing.

3.2 Field Handling of Samples at the Surface

1. Immediately place harvested mussels in a fresh bucket of site water. It is important to complete the sampling as quickly as possible.
    a. Note on the log sheet the time that the sample(s) are brought to the surface.
    b. After processing, note the time of completed processing.
2. While submerged, separate into individual mussels and gently cut off remaining byssal threads and substrate with scissors (or pull gently to remove?).
3. While submerged or in a stream of site water, gently massage mud/algae/sand from the shell with a gloved hand.
4. All collected mussels must be essentially free of external detritus before freezing.
5. Do not add water to the mussels in the collection bags.

6. Prepare Teflon bag (small for individuals, large for multiple mussels), label ID tags with date and sample location code and place ID tag on a zip tie.
7. Individual samples: place into small Teflon bag, pinching the top closed and tightly secure with an ID tag/zip tie. Grouped samples: place into large Teflon bag, pinching the top closed and tightly secure with an ID tag/zip tie.
8. Immediately place in dry shipper to freeze, taking care to minimize the amount of time the shipper is open.
9. Enter pertinent information on the data sheet.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

- Personnel should follow good laboratory practices in order to ensure that all materials are contaminant free and ready to use.
- Please make note of any deviations during the collection no matter how trivial they seem.

Standard Operating Procedure 004
Collection of Freshwater Mussels for Impact Analysis

1.0 OBJECTIVE

To collect mussels from a series of experimental sites during one field trip to gauge the metabolomic variability.

2.0 REQUIRED MATERIALS

- No materials required other than appropriate data log sheets.

3.0 PROCEDURE

3.1 Identification of suitable sites

1. The dive sites should have a large number of mussels available.
2. The dive sites should represent a hypothesis to be tested and the experimental design should include a 'baseline' site.
3. Mark the sample site for accurate return to the sample site in subsequent trips or photo-document the site with appropriate landmarks.
4. The 'baseline' site could be identical to the site identified in SOP-001.

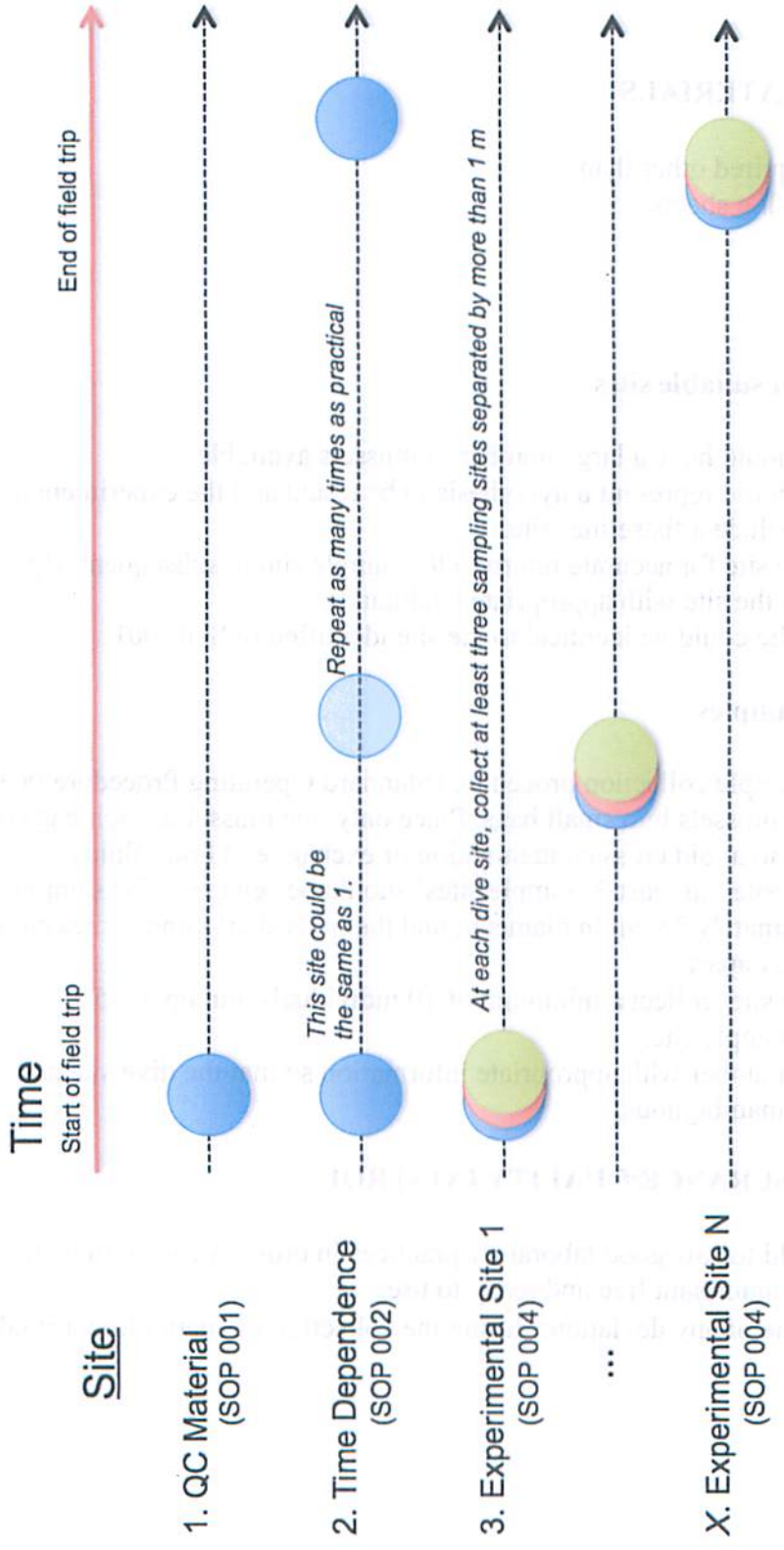
3.2 Collection of Samples

1. Following the sample collection procedure (Standard Operating Procedure 003), place individual mussels into small bags. Place only one mussel in each bag (with no added water) to avoid cross contamination or exchange of body fluids.
2. At a given 'dive site', at least 3 'sample sites' should be selected. The sample sites are approximately 25 cm in diameter, and the individual sample sites should be at least 1 meter apart.
3. For each sample site, collect a minimum of 10 individuals, but up to 15 individuals per sample site.
4. Log the sample number with appropriate information so that the dive site and sample site are unambiguous.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

- Personnel should follow good laboratory practices in order to ensure that all materials are contaminant free and ready to use.
- Please make note of any deviations during the collection no matter how trivial they seem.

Schematic of Experimental Proposal



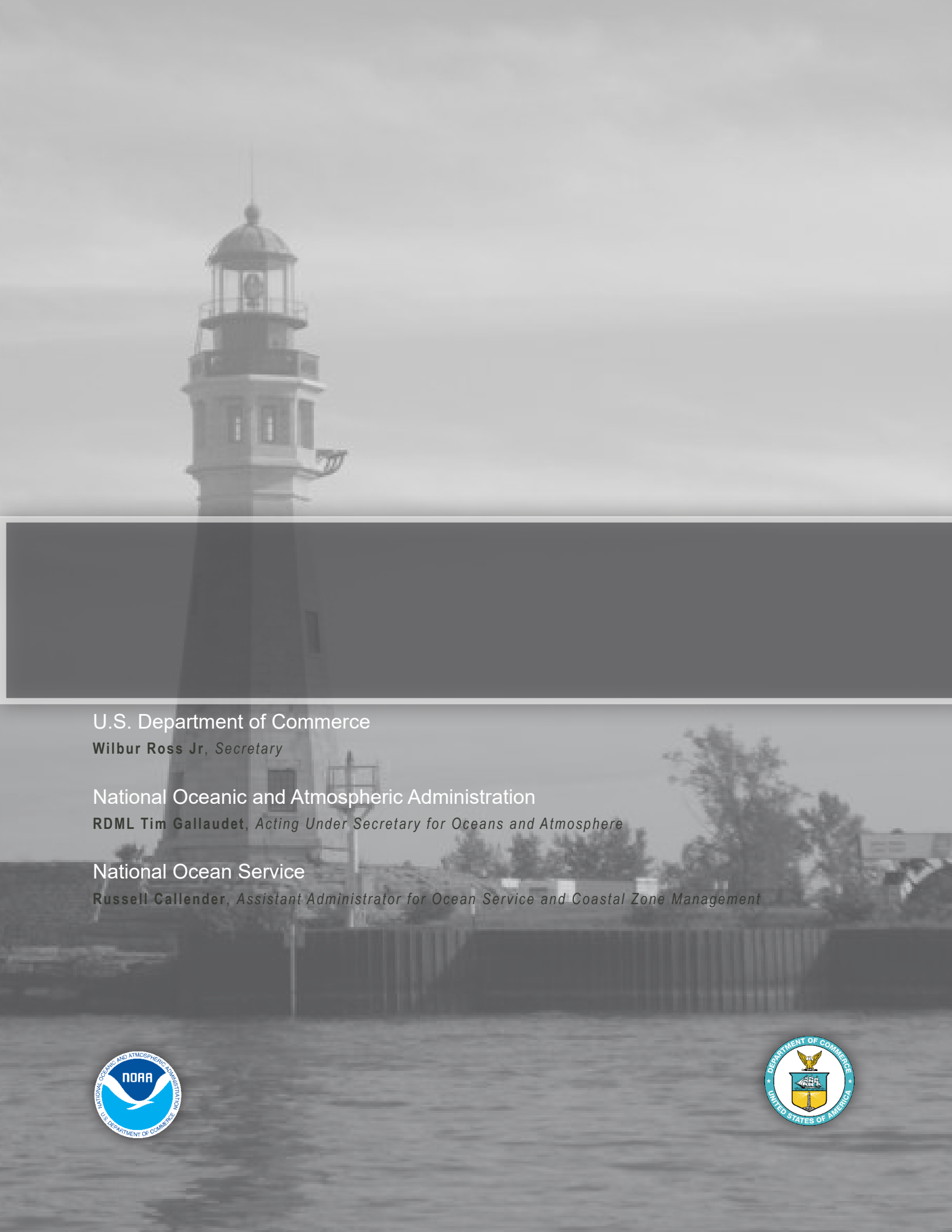
METABOLOMICS- MASS SPECTROMETRY

AXYS Analytical Services Ltd. provided the analytical services for targeted metabolomics on dreissenid mussels and clams. The methods are proprietary and confidential. Both mussels and clams were extracted from their shells, exterior water removed and the whole body homogenized. Extraction with methanol and chloroform used a bead blender and a portion of the extract was subject to further workup and analysis for the six lists of metabolites:

· MABA: Amino acids and Biogenic amines (43 metabolites)
· MFAAHEX: Fatty acids (18 metabolites) and Hexose (1 metabolite)
· MLIP: Phospholipids and Acylcarnitines (144 metabolites),
· MNRG: Metabolites associated with energy pathways (17 metabolites).

The point of contact at AXYS:

Bharat Chandramouli
AXYS Analytical Services Ltd, 2045 Mills Road W.,
Sidney, BC, Canada, V8L 5X2.
Tel. (250) 655-5800, fax (250) 655-5811



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