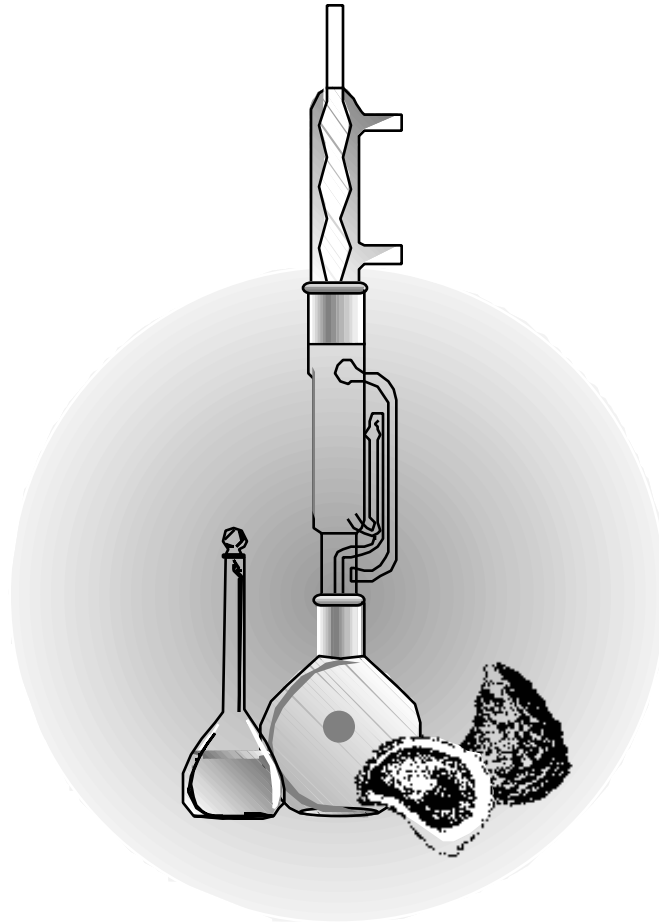


National Status and Trends Program
for Marine Environmental Quality

**Sampling and Analytical Methods of the
National Status and Trends Program
Mussel Watch Project: 1993-1996 Update**



Silver Spring, Maryland
March 1998

US Department of Commerce

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

Coastal Monitoring and Bioeffects Assessment Division
Office of Ocean Resources Conservation and Assessment
National Ocean Service
National Oceanic and Atmospheric Administration
U.S. Department of Commerce
N/ORCA2, SSMC4
1305 East-West Highway
Silver Spring, MD 20910

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**Sampling and Analytical Methods of the
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1993-1996 Update**

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



Silver Spring, Maryland
March 1998

United States
Department of Commerce

National Oceanic and
Atmospheric Administration

National Ocean Service

William M. Daley
Secretary

D. James Baker
Under Secretary

Nancy Foster
Assistant Administrator

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LIST OF ACRONYMS

ACS	American Chemical Society
Alumina	Used as an adsorbent in liquid-solid chromatography made by dehydrating alumina trihydrate
AMU	Atomic Mass Unit
CAS	Chemical Abstract Service
CRM	Certified Reference Material, prepared by the National Research Council of Canada
CRS	Clean-up Recovery Standard
CVAA	Cold vapor atomic absorption spectrometry
DB5	Capillary column, internal coating 5% phenyl silicone and 95% methyl silicone
DBT	Dibutyltin
DDT	1,1,1-Trichloro-2,2-bis[<i>p</i> -chlorophenyl]ethane
ECD	Electron capture detector
EDL	Electrodeless discharge lamp
EPA	U.S. Environmental Protection Agency
FAAS	Flame atomic absorption spectrometry
GC	Gas Chromatography
GERG	Geochemical and Environmental Research Group, Texas A&M University
GFAA	Graphite furnace atomic absorption spectrometry
GPC	Geopermeation chromatography
HCH	1,2,3,4,5,6-Hexachlorocyclohexane. Lindane is gamma-HCH
HCL	Hollow cathode lamp
HEPA	High efficiency particulate attenuator
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma - mass spectrometry
i.d.	Internal diameter
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LBS	Laboratory Spike Sample
LC-FL	Liquid chromatography with fluorescence detection
LCSS	Labeled compound spiking solution
MBT	Monobutyltin
MDL	Method detection limit
ML	Minimum level
MS	Mass spectrometry in discussions of analytical methodology Matrix spike in discussions of Quality Assurance/Quality Control
MSD	Matrix spike duplicate
NIST	National Institute of Standards and Technology
NMFS	National Marine Fisheries Service, NOAA
NOAA	National Oceanic and Atmospheric Administration, Department of Commerce
NOS	National Ocean Service, NOAA
NRC	National Research Council (Canada)
Nsec	Nanosecond
NS&T	National Status and Trends Program
NWFSC	NOAA/NMFS/Northwest Fisheries Science Center
OPR	Ongoing precision and recovery, sample
PAH	Polycyclic aromatic hydrocarbons
PAR	Precision and recovery spiking solution
PCB	Polychlorinated biphenyl
PFTBA	Perfluorotributylamine

PPB	Concentrations of parts per billion, ng/g (Mussel Watch Project data are reported on a dry weight basis)
PPM	Concentrations of parts per million, µg/g (Mussel Watch Project data are reported on a dry weight basis)
PPQ	Concentrations of parts per quadrillion, pg/L (for water samples, not regularly quantified in the Mussel Watch Project)
PPT	Concentrations of parts per trillion, pg/g (used to report low level contaminants such as dioxins and furans on a dry weight basis)
QA	Quality assurance
QC	Quality control
RM	Reference Material
RPD	Relative percent difference
RPM	Revolutions per minute
SICP	Selected ion current profile
SIM	Selected ion monitoring
SOP	Standard Operating Procedure
SRM	Standard reference material, prepared by the National Institute of Standards and Technology
TAMU	Texas A&M University
TERL	Trace Element Research Laboratory, Department of Oceanography, Texas A&M University
TBT	Tributyltin
XRF	X-ray fluorescence spectrometry

Introduction

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

Coastal Monitoring and Bioeffects Assessment Division
Office of Ocean Resources Conservation and Assessment
National Ocean Service
National Oceanic and Atmospheric Administration
Silver Spring, MD

ABSTRACT

Polycyclic aromatic hydrocarbons, butyltins, polychlorinated biphenyls, DDT and metabolites, other chlorinated pesticides, trace and major elements, and a number of measures of contaminant effects are quantified in bivalves and sediments collected as part of the NOAA National Status and Trends (NS&T) Program. This document contains descriptions of some of the sampling and analytical protocols used by NS&T contract laboratories from 1993 through 1996.

1. DISCUSSION

The quantification of environmental contaminants and their effects by the National Oceanic and Atmospheric Administration's National Status and Trends (NS&T) Program began in 1984. Polycyclic aromatic hydrocarbons, butyltins, polychlorinated biphenyls, DDT and metabolites, other chlorinated pesticides, trace and major elements, and a number of measures of contaminant effects are quantified in estuarine and coastal samples. There have been two major monitoring components within this program: the National Benthic Surveillance Project which was responsible for quantification of contamination in fish tissue and sediments, and developing and implementing new methods to define the biological significance of environmental contamination; and the Mussel Watch Project which currently monitors pollutant concentrations by quantifying contaminants in bivalve mollusks and sediments. Methods for sample collection, preparation, and quantification through 1992 were described in NOAA Technical Memorandum NOS ORCA 71 (Vols. 1 - 4). Part of the NS&T Program is the performance-based Quality Assurance Project which allows for the documentation of methodology and laboratory performance through time as analytical procedures change. Methods in the following pages are those used by laboratories that have worked on the NS&T Project since 1992 as well as the methods from the National Institute of Standards and Technology, the group that oversees the quality of organic contaminant analyses. Summaries of the methods used by the National Research Council of Canada, which is responsible for the assurance of quality for trace element analyses, are available in other NOS ORCA technical memoranda.

Table 1 shows the time periods laboratories were responsible for various aspects of the Mussel Watch Project (since 1992), and the authors of the following chemistry chapters. Table 2 lists the major and trace elements, and organic contaminants measured as part of the core Mussel Watch Project.

Not only have analytical methods used within the Mussel Watch Project changed with time but the analytes measured have also changed. The core list of trace elements and PAHs has remained the same but since 1995, the NS&T Project no longer regularly reports PCB 77 and 126. While these two congeners are quantifiable during the analyses for the other 18 PCB congeners measured by the NS&T Project, PCB 77 and PCB 126 are only a small percentage of the co-eluting PCBs with which they are associated. Because planar PCBs are of interest to the NS&T Project, PCBs 77, 126, and 169 have been measured in select samples since 1995. While

the core list of PAHs measured by the NS&T Project has remained the same the value of also measuring alkylated PAHs has become apparent and these compounds have been measured aperiodically since 1993. Beginning in 1995, furans, dioxins and other water soluble contemporary pesticides have also been measured at selected sites.

Detection limits do change as a function of analytical techniques used, detection limits for the year 1993 -1996 for each laboratory participating in the Mussel Watch Project are found in Tables 3 - 15. For information on the analytical evolution of the NS&T Program (both the National Benthic Surveillance and Mussel Watch Projects), early analytical methods, and early detection limits (and how they were derived) see NOAA Technical Memorandum NOS ORCA 71.

Table 1. Laboratories analyzing National Status and Trends Program Mussel Watch Project samples for trace organics and trace elements.

Trace Organic Analyses.....		
Year	1992-1994	1995-1996
East Coast*	Battelle* (Peven-MacCarthy and Uhler)	TAMU/GERG (Wade <i>et al.</i>)
Gulf Coast	TAMU/GERG (Wade <i>et al.</i>)	TAMU/GERG (Wade <i>et al.</i>)
West Coast	Battelle (Peven-MacCarthy and Uhler)	TAMU/GERG (Wade <i>et al.</i>)
Trace Element Analyses.....		
Year	1992-1994	1995-1996
East Coast	Battelle (Crecelius <i>et al.</i>)	TAMU/TERL (Taylor and Presley)
Gulf Coast	TAMU/TERL (Taylor and Presley)	TAMU/TERL (Taylor and Presley)
West Coast	Battelle (Crecelius <i>et al.</i>)	TAMU/TERL (Taylor and Presley)

* East Coast samples include those samples collected in the Great Lakes. Gulf coast sites include those sites collected from Puerto Rico. West Coast sites include sites in Alaska and Hawaii.

• Battelle - Battelle, Duxbury, MA (trace organic analyses) and Sequim, WA (trace element).
 TAMU/GERG - Geochemical and Environmental Research Group of Texas A&M University, College Station, TX.
 TAMU/TERL - Trace Element Research Laboratory, Department of Oceanography, Texas A&M University, College Station, TX.

Table 2. Organic contaminants, major and trace elements and organometallics determined as part of the NS&T Program.

Polycyclic aromatic hydrocarbons

Low molecular weight PAHs
(2- and 3-ring structures)

1-Methylnaphthalene
1-Methylphenanthrene
2-Methylnaphthalene
2,6-Dimethylnaphthalene
1,6,7-Trimethylnaphthalene
Acenaphthene
Acenaphthylene
Anthracene
Biphenyl
Dibenzothiophene
C₁-Dibenzothiophenes
C₂-Dibenzothiophenes
C₃-Dibenzothiophenes
Fluorene
C₁-Fluorenes
C₂-Fluorenes
C₃-Fluorenes

Naphthalene
C₁-Naphthalenes
C₂-Naphthalenes
C₃-Naphthalenes
C₄-Naphthalenes
Phenanthrene
C₁-Phenanthrenes/
Anthracenes
C₂-Phenanthrenes/
Anthracenes
C₃-Phenanthrenes/
Anthracenes
C₄-Phenanthrenes/
Anthracenes

High molecular weight PAHs
(4-, 5-, and 6-rings)

Benz[*a*]anthracene
Benzo[*a*]pyrene
Benzo[*b*]fluoranthene
Benzo[*e*]pyrene
Benzo[*ghi*]perylene
Benzo[*k*]fluoranthene
Chrysene
C₁-Chrysenes
C₂-Chrysenes
C₃-Chrysenes
C₄-Chrysenes
Dibenz[*a,h*]anthracene
Fluoranthene
C₁-Fluoranthenes/Pyrenes
Indeno[1,2,3-*cd*]pyrene
Perylene
Pyrene

Chlorinated pesticides (* - determined since 1995)

2,4'-DDD
4,4'-DDD
2,4'-DDE
4,4'-DDE
2,4'-DDT
4,4'-DDT
Aldrin
Chlorpyrifos *

cis-Chlordane
Dieldrin
Endosulfan-I *
Endosulfan-II *
delta-Hexachlorohexane *
gamma-Chlordane *
gamma-Hexachlorohexane *
Heptachlor

Heptachlor epoxide
Hexachlorobenzene
alpha-Hexachlorohexane
beta-Hexachlorohexane *
Mirex
cis-Nonachlor
trans-Nonachlor
Oxychlordane *

Polychlorinated biphenyl congeners (IUPAC numbering system)

PCB 8, PCB 18, PCB 28, PCB 44, PCB 52, PCB 66, PCB 101, PCB 105, PCB 118, PCB 128,
PCB 138, PCB 153, PCB 170, PCB 180, PCB 187, PCB 195, PCB 206, PCB 209

Planar PCBs (PCB 77, PCB 126, PCB 169)

Table 2. Organic contaminants, and major and trace elements determined as part of the NS&T Program (cont.).

Chlorinated dibenzofurans (determined since 1995)	Chlorinated dioxins (determined since 1995)
2,3,7,8-Tetrachlorodibenzofuran	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
1,2,3,7,8-Pentachlorodibenzofuran	1,2,3,7,8-Pentachlorodibenzo- <i>p</i> -dioxin
2,3,4,7,8-Pentachlorodibenzofuran	1,2,3,4,7,8-Hexachlorodibenzo- <i>p</i> -dioxin
1,2,3,4,7,8-Hexachlorodibenzofuran	1,2,3,6,7,8-Hexachlorodibenzo- <i>p</i> -dioxin
1,2,3,6,7,8-Hexachlorodibenzofuran	1,2,3,7,8,9-Hexachlorodibenzo- <i>p</i> -dioxin
2,3,4,6,7,8-Hexachlorodibenzofuran	1,2,3,4,6,7,8-Heptachlorodibenzo- <i>p</i> -dioxin
1,2,3,7,8,9-Hexachlorodibenzofuran	Octachlorodibenzo- <i>p</i> -dioxin
1,2,3,4,6,7,8-Heptachlorodibenzofuran	
1,2,3,4,7,8,9-Heptachlorodibenzofuran	
Octachlorodibenzofuran	
Major and trace elements	
Al, Si, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Sn, Sb, Ag, Cd, Hg, Tl, Pb	
Organotins	
Monobutyltin ³⁺ , dibutyltin ²⁺ , tributyltin ⁺ , tetrabutyltin	

Only the cation portion of the molecule is quantified because many anions can combine with the tin-containing cation. Tributyltin is the primary biocide; dibutyltin and monobutyltin are metabolites of tributyltin; and tetrabutyltin is an unintended manufacturing by-product.

Table 3. Mussel Watch Project East and West Coasts tissue polycyclic aromatic hydrocarbons, method limits of detection (ng/g dry weight).

Compound	1993 and 1994	Compound	1993 and 1994
Acenaphthene	2.7	2-Methylnaphthalene	2.3
Acenaphthylene	2.1	1-Methylphenanthrene	3.0
Anthracene	2.4	Naphthalene	19
Benz[<i>a</i>]anthracene	1.8	Perylene	6.7
Dibenz[<i>a,h</i>]anthracene	3.8	Phenanthrene	5.5
Benzo[<i>b</i>]fluoranthene	2.1	Pyrene	5.2
Benzo[<i>k</i>]fluoranthene	4.7	1,6,7-Trimethylnaphthalene	1.8
Benzo[<i>ghi</i>]perylene	5.1		
Benzo[<i>a</i>]pyrene	5.0	C ₁ to C ₄ - Naphthalenes	19
Benzo[<i>e</i>]pyrene	4.0	C ₁ to C ₃ - Fluorenes	2.7
Biphenyl	4.8	C ₁ to C ₄ - Phenanthrenes +	
Chrysene	1.7	anthracenes	5.5
2,6-Dimethylnaphthalene	3.6	Dibenzothiophene	2.7
Fluoranthene	5.0	C ₁ to C ₃ - Dibenzothiophenes	2.7
Fluorene	2.7	C ₁ - Fluoranthenes + pyrenes	5.2
Indeno[1,2,3- <i>cd</i>]pyrene	2.8	C ₁ to C ₄ - Chrysenes	1.7
1-Methylnaphthalene	2.6		

Table 4. Mussel Watch Project East and West Coasts tissue pesticides and PCBs, method limits of detection (ng/g dry weight).

Compound	1993	1994	Compound	1993	1994
Aldrin	1.8	1.3	PCB 8	3.9	4.7
<i>cis</i> -Chlordane	1.4	1.8	PCB 18	2.2	1.5
Dieldrin	2.3	2.2	PCB 28	1.8	0.85
Heptachlor	3.3	0.85	PCB 44	1.4	1.3
Heptachlor epoxide	1.6	0.75	PCB 52	1.8	1.1
Hexachlorobenzene	1.0	0.74	PCB 66	3.3	0.67
gamma-HCH	1.8	0.92	PCB 101	1.5	0.72
Mirex	2.1	2.1	PCB 105	2.3	0.37
<i>trans</i> -Nonachlor	2.2	0.97	PCB 118	2.6	0.88
2,4'-DDD	4.1	0.89	PCB 128	1.9	1.3
4,4'-DDD	3.2	0.69	PCB 138	2.1	0.76
2,4'-DDE	1.5	0.94	PCB 153	4.5	1.1
4,4'-DDE	2.6	2.5	PCB 170	2.2	2.2
2,4'-DDT	3.1	0.97	PCB 180	2.5	2.1
4,4'-DDT	3.1	1.3	PCB 187	2.5	0.86
			PCB 195	1.7	1.2
			PCB 206	1.6	1.9
			PCB 209	1.9	3.3

Table 5. Mussel Watch Project East and West Coasts tissue organotin, method limits of detection (ng/g cation, dry weight).

Compound	1993	1994
Monobutyltin (MBT)	12	7.6
Dibutyltin (DBT)	12	7.5
Tributyltin (TBT)	19	7.6
Tetrabutyltin	6.7	7.6

Table 6. Mussel Watch Project Gulf Coast (until 1995 and all coasts thereafter) sediment aromatic hydrocarbons, method limits of detection (ng/g dry weight).

Compound	1993 and 1994	1995 and 1996	Compound	1993 and 1994	1995 and 1996
Acenaphthene	0.6	0.5	Perylene	2.7	0.6
Acenaphthylene	0.5	0.3	Phenanthrene	0.6	0.8
Anthracene	0.5	0.5	Pyrene	0.4	1.1
Benz[<i>a</i>]anthracene	0.4	0.2	1,6,7-Trimethyl= naphthalene	0.7	0.4
Benzo[<i>b</i>]fluoranthene	0.3	1.3	C ₁ - Naphthalenes	2.3	2.6
Benzo[<i>k</i>]fluoranthene	0.3	0.5	C ₂ - Naphthalenes	1.2	1.2
Benzo[<i>ghi</i>]perylene	3.0	1.3	C ₃ to C ₄ - Naphthalenes	1.3	0.9
Benzo[<i>a</i>]pyrene	0.3	0.6	C ₁ to C ₃ - Fluorenes	1.2	1.0
Benzo[<i>e</i>]pyrene	0.2	0.6	C ₁ to C ₄ - Phenanthrenes + anthracenes	1.1	0.5
Biphenyl	0.4	0.3	Dibenzothiophene	0.5	0.3
Chrysene	0.3	0.7	C ₁ to C ₃ - Dibenzo= thiophenes	1.0	0.6
Dibenz[<i>a,h</i>]anthracene	0.4	0.5	C ₁ - Fluoranthenes + pyrenes	0.7	2.1
2,6-Dimethylnaphthalene	0.6	0.6	C ₁ to C ₄ - Chrysenes	0.7	1.4
Fluoranthene	0.3	1.0			
Fluorene	0.6	0.5			
Indeno[1,2,3- <i>cd</i>]pyrene	0.3	0.3			
1-Methylnaphthalene	1.1	1.0			
2-Methylnaphthalene	1.2	1.7			
1-Methylphenanthrene	0.5	0.2			
Naphthalene	1.1	2.2			

Table 7. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) sediment pesticides and PCBs, method limits of detection (ng/g dry weight).

Compound	1993 - 1995	1996	Compound	1993 - 1995	1996
Aldrin	0.04	0.13	PCB 8	0.14	0.12
<i>cis</i> -Chlordane	0.04	0.08	PCB 18	0.07	0.82
Dieldrin	0.42	0.04	PCB 28	0.06	0.09
Heptachlor	0.19	0.05	PCB 44	0.08	0.10
Heptachlor epoxide	0.32	0.04	PCB 52	0.07	0.42
Hexachlorobenzene	0.05	0.07	PCB 66	0.10	0.07
gamma-HCH	0.04	0.08	PCB 101	0.09	0.15
Mirex	0.04	0.11	PCB 105	0.17	0.06
<i>trans</i> -Nonachlor	0.04	0.10	PCB 118	0.32	0.07
			PCB 128	0.22	0.14
2,4'-DDD	0.04	0.18	PCB 138	0.17	0.07
4,4'-DDD	0.11	0.07	PCB 153	0.15	0.08
2,4'-DDE	0.05	0.08	PCB 170	0.12	0.17
4,4'-DDE	0.06	0.06	PCB 180	0.09	0.05
2,4'-DDT	0.33	0.05	PCB 187	0.11	0.08
4,4'-DDT	0.08	0.09	PCB 195	0.11	0.09
			PCB 206	0.13	0.05
			PCB 209	0.22	0.10

Table 8. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) sediment contemporary pesticides, method limits of detection (ng/g dry weight).

Compound	1995*	1996
1,2,4,5-Tetrachlorobenzene	-	0.71
1,2,3,4-Tetrachlorobenzene	-	0.98
Pentachlorobenzene	-	0.11
Pentachloroanisole	-	0.05
Chlorpyrifos	-	-
Dicofol	-	-
alpha-HCH	0.09	0.37
beta-HCH	0.03	0.17
delta-HCH	0.17	0.05
Oxychlorthane	0.18	0.07
gamma-Chlordane	0.05	0.15
cis-Nonachlor	0.03	0.04
Endosulfan I	-	-
Endosulfan II	0.08	0.06

* These additional analytes were first quantified at selected sites on the three major U.S. coasts beginning in 1995.

Table 9. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) tissue aromatic hydrocarbons, method limits of detection (ng/g dry weight).

Compound	1993 and 1994	1995 and 1996	Compound	1993 and 1994	1995 and 1996
Acenaphthene	8.5	12	Perylene	4.8	10.1
Acenaphthylene	7.3	4.8	Phenanthrene	3.5	6.6
Anthracene	2.3	4.5	Pyrene	4.4	5.6
Benz[a]anthracene	5.1	2.5	1,6,7-Trimethyl= naphthalene	12	8.4
Benzo[b]fluoranthene	4.0	8.7	C ₁ - Naphthalenes	20	15
Benzo[k]fluoranthene	4.0	5.4	C ₂ - Naphthalenes	17	21
Benzo[ghi]perylene	3.0	4.1	C ₃ to C ₄ - Naphthalenes	25	17
Benzo[a]pyrene	4.9	4.7	C ₁ to C ₃ - Fluorenes	19	14
Benzo[e]pyrene	2.5	4.3	C ₁ to C ₄ - Phenanthrenes + anthracenes	30	18
Biphenyl	9.2	10	Dibenzothiophene	2.2	8.7
Chrysene	11	5.8	C ₁ to C ₃ - Dibenzo= thiophenes	4.4	17
Dibenz[a,h]anthracene	2.8	6.6	C ₁ 1 - Fluoranthenes + pyrenes	8.7	11
2,6-Dimethylnaphthalene	8.7	10	C ₁ to C ₄ - Chrysenes	22	12
Fluoranthene	4.2	5.8			
Fluorene	9.3	6.8			
Indeno[1,2,3-cd]pyrene	5.6	6.5			
1-Methylnaphthalene	9.0	6.4			
2-Methylnaphthalene	11	8.5			
1-Methylphenanthrene	15	9.1			
Naphthalene	6.2	8.3			

Table 10. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) tissue pesticides and PCBs, method limits of detection (ng/g dry weight).

Compound	1993	1994 and 1995	1996
Aldrin	0.49	0.41	1.1
<i>cis</i> -Chlordane	0.75	0.49	0.39
Dieldrin	0.66	1.0	1.7
Heptachlor	0.52	0.53	0.54
Heptachlor epoxide	0.57	0.09	0.31
Hexachlorobenzene	0.54	0.40	0.75
gamma-HCH	0.33	0.25	0.77
Mirex	0.54	0.28	0.62
<i>trans</i> -Nonachlor	1.9	0.25	0.35
2,4'-DDD	0.64	0.17	0.37
4,4'-DDD	3.7	0.28	0.92
2,4'-DDE	0.30	0.44	0.35
4,4'-DDE	0.76	0.92	1.3
2,4'-DDT	0.47	0.31	0.53
4,4'-DDT	0.38	0.83	1.1
PCB 8	0.84	1.4	0.82
PCB 18	0.52	1.1	1.0
PCB 28	0.35	0.75	0.88
PCB 44	0.24	0.62	0.75
PCB 52	0.92	1.0	1.1
PCB 66	0.39	0.65	0.45
PCB 101	0.51	0.47	0.47
PCB 105	1.1	1.2	0.55
PCB 118	0.47	1.3	0.96
PCB 128	0.40	0.62	3.6
PCB 138	5.9	1.0	1.1
PCB 153	1.6	2.6	1.1
PCB 170	0.36	2.4	1.1
PCB 180	0.36	0.76	0.53
PCB 187	0.71	0.38	0.62
PCB 195	0.89	0.82	0.69
PCB 206	0.59	0.48	0.67
PCB 209	0.59	1.1	0.77

Table 11. Mussel Watch Project tissue contemporary pesticides, method limits of detection (ng/g dry weight).

Compound	1995*	1996
1,2,4,5-Tetrachlorobenzene	-	0.83
1,2,3,4-Tetrachlorobenzene	-	1.7
Pentachlorobenzene	-	0.89
Pentachloroanisole	-	0.63
Chlorpyrifos	-	-
Dicofol	-	-
alpha-HCH	0.09	0.49
beta-HCH	0.03	0.92
delta-HCH	0.17	0.83
Oxychlordane	0.18	0.72
gamma-Chlordane	0.04	0.39
cis-Nonachlor	0.03	0.62
Endosulfan I	-	-
Endosulfan II	0.08	1.1

* These additional analytes were first quantified at selected sites on the three major U.S. coasts beginning in 1995.

Table 12. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) tissue organotin, method limits of detection (ng/g Sn, dry weight).

Compound	1993 - 1994		1995 - 1996	
	Bivalves	Sediments	Bivalves	Sediments
Monobutyltin (MBT)	6.5	1.0	8.8	15
Dibutyltin (DBT)	5.6	1.0	8.6	15
Tributyltin (TBT)	5.5	1.0	12	15
Tetrabutyltin	4.7	1.0	11	15

Table 13. Mussel Watch Project East and West Coasts tissue major and trace elements, method limits of detection ($\mu\text{g/g}$ dry weight).

Elements	1993	1994
Al	18	120
Si	-	300
Cr	0.58	0.41
Mn	6.7	21
Fe	34	920
Ni	0.79	0.74
Cu	2.2	110
Zn	39	1400
As	2.6	1.8
Se	1	3.7
Ag	0.14	0.42
Cd	0.54	0.33
Sn	0.35	0.60
Sb	-	0.090
Hg	0.021	0.076
Tl	-	
Pb	0.059	0.19

Table 14. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) sediment major and trace elements, method limits of detection ($\mu\text{g/g}$ dry weight).

Elements	1993	1994	1995	1996
Al	200	200	360	106
Cr	0.05	0.10	0.50	0.64
Mn	3.5	3.5	7.8	2.5
Fe	11	100	480	290
Ni	0.4	0.40	0.33	0.19
Cu	0.44	0.44	0.47	0.30
Zn	1.5	1.5	11	0.78
As	0.24	0.25	0.10	0.31
Se	0.10	0.10	0.01	0.02
Ag	0.013	0.013	0.01	0.011
Cd	0.018	0.018	0.013	0.003
Sn	0.10	0.10	0.093	0.11
Sb	0.10	0.10	0.08	0.15
Hg	0.006	0.01	0.005	0.005
Tl	---	---	0.20	0.04
Pb	0.15	0.15	0.44	0.35

Table 15. Mussel Watch Project Gulf Coast (through 1994 and all coasts thereafter) tissue inorganic method limits of detection ($\mu\text{g/g}$ dry weight).*

Elements	1993	1994	1995	1996
Al	0.70	120	120	10
Cr	0.15	0.50	0.50	0.40
Mn	---	3.8	3.8	1.7
Fe	8.5	490	100	15
Ni	0.24	0.15	0.15	0.14
Cu	0.18	0.2	0.16	0.6
Zn	1.2	2.3	6.5	5.7
As	0.2	0.41	0.40	0.35
Se	0.15	0.60	0.50	0.41
Ag	0.035	0.10	0.10	0.29
Cd	0.012	0.00	0.003	0.008
Sn	0.12	0.14	0.14	0.28
Sb	-	-	0.08	0.15
Hg	0.016	0.012	0.012	0.015
Tl	-	-	0.20	0.038
Pb	0.10	0.11	0.11	0.10

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ANCILLARY METHODS

Dry Weight Determination of Sediments

S. T. Sweet and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

The percent dry weight determination procedure used for the NOAA National Status and Trends Mussel Watch Project sediment samples is described. Sediment dry weight was obtained by drying the samples to a constant weight.

1. INTRODUCTION

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

2. APPARATUS AND MATERIALS

2.1. Equipment

Balance, analytical, capable of measuring milligram accuracy.
Calibrated weights
Oven, drying capable of maintaining 63 - 65 °C.
Spatula
Vials, scintillation, combusted glass, 20-dram

2.2. Reagents

Methanol (CH₃OH) [67-56-1] (pesticide quality or equivalent).
Dichloromethane (CH₂Cl₂) [75-09-2] (pesticide quality or equivalent).

3. PROCEDURE

Sediments were collected in pre-cleaned and/or pre-combusted glass jars and stored frozen (-20 °C). Sediments were thawed and homogenized using a solvent rinsed spatula. The spatula was triple rinsed, first using methanol to remove traces of moisture and then using dichloromethane to remove organics. The scintillation vials were combusted for 4 hr at 400 °C and stored in a dessicator prior to use. The analytical balance was calibrated with standard weights prior to use. A scintillation vial was removed from the dessicator, labeled, and weighed. Approximately five (5) grams of sample was placed in the vial and the weight recorded. This procedure was repeated for all samples in the sample set. The samples were dried for 24 hr in a drying oven set at 63 - 65 °C. The samples were removed from the oven after the initial 24 hr and allowed to cool to room temperature in a dessicator for at least 30 min. The samples were weighed and the weight recorded. The samples were put back in the oven for at least 2 hr after which they were removed from the oven and allowed to cool for at least 30 min in a dessicator. The sample set was reweighed and the weight recorded. If the difference between the first and the second weighing was less than ± 0.02 g, the dry weight

percent was calculated based on the last weighing. The samples were reheated and reweighed until the difference between weighings was less than ± 0.02 g.

4. CALCULATIONS

4.1. Percent dry weight

$$\text{Percent dry weight} = \frac{[\text{Vial wt.} + \text{Dry sample wt.}] - [\text{Vial wt.}]}{[\text{Vial wt.} + \text{Wet sample wt.}] - [\text{Vial wt.}]} \times 100$$

4.2. Relative Percent Difference between duplicates

$$\text{RDP} = \frac{|\text{First value} - \text{Second value}|}{(\text{First value} + \text{Second value})/2} \times 100$$

5. QUALITY CONTROL

A method blank and a duplicate were processed for every 20 samples or less. The method blank was an empty vial. The sample identified for the matrix spike/matrix spike duplicate sample or the original/duplicate sample for trace organic extraction was used as the percent dry weight duplicate. For the blank, the absolute difference between the vial weight before and after the drying was no more than ± 0.02 g.

The Relative Percent Difference (RPD) was determined for the calculated percent dry weights for the original and duplicate. The RPD should agree within $\pm 25\%$. If the RPD criteria was not met, the samples were reweighed. If the RPDs were still not within specifications, subsamples for percent dry weight were redone.

6. CONCLUSIONS

Sediment samples were normally 40 to 70% dry weight. Dry weight measurements were used to calculate sediment analyte concentrations on a dry weight basis.

Determination of Percent Dry Weight for Tissues

Y. Qian and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

The percent dry weight of a tissue is the weight of solid material (in percent) in the sample compared to the total weight of the sample (dry material plus water). A procedure used to determine the percent dry weight of biological tissue samples is described. An aliquot of 0.5 - 1 g of sample was brought to constant weight at 63 - 65 °C. The difference between the weight of the dried sample and that of the wet sample was used to calculate the percent dry weight of the sample. Quality controls used to ensure the accuracy and precision in percent dry weight determination are also described.

1. INTRODUCTION

The percent dry weight of a tissue is the weight of solid material (in percent) in a sample of biological tissue compared to the total sample weight.

A subsample of tissue was weighed. The subsample was then dried at 63 - 65 °C (approximately 24 hr) to constant weight. The dry subsample was reweighed.

2. APPARATUS AND MATERIALS

2.1. Apparatus

Balance, top loading with an accuracy of 0.001 g, calibrated daily
Convection oven, 63 - 65 °C
Electrobalance, Cahn or equivalent, with an accuracy of 0.0001 mg, set on the 250 mg - 1 µg scale, calibrated daily
Freeze dryer, LabConco 8 or equivalent

2.2. Labware

Aluminum foil, heavy duty
Beakers, 10 mL capacity, borosilicate glass, heated at 103 - 105 °C for 1 hr and cooled to room temperature in a dessicator before use.
Cutting tools (scissors, scalpels, etc.), stainless steel, washed, dried and rinsed with dichloromethane
Forceps, stainless steel, washed, dried and rinsed with dichloromethane
Jars, glass, 1 pint capacity,
Spatulas, stainless steel, washed, dried and rinsed with dichloromethane
Tweezers, stainless steel, anti-magnetic for microbalance

All glassware was washed and solvent rinsed or combusted at 440 °C for 4 hr.

2.3. Solvents and reagents

Dichloromethane, Burdick and Jackson pesticide grade or equivalent, lot tested.

3. PROCEDURE

Combusted beakers were prepared for use by heating at 440 °C for 4 hr and then cooling to room temperature in a dessicator.

It was essential that the dried beakers and dried samples be stored in the dessicator during this procedure to minimize condensation of moisture which would effect the final weight.

A dried beaker was removed from the dessicator using forceps and was immediately placed on the top loading balance and weighed. The sample was mixed until it was homogeneous. A subsample (0.5 - 1 g) of the homogenized tissue was weighed into the beaker. Any unusual sample characteristics (i.e., odor, oil) was noted in the comment section of the dry weight bench sheet.

A method blank was prepared by using an empty beaker. The duplicate samples for this method were the samples designated as matrix spike (MS) and the matrix spike duplicate (MSD) samples for organic extraction. If MS/MSD samples were not being prepared, then the samples designated as original/duplicate samples for organic extraction may serve as the quality control duplicate required for this procedure.

All samples were placed in the a cardboard box lined with aluminum foil using forceps. The box was loosely covered with aluminum foil and placed in a convection oven at 63 - 65 °C for 24 hr.

The box containing the samples was removed from the oven. The samples were placed in a dessicator using forceps. The samples were allowed to cool to room temperature in the dessicator for 30 min.

The dried beakers and dried samples were stored in the dessicator during this procedure to minimize condensation of moisture which would effect the final weight.

The dried sample in the beaker was removed from the dessicator using forceps and immediately placed on the top loading balance and weighed. The samples were reheated to 63 - 65 °C for at least 2 hr, and again cooled in a dessicator for 30 min to room temperature.

The dried sample in the beaker was removed from the dessicator using forceps and immediately placed on the top loading balance and weighed.

If the difference between the first and second weight of the 10-mL beaker with the dried sample was less than ± 0.02 g, the percent dry weight was calculated according to Section 4.1 using the second weight. If the difference was greater than 0.02 g, the heating, cooling and weighing process was continued until the difference in the last two weights was less than ± 0.02 g, and the percent dry weight was calculated based on the last weighing.

The relative percent difference (RPD) for the percent dry weight between MS and MSD or Sample and Duplicate was calculated. The acceptable limits for the RPD was within $\pm 25\%$. If the RPD was not within specifications, the samples were reweighed. If the RPDs were still not within specifications, the percent dry weights were redetermined. For the blank, the acceptable

limit for the absolute difference between the vial weight before and after drying was less than ± 0.02 g.

4. CALCULATIONS

4.1. Percent dry weight

$$\text{Percent dry weight} = \frac{[\text{Vial wt.} + \text{Dry sample wt.}] - [\text{Vial wt.}]}{[\text{Vial wt.} + \text{Wet sample wt.}] - [\text{Vial wt.}]} \times 100$$

4.2. Relative percent difference between duplicates

$$\text{RDP} = \frac{|\text{First value} - \text{Second value}|}{(\text{First value} + \text{Second value})/2} \times 100$$

5. QUALITY CONTROL

All quality control samples were processed in a manner identical to actual samples.

A method blank and a duplicate were processed with every sample set. The method blank was an empty 10-mL beaker. For the blank, the absolute difference between the vial weight before and after drying should be less than ± 0.02 g for the toploading balance. The RPD for the percent dry weights should agree within $\pm 25\%$.

6. CONCLUSIONS

Bivalve tissues are normally about 20% dry weight and 80% water. Dry weight measurements were used to calculate tissue analyte concentrations on a dry weight basis.

Sediment Grain Size Analysis - Gravel, Sand, Silt and Clay

S. T. Sweet, S. Laswell, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Contaminants generally are found in higher concentrations on small-sized particles. Grain size is an important characteristic of sediments that may be correlated with contaminant concentrations. This procedure describes the method used to determine grain size for the NOAA National Status and Trends Program (NS&T).

1. INTRODUCTION

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

In the NOAA NS&T program, attempts are made to collect only fine-grained sediments. Sand (plus any gravel) is determined by wet sieving with a 62.5 μ screen.

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

2. SAMPLE COLLECTION, PRESERVATION AND STORAGE

2.1. Sample collection

Sediment was collected and placed in plastic Ziploc or Whirl-Pak bags, sealed and labeled.

2.2. Sample preservation and storage

Samples were refrigerated. It is recommended that samples not be frozen or freeze-dried as these processes can cause changes in the grain size distribution.

3. APPARATUS AND MATERIALS

3.1. Labware and apparatus

The following labware and equipment is needed to perform the grain size analyses:

Bags, Ziploc, gallon size. Dow, Indianapolis, IN.
Balance, analytical, 0.1 mg accuracy, Fisher Scientific, Pittsburg, PA.
Beakers, 50-mL

Cylinders, graduated, 1-L
Dessicator, Boekel, Philadelphia, PA.
Mason jars, 1 pint, 70610-00518. Kerr Manufacturing Corp., Los Angeles, CA.
Ovens, drying, maintained at 40 - 50 °C and 100 - 130 °C. 1305M. VWR Scientific, Westchester, PA.
Pipette, 20-mL capacity
Rods, stirring, glass.
Shaker table, H4325, Humboldt Mfg. Co., Norridge, IL.
Sieve, 8-in diameter, 63 mm, ASTM-E-11 specification. Scientific Products, McGraw Park, IL.
Sieve, size 10, 2000 mm, -1 phi for gravel, ASTM-E-11 specification. Scientific Products, McGraw Park, IL.
Sieve, size 230, 63 mm, +4 phi for sand, ASTM-E-11 specification. Scientific Products, McGraw Park, IL.
Timer, 1 sec intervals
Whirl-Paks, 18-oz. NASCO, Ft. Atkinson, WI.

Volumetric glassware and analytical balances were calibrated.

3.2. Reagents

Sodium hexametaphosphate solution, 2.5 g/L. $[(\text{NaPO}_3)_6]$ [10124-56-8] Mallinckrodt, Paris, KY.
Distilled water
Hydrogen peroxide (30%) (H_2O_2) [7722-84-1] Mallinckrodt, Paris, KY.

4. PROCEDURE

4.1. Preparation of samples for dry-sieving and pipette analysis

The samples were homogenized by kneading the sample bag by hand. Approximately 15 - 20 g of sample was placed in a large glass jar. This sample size was chosen to minimize the interaction of individual grains with each other during settling and lessening the probability of flocculation; as well as to maximize the amount of material weighed (i.e., with small samples the error in weighing becomes large with respect to the sample weight). The sample was treated with approximately 50 - 100 mL of 30% hydrogen peroxide (volume varies with amount of organic matter present) for 12 hr prior to analyses to oxidize the organic matter present in the sediment. The sample was washed with distilled water to remove soluble salts. Four hundred mL of sodium hexametaphosphate solution (approximately 2.5 g/L) was added to disperse the particles in the sample. The samples were shaken in the shaker table for approximately 24 hr.

4.2. Size analysis of sand/gravel fraction by wet-sieving

A 62.5 μ screen was placed over a 1-L graduated cylinder. The sample containing the dispersed sediment was poured over the screen and washed with dispersant to rinse any remaining fine-grained sediment into the cylinder. This process separated the gravel/sand fraction (on the screen) from the silt/clay fraction (in the cylinder). The coarse fraction was washed into a pre-weighed beaker with distilled water and placed in an oven (100 - 130 °C) to dry for 24 hr. The beaker was removed from the oven and left to cool to room temperature. The beaker was left open for several hours to allow equilibration with the moisture content of the atmosphere. The beaker was weighed to 0.1 mg with an analytical balance. This is the gravel/sand fraction.

4.3. Silt/clay sized material by settling

The volume of liquid in the graduated cylinder containing the silt/clay material was taken to exactly one liter volume with dispersant solution. The cylinder was stirred vigorously and left to stand for one day. When no flocculation was observed, analyses continued. If flocculation was observed, the sample was discarded and the procedure restarted.

The fine fraction subsamples were taken at the specific times given below to produce the 4 and 8 interval values. Two labeled beakers were pre-weighed to 0.1 mg for use with these subsamples.

The cylinder was stirred vigorously starting at the bottom and working up until all the sediment was distributed uniformly throughout the cylinder. At the end of the vigorous stirring, long, smooth strokes the full length of the cylinder (from the bottom until the stirring rod breaks the surface) were used. As soon as the rod emerged for the last time, the timer was started. The pipette was inserted to a depth of 20 cm, and at the end of exactly 20 sec, 20 mL (this is the 4 aliquot) was withdrawn. This was the most important single step, since this is the basis for the determination of total weight of the silt and clay. The suspension was pipetted into a preweighed beaker. The pipette was rinsed with 20 mL of distilled water and the rinse water was added to the same beaker.

After exactly 2 hr and 3 min, a 20-mL aliquot at a depth of 10 cm was withdrawn. This aliquot represents the clay fraction (8). The aliquot of the suspension was pipetted into a different pre-weighed beaker, rinsed with 20 mL of distilled water and added to the beaker. After 2 hr and 3 min all the silt had settled below 10 cm depth leaving only clay.

The beakers were placed in an oven and the suspensions evaporated to dryness for at least 24 hr at 100 - 130 °C. After 24 hr, the beakers were removed from the oven and left to cool to room temperature for approximately 4 hr to equilibrate with the moisture content of the atmosphere. The beakers were weighed to 0.1 mg with an analytical balance, and the weights recorded on a data sheet.

5. CALCULATIONS

The 4 and 8 dry weight aliquots included the weight of the added dispersant. The dispersant weight (2.5 g/L) was multiplied by the fraction of the total solution removed (20 mL/1000 mL) to calculate the weight of dispersant (0.05 g) in the 4 and 8 aliquots. The weight of the dispersant was then subtracted from the 4 and 8 aliquot weight. This total was then multiplied by 50 (1000 mL/20 mL) to yield the sample weight of the silt plus clay fraction (4) or the clay fraction (8). The sample weight of the silt fraction was calculated by subtracting the sample weight of the clay fraction (8) from the sample weight of the silt plus clay fraction (4).

$$\text{Total dry weight} = \text{wt. sand} + \text{wt. silt} + \text{wt. clay}$$

where "wt. sand" is the sum of the sand and gravel fractions (particles greater than 62.5 μ).

6. CONCLUSIONS

Results were reported as percent sand, silt, and clay on a dry weight basis. The minimum method performance standard for the method was detection of 0.5% of each fraction. Duplicate

samples were analyzed with every 20 samples. Results were reported to three significant figures. The grain size analyses is important because there is sometimes a correlation between sediment contaminant concentrations and percent of fine sediments.

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Total Organic and Carbonate Carbon Content of Sediments

S. T. Sweet and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

This chapter describes the analytical method used to determine total organic and carbonate carbon in sediments collected as part of the NOAA National Status and Trends Mussel Watch Project.

1. INTRODUCTION

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

Total carbon compounds in samples were decomposed by pyrolysis in the presence of oxygen and the CO₂ that was formed was quantified by infrared detection. Total organic carbon (TOC) was determined after sample acidification which converted carbonate carbon in samples to carbon dioxide. The CO₂ produced was purged from the acidified sample prior to analysis. Carbonate carbon or total inorganic carbon (TIC) was determined as the difference between total carbon and total organic carbon.

2. APPARATUS AND MATERIALS

2.1 Equipment

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

2.2 Reagents

Accelerator, copper metal, 501-263. LECO Corp., St. Joseph, MI.
Accelerator, iron chip, 501-077. LECO Corp., St. Joseph, MI.
Catalyst Pellets, platinized silica, 501-587. LECO Corp., St. Joseph, MI.
Hydrochloric acid (HCl) [7647-01-0], ACS reagent grade, A144-212. Fisher Scientific, Pittsburgh, PA.
Magnesium perchlorate (anhydrous) [$\text{Mg}(\text{ClO}_4)_2$] [10034-81-8], 501-171. LECO Corp., St. Joseph, MI.
Manganese dioxide (MnO_2) [1313-13-9], 501-060. LECO Corp., St. Joseph, MI.
Molecular sieve 5A Supelco #2-0301. Bellefonte, PA.
Standards, pin and ring carbon, range 0.1 to 1.0% carbon, 501-502, 501-503, 501-504. LECO Corp., St. Joseph, MI.
Water, HPLC grade, 6795-09. Malinkrodt, Paris, KY.

3. Procedure

3.1. LECO system preparation

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

3.2. Total carbon determination

3.2.1. Sample preparation

Between 0.1 to 0.5 ± 0.001 g of oven-dried, finely ground homogenized sediment was weighed on a calibrated balance into a tared, carbon-free combustion crucible. The amount of sample depended on the expected carbon concentration. Between 0.5 and 8.6 mg of carbon were required for a response within the range of the standard curve.

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

3.2.2. Sample analyses

The crucible was placed on the oven pedestal and sealed within the oven combustion tube. The oxygen flow was allowed to stabilize for about 15 sec, the flow rate checked on the rotameter, and adjusted to 800 mL/min. The induction furnace was turned on. The carbon present in the sample began to combust after about 20 sec and the evolved CO_2 was trapped on a 5A 45/60 mesh molecular sieve. The Variac transformer was set at 40% and switched on. After the sieve had heated for one minute, the Hewlett Packard integrator was started. Once the signal returned to baseline, the Variac was shut off, the cooling fan turned on, and the integrator stopped. The peak area for the sample was recorded. The oven was opened and the hot crucible was removed. This procedure was repeated for all samples in each analytical batch.

3.2.3. Standard analyses

Standard LECO pin and ring carbon standards were placed into an empty carbon free combustion crucibles and one scoop of the copper accelerator was added. LECO calibration standards consisted of 1 g steel rings or pins of precisely known carbon concentration. These calibration standards were traceable to NIST standard reference materials. Different LECO carbon standards were chosen to cover the range of 0.1 to 1.0% carbon and at least five different carbon standards were analyzed with each sample set. Standards were analyzed using the same procedure as outlined in Section 3.2.2.

3.3. Total Organic Carbon determination

3.3.1. Sample preparation

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

3.3.2. Sample analyses

These samples were analyzed as described in Section 3.2.2.

3.4. Total carbonate carbon content

Carbonate carbon was determined by difference between total carbon and total organic carbon in a sample.

4. STANDARDIZATION AND CALCULATIONS

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

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

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

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

$$C_s = \frac{M_1 A + M_2 A^2 + b}{wt.}$$

where A was the area of the sample peak, b was the intercept of the second order calibration equation, M_1 and M_2 were the coefficient from the second order calibration equation, C_s was the percent carbon in the sample, and wt was the sample dry weight.

$$\text{Percent carbonate carbon} = \text{Percent total carbon} - \text{Percent TOC}$$

5. QUALITY CONTROL

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

A method blank, consisting of approximately 1.4 g each of copper and iron chip accelerators, was run with every 20 samples or every sample set, whichever was more frequent. Blank levels were less than three times method detection limit (MDL).

Duplicate samples were run for every 20 samples or at least with every sample set. Duplicates agreed within $\pm 20\%$ for low level ($< 1.0\%$ carbon) samples and $\pm 10\%$ for normal/high level (1.0% carbon) samples. Duplicates were less precise for very inhomogeneous samples (i.e., peats, samples containing twigs, grasses, etc.).

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

6. REPORTING AND PERFORMANCE CRITERIA

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

The minimum method performance standard was detection of 0.02% carbon in a sample.

7. CONCLUSIONS

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

Determination of Percent Lipid in Tissue

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

The percent lipid (weight/weight basis) of a tissue is operationally defined as the weight (in percent) of the material extracted by dichloromethane from the tissue sample. The procedure used to determine the percent lipids of biological tissue samples is described. An appropriate amount of sodium sulfate-dried tissue sample was extracted three times with dichloromethane (100 mL each time). An aliquot of 20 mL of the extract was quantitatively removed for lipid determination. This aliquot was further dried with sodium sulfate and brought to a final volume of 1.0 mL in dichloromethane. An aliquot of 100 μ L was taken and evaporated to constant weight. The residual weight of this dried 100 μ L portion was used to calculate the percent lipids of the sample based on the dry weight. Quality control measures used to ensure the accuracy and precision of percent lipids determination are also described.

1. INTRODUCTION

The percent lipid content of a tissue is operationally defined as the weight (in percent) of material extracted from a tissue with dichloromethane.

The tissue sample was extracted with dichloromethane in the presence of anhydrous sodium sulfate. An aliquot of the extract was removed for lipid determination. This aliquot was filtered and concentrated to a known volume. A known volume of the aliquot was taken, evaporated to dryness, weighed, and the percent lipids for the sample was determined on a dry weight basis.

2. APPARATUS AND MATERIALS

2.1. Apparatus

Electrobalance, Cahn Electrobalance or equivalent, with an accuracy of 0.0001 mg, set on the 250 mg - 1 μ g scale
Hot plate, set on low heat
Micropipette, 100 μ L
Rotovaporator, Brinkmann Rotovapor R110, or equivalent
Vortex mixer

2.2. Labware

Aluminum foil, heavy duty
Flat bottom flasks, 125-mL capacity, borosilicate glass
Funnels, powder, 65 cm, borosilicate glass
Glass fiber filters, 11.0 cm, Whatman GF/C or equivalent
Graduated cylinders, 25-mL and 500-mL capacity, borosilicate glass
Syringe, 1-mL volumetric
Tweezers, stainless steel, anti-magnetic
Vials, 7 dram capacity, borosilicate glass, with Teflon-lined, solvent rinsed caps

All glassware was washed and then solvent rinsed or combusted at 440 °C for 4 hr.

2.3. Solvents and reagents

Dichloromethane (CH₂Cl₂) [75-09-2], Burdick and Jackson pesticide grade or equivalent, lot tested.

Sodium sulfate (Na₂SO₄) [7757-82-6], granular, anhydrous, J. T. Baker, ACS reagent grade or equivalent, combusted at 440 °C for 4 hr; stored at 120 °C and cooled to room temperature in a desiccator before use.

3. PROCEDURE

After tissue samples were extracted (see tissue extraction method), the solvent level of the sample extract was marked on the 500-mL flat-bottom flask. The tissue extract was mixed by swirling the 500-mL flat-bottom flask and a 20-mL aliquot of the tissue extract was removed using a 25-mL graduated cylinder for lipid determination. A glass fiber filter containing approximately 10 g of anhydrous sodium sulfate was pre-wetted with dichloromethane and the 20-mL aliquot was filtered into the 125-mL flask. The graduated cylinder was rinsed three times with 3 - 5 mL of dichloromethane and the rinses poured through the filter. The total volume of the sample extract was determined after the sample extract was evaporated and transferred from the 500-mL flat-bottom flask. Each of the 500-mL flasks was filled with tap water to the previously marked line, and the volume of the water measured with a 500-mL graduated cylinder.

A method blank and a duplicate were processed with every sample set. Twenty (20) mL of the extraction method blank was used as the lipid method blank. The sample extracts for the extraction batch matrix spike (MS)/matrix spike duplicate (MSD) was used as the original/duplicate.

The filtered aliquot was evaporated using the Rotoevaporator to near dryness. The residue was quantitatively transferred with dichloromethane to a 7-dram vial. The solvent in the 7 dram vial was evaporated to dryness under a stream of nitrogen. Alternatively, the solvent was allowed to evaporate by loosely capping the vials.

A 1-mL syringe was rinsed three times with dichloromethane. Dichloromethane was added to the lipid vial so that the final volume was 1 mL. The cap was replaced and the sample vial was shaken by a vortex mixer for 10 sec. The vial was allowed to sit for at least 30 min and was mixed using the vortex mixer again. For samples that were very high in lipid, it was necessary to adjust the final volume of 2 mL.

The 100 µL micropipette was calibrated according to the manufacturer's instructions. The micropipette was pre-rinsed at least five times with dichloromethane. The electrobalance was calibrated with the standard 200 mg weight according to the manufacturer's instructions.

Using tweezers, a small piece (approximately 1 cm x 2 cm) of glass fiber filter was placed on a corrugated aluminum foil support on the hot plate for a few minutes. The filter was transferred to the electrobalance weighing pan and the balance tared. The filter was placed back on the hot plate.

A 100-µL aliquot of the lipid extract was removed using a 100 µL and slowly dotted onto the warm filter. The micropipette was rinsed again at least five times with dichloromethane. The 7-dram vial was tightly capped and stored in the freezer at -20 °C.

When the solvent had evaporated from the filter, the filter was placed on the weighing pan of the tared electrobalance using tweezers. When the reading was stable, the weight (in mg) was recorded.

The percent lipids in the extract was calculated on a dry weight basis.

The relative percent difference (RPD) for the percent lipid values for the original and duplicate sample were calculated. The acceptable RPDs for the sample and Duplicate or MS and MSD was required to be within $\pm 25\%$. If the RPD was not within $\pm 25\%$, the original and duplicate sample extracts were reweighed. If the RPDs were still not within specifications, the samples were re-extracted for lipids. The lipid weight (in mg) for the blank was less than 0.005 mg.

4. CALCULATIONS

4.1. Percent lipid

$$\text{Percent lipid} = \frac{TV}{AV} \times \frac{FV}{VW} \times \frac{LW}{SW} \times 100$$

where TV is the total volume of the extract (mL), AV is the volume of the aliquot (mL), FV is the final volume (mL), VW is the volume weighed (mL), LW is the lipid weight (grams), and SW is the sample weight (grams).

4.2. Relative percent difference (RPD) for duplicate analysis

$$\text{RDP} = \frac{|\text{First value} - \text{Second value}|}{(\text{First value} + \text{Second value})/2} \times 100$$

5. QUALITY CONTROL

All quality control samples were processed in a manner identical to the actual samples.

A method blank and a duplicate were processed with every sample set. The lipid method blank was a 20-mL aliquot of the extraction method blank. The extraction batch sample extract designated as the MS/MSD or as original/duplicate was used as the QC duplicate required for this procedure. The RPD for the calculated percent lipid values for the duplicates was determined. The RPD agreed within $\pm 25\%$. The lipid weight (in mg) for the method blank was less than 0.005 mg.

6. CONCLUSIONS

This method operationally defined the percent lipid in tissue by extraction with dichloromethane. Lipid content has been found to be correlated to contaminant concentrations for specific tissues and whole organisms.

TRACE ELEMENT ANALYSES

TERL Trace Element Quantification Techniques

B. J. Taylor and B. J. Presley
Oceanography Department
Texas A&M University
College Station, TX

ABSTRACT

Sample preparation and analysis methods have been developed and refined that allow the accurate and precise determination of major and trace elements in sediment and biological tissue samples. Sample preparation emphasizes homogenization and total digestion steps that minimize contamination. Analysis utilizes atomic absorption and neutron activation techniques, and includes a full suite of quality assurance samples (with an emphasis on certified reference materials) in order to produce reliable data. These methods allow measurement of both background and elevated concentrations within NOAA's Status and Trends Program, permitting subtle temporal and spatial differences to be detected.

1. INTRODUCTION

This chapter documents the analytical procedures used for major and minor element analysis of marine sediments and tissue samples collected from the Gulf of Mexico coast of the United States as part of the Mussel Watch Project of NOAA's National Status and Trends Program. These procedures were used by the Trace Element Research Laboratory (TERL), Department of Oceanography, Texas A&M University to analyze samples collected from 1991 to 1997.

2. EQUIPMENT AND SUPPLIES

2.1. Instrumentation

Perkin-Elmer model Z/3030 atomic absorption spectrophotometer. Perkin-Elmer, Norwalk, CT.
Printer, Okidata Microliner 184
Graphite furnace, Perkin-Elmer HGA-600
Graphite furnace cooling unit:
Constant Temperature Circulator Model FK. Haake, Paramus, NJ.
IC-6 refrigeration unit, Lauda water bath. Curtin-Matheson, Houston, TX.
Autosampler, Perkin-Elmer AS-60
EDL power supply, Perkin-Elmer Model 040-0354

Perkin-Elmer model 4100-ZL atomic absorption spectrophotometer. Perkin-Elmer, Norwalk, CT
Printer, Okidata Microliner 320
Graphite furnace, transversely-heated stabilized temperature platform
Autosampler, Perkin-Elmer AS-70
EDL power supply, Perkin-Elmer System 2

Perkin-Elmer model 3110 atomic absorption spectrophotometer. Perkin-Elmer, Norwalk, CT
Background correction, deuterium arc
Burner (0040-0146) with standard nebulizer, flow spoiler, and single-slot acetylene (0040-0266) and nitrous oxide (0040-0277) burner heads
Digital absorbance readout

Mercury Monitor, Model 1235. Laboratory Data Control Analytical, Riviera Beach, FL.
Wavelength setting, 254 nm
Absorbance cell, 30 cm
Recorder, OmniScribe Model A5101-2. Houston Instruments, Houston, TX.

Ortec pure germanium large volume co-axial detectors, GEM-22170S and 23185-P, with 1.68 KeV and 1332.5 KeV resolution, 22% efficient compared to NaI detector. EG&G Ortec, Oak Ridge, TN.

Nuclear Data model 9900 MCA, implemented on a VAX station II-GPX.

2.2. Supplies

Hollow cathode lamps (HCL). Perkin-Elmer, Norwalk, CT.
Electrodeless discharge lamps (EDL). Perkin-Elmer, Norwalk, CT.
Argon, high-purity (99.999%)
Acetylene, industrial
Nitrous oxide
Graphite tubes, pyrolytically coated, grooved, Perkin-Elmer B010-9322
Graphite tubes, pyrolytically coated, ungrooved, Perkin-Elmer B010-9322
L'vov graphite platforms, pyrolytically coated, Perkin-Elmer B010-9324
Autosampler cups, 2-mL, polystyrene, B2713-2. Baxter Scientific Products, McGaw Park, IL.

2.3. Labware

Balance, 0.01 g, Fisher 200 Ainsworth Jars, large, Teflon
toploader. Fisher Scientific, Pittsburgh, PA. Pipette tips, for Finnpette, Finntip 62. Labsystems
Balance, 0.01 g, Mettler PC2000. Pipette tips, polypropylene for Eppendorf
Balance, analytical, 0.0001 g, Mettler H10. pipettes, 10 - 100-mL Model 22 34190-1
Balls, 1-cm diam., Teflon and 200-1000 mL, 22 35 090-1.
Balls, 3.5-cm diam, Teflon Pipette, Finnpette, adjustable, 1000 - 5000-
Bench, clean, with HEPA filter. Liberty mL 9402020. Curtin-Matheson Scientific,
Industries, East Berlin, CT. Houston, TX.
Bottles, screw-cap bottles, polyethylene, Pipette, transfer, polyethylene.
wide-mouth, 1-oz., Nalgene 2104-0001 Pipettes, Eppendorf, fixed volume: 10-mL,
Digestion vessels, 50-mL, Teflon (PFA), 561- 22350102; 25-mL, 22350307; 50-mL,
R. Saville, Minnetonka, MN. 22350404; 100-mL, 22350501; 200-
Drying oven, 60 °C, NAPCO 332. Curtin- mL, 22350609; 500-mL, 22350706;
Matheson Scientific, Houston, TX. 1000-mL, 22350803.
Drying oven, 130 °C, Thelco Vials, snap-cap vials, polystyrene, 5-, 15-,
Freeze dryer (Virtis 10-100) and vacuum and 40-dram. Baxter Scientific Products,
pump (Welch Duo-Seal 1402B80). Virtis McGaw Park, IL.
Co., Gardner, NY, and Welch, Skokie, IL.

2.4. Reagents

Ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) Ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) [50-81-7], A-7506.
[7722-76-1], Spectropure Grade, P30. Sigma, St. Louis, MO.
Spex, Edison, NJ.

Boric acid (H_3BO_3) [10043-35-3], 10659, Grade 1. Johnson Matthey, West Chester, PA.

Citric acid ($\text{C}_6\text{H}_8\text{O}_7$) [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 [$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$] [13446-18-9], MG60-50. Spex, Edison, NJ.

Nickel oxide (NiO) [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.

Perchloric acid (HClO_4) [7601-90-3], concentrated (70%), Ultrex 4805-01. J. T. Baker, Phillipsburg, NJ.

Stannous chloride, ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) [10025-69-1], 8176. Mallinckrodt, Paris, KY.

Water, redistilled in quartz sub-boiling still.

2.5. Matrix modifiers

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

2.6. Standards

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

Element	Stock number
Ag	7100-16UK
Al	600-16UK
As	800-16UK
Cd	1700-16UK
Cr	2100-16UK
Cu	2300-16UK
Fe	4200-16UK
Hg	4800-16UK
Mn	4600-16UK
Ni	5300-16UK
Pb	4300-16UK
Sb	700-16UK
Se	6700-16UK
Sn	8500-16UK
Zn	9500-16UK

3. SAMPLE TREATMENT

3.1. Oyster and mussel tissue

3.1.1. Bivalve shucking

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). 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 is recorded. When all individuals from a site had been shucked, they were weighed on a top loading balance to measure the total sample wet weight. The pooled samples were placed in a freezer to await further processing.

3.1.2. 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.

3.1.3. 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.

3.1.4. Homogenization of dry aliquot

When samples have been 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.

3.1.5. 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. Three mL of HNO₃ were added and the bombs capped loosely and allowed to stand overnight at room temperature. The bombs were then tightened to 18 foot-lbs and placed in a 130 °C oven for a total of approximately 20 hr. During this time, the bombs were periodically removed from the oven, allowed to cool, and vented to release excess pressure. When digestion was complete, the samples were allowed to cool and 18 mL of quartz distilled water added to each sample. 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) sample was weighed into a 50-mL polypropylene centrifuge tube. Concentrated H₂SO₄ (2.5 mL) and 1.5 mL of concentrated HNO₃ were added and the samples heated in a water bath 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 $\text{K}_2\text{S}_2\text{O}_8$ were added to each tube, and the samples left overnight without heating. Before analysis, 5 mL of 10% (w/w) $\text{NH}_2\text{OH} \cdot \text{HCl}$ were added to reduce excess permanganate and the volume brought to 40 mL with distilled water.

3.1.6. Displacement volume

Bivalve shells were removed from the refrigerator and placed in a displacement cylinder containing distilled water. Water escaping from the cylinder as shells were added was captured in a graduated cylinder. When the water flow ceased the volume of displaced water was recorded. Shells were then removed from the cylinder, placed in plastic bags, and returned to the refrigerator.

3.2. Bottom sediment

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

3.2.1. Homogenization

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

3.2.2. Freeze drying

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

3.2.3. Homogenization of dry aliquot

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

3.2.4. Digestion

Approximately 0.2 g of homogenized, dried sediment were weighed to the nearest 0.0001 g and transferred to tared, acid-washed Teflon bombs. Three mL HNO_3 were added and the bombs tightened to 18 foot-lbs and placed in a 130 °C oven for a total of approximately 12 hr. During this time, the bombs were periodically removed from the oven, allowed to cool, and vented to release excess pressure. After this period, the bombs were removed from the oven and allowed to cool. Two mL of concentrated HF were added and the bombs retightened and returned to the oven for 12 hr. After cooling, 18 mL of 4% boric acid were added and the bombs retightened and returned to the oven for another 12 hr. After the samples were allowed to cool, the content of the bombs were mixed by shaking, and the bombs weighed to 0.01 g to determine the total solution weight. The digest solution was then transferred to labeled 1 oz polyethylene bottles. Solution density was determined by weighing known volumes with calibrated Eppendorf pipettes in order to determine solution volume. At this point, a 20-fold dilution was made for FAAS analysis of Al, Fe, Mn, Si, and Zn. One mL of digest solution was

diluted with 19 mL of an acidified seawater solution containing 2:1:17 proportions of seawater:HNO₃:deionized water.

For analysis of Hg, sediment samples were digested using a modified version of EPA method 245.5. Approximately 0.1 to 1.0 g (dry weight) sample was weighed into a 50-mL polypropylene centrifuge tube. Concentrated H₂SO₄ (2.5 mL) and 1.5 mL of concentrated HNO₃ were added and the samples heated in a water bath at 90 - 95 °C for 30 min. After cooling, 10 mL of distilled water, 10 mL of 5% (w/w) KMnO₄, and 5 mL of 5% (w/w) of K₂S₂O₈ were added to each tube, and the samples again heated in a water bath at 90 - 95 °C for 30 min. Before analysis, 5 mL of 10% (w/w) NH₂OH · HCl were added to reduce excess permanganate and the volume brought to 40 mL with distilled water.

4. CALIBRATION AND ANALYSIS

Calibration standards were prepared by serial dilution of commercially available atomic absorption standards using calibrated micropipettes, new snap-cap vials, a top loading balance, and 2 N HNO₃. Concentrations of working standards were verified by comparison with independent standards traceable to National Institute of Standards and Technology (NIST) Standard Reference Materials.

In all cases, final working standards were prepared in an acid matrix that matches 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.

For graphite furnace atomic absorption analysis, standards were placed in positions 1 - 4 of the autosampler tray, and samples and associated quality control samples in positions 5 - 40. Matrix modifiers were placed in positions 0 and 40 if necessary. Analysis begins with position 1, thus standards were analyzed first. After the samples in positions 16, 28, and 40, the standards were rerun before sample analysis continues. After one tray is finished, another tray was placed on the autosampler and analysis was begun in position 5.

5. 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) as the dependent variable ("y"), and concentration as the dependent variable ("x"). If the concentration range extends 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.

$$\text{Abs} = a + b (\text{conc}_{\text{obs}})$$

$$\text{conc}_{\text{obs}} = \frac{\text{Abs} - a}{b}$$

5.1. Concentration

The Perkin-Elmer Z3030 used in much of this project incorporates a patented curve-fitting routine described here. In this method, the concentration was determined as

$$C = \frac{K_1 A + K_3 A^2}{K_2 A - 1}$$

where C is the concentration, A is the absorbance-sec, and K_1 , K_2 , and K_3 are the coefficients determined by solution of simultaneous equations or by the method of least squares.

In our laboratory, the instrument was allowed to auto-select the appropriate equation to fit the data. In all cases, the standard curve was computed from a blank and three standards that were equally spaced from zero to the maximum concentration. For example, concentrations might be 0, 1, 2, and 3 ppb. If the highest standard was within 15% of the value expected from extrapolation of the lowest standard, a 2-coefficient equation was used. If the highest standard was not within 15% of the value expected from extrapolation of the lowest standard, a 3-coefficient equation was used. Because of the number of standards analyzed, the 2-coefficient equation was calculated via least squares regression and the 3-coefficient equation via solution of simultaneous equations. The instrument had the capability to perform a "reslope" based on a single point. However, our recalibrations always involved complete re-analysis all standards. Comparison of observed values predicted by the Perkin-Elmer curve fitting routine with those calculated independently by least squares showed insignificant differences that were within rounding errors of the printout.

5.2. Dilution factor

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

$$DF = \frac{[(\text{bomb tot.}) - (\text{bomb tare})]}{(\text{spl. wt.}) \times (\text{soln. dens.})}$$

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).

5.3. Concentration

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

$$\text{If } \text{conc}_{\text{obs}} < \text{DL, final concentration} = (\text{DL}) (DF_{\text{instr}}) (DF_{\text{dign}})$$

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

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. CONCLUSIONS

Through a consistent effort to eliminate sample contamination, increase accuracy, and improve precision, sample preparation and analysis methods evolved to a level where reliable data can be generated even at low background levels. Critical points within the overall process include:

- a scrupulous effort to minimize contamination from laboratory dust;
- minimal reuse of materials such as bottles, sample cups, and pipette tips;
- utilization of ultrapure reagents;
- closed digestion systems;
- meticulous attention to detail in both sample preparation and analytical stages; and
- personnel with sufficient experience to make necessary method adjustments.

Generating accurate and precise data on environmental samples requires continuous scrutiny of instrumental operation and data quality, and is not consistent with a “production”, “just run the method” mentality. Unless sample concentrations were “known” beforehand, such as from previous years’ data, the best measures of data quality were certified reference materials and performance in blind intercalibration exercises.

7. INSTRUMENTAL ANALYSIS

Table 1 provides information for which analytical method was used for each sample matrix.

Table 1. Elemental quantification techniques by matrix.

Section	Analyte	Method	Matrix		Section	Analyte	Method	Matrix	
			Tissue	Sediment				Tissue	Sediment
7.1	Mercury	CVAA	X	X	7.14	Selenium	GFAA	X	X
7.2	Aluminum	FAA		X	7.15	Tin	GFAA	X	X
7.3	Copper	FAA	X		7.16	Aluminum	INAA		X
7.4	Iron	FAA	X	X	7.17	Chromium	INAA		X
7.5	Manganese	FAA		X	7.18	Iron	INAA		X
7.6	Zinc	FAA	X	X	7.19	Manganese	INAA		X
7.7	Silver	GFAA	X	X	7.20	Arsenic	INAA	X	
7.8	Arsenic	GFAA	X	X	7.21	Chromium	INAA	X	
7.9	Cadmium	GFAA	X	X	7.22	Iron	INAA	X	
7.10	Chromium	GFAA	X	X	7.23	Selenium	INAA	X	
7.11	Copper	GFAA	X	X	7.24	Silver	INAA	X	
7.12	Nickel	GFAA	X	X	7.25	Zinc	INAA	X	
7.13	Lead	GFAA	X	X					

CVAA - Cold vapor atomic absorption

FAA - Flame atomic absorption

GFAA - Graphite furnace atomic absorption

INAA - Instrumental neutron activation analysis

7.1. Mercury

METHOD: Cold vapor atomic absorption

INSTRUMENTATION: Laboratory Data Control Model 1205 Spectrophotometer with 30-cm path length gas cell.

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 254 nm
Lamp: Low pressure, hot cathode Hg lamp
Peak measurement: Peak height (absorbance)
Range: 0.2 absorbance units
Output: To strip chart recorder, 10 mV full scale

Reaction Conditions:

Sample volume: 1 mL
Reductant: 10% SnCl₂
Reductant volume: 0.1 mL
Reaction vessel: 25-mL Erlenmeyer flask

STANDARDS: 0, 0.5, 1.0, and 2.0 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃ / 0.1 M HCl.

APPROXIMATE SENSITIVITY: Approximately 0.160 Abs for 1 mL of 2 ng/mL solution.

7.2. Aluminum

METHOD: Flame atomic absorption

INSTRUMENTATION: Atomic absorption spectrophotometer Perkin-Elmer 3110

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength:	309.3 nm
Slit width:	0.7 nm
Lamp:	Al HCL, 10 mA
Background correction:	Deuterium arc
Peak measurement:	Peak height (absorbance)
Read mode:	Peak

FLAME SETTINGS:

Fuel:	Acetylene
Oxidant:	Nitrous oxide
Flame:	Oxidizing
Burner:	Single slot, 5.5 cm, parallel

STANDARDS: 0, 10, 30, and 50 ppm prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: Samples and standards are spiked with La (prepared from LaCl₃) to a final concentration of 1000-2000 ppm La to suppress ionization interferences.

APPROXIMATE SENSITIVITY: 50 µg/mL gives approximately 0.200 Abs.

7.3. Copper

METHOD: Flame atomic absorption

INSTRUMENTATION: Atomic absorption spectrophotometer Perkin-Elmer 3110

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength:	324.7 nm
Slit width:	0.7 nm
Lamp:	Cu HCL, 15 mA
Background correction:	deuterium arc
Peak measurement:	Peak height (absorbance)
Read mode:	Peak

FLAME SETTINGS:

Fuel:	Acetylene
Oxidant:	Air
Flame:	Oxidizing
Burner:	Single slot, 10 cm, parallel

STANDARDS: 0, 1, 2.5, and 5 ppm prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

APPROXIMATE SENSITIVITY: 2 µg/mL gives approximately 0.125 Abs.

7.4. Iron

METHOD: Flame atomic absorption

INSTRUMENTATION: Atomic absorption spectrophotometer Perkin-Elmer 3110

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength:	248.3 nm
Slit width:	0.2 nm
Lamp:	Fe HCL, 18 mA
Background correction:	Deuterium arc
Peak measurement:	Peak height (absorbance)
Read mode:	Peak

FLAME SETTINGS:

Fuel:	Acetylene
Oxidant:	Air
Flame:	Oxidizing
Burner:	Single slot, 10 cm, parallel

STANDARDS: 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 ppm prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

APPROXIMATE SENSITIVITY: 3 µg/mL gives approximately 0.100 Abs.

7.5. Manganese

METHOD: Flame atomic absorption

INSTRUMENTATION: Atomic absorption spectrophotometer Perkin-Elmer 3110

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength:	279.5 nm
Slit width:	0.2 nm
Lamp:	Mn HCL, 20 mA
Background correction:	Deuterium arc
Peak measurement:	Peak height (absorbance)
Read mode:	Peak

FLAME SETTINGS:

Fuel:	Acetylene
Oxidant:	Air
Flame:	Oxidizing
Burner:	Single slot, 10 cm, parallel

STANDARDS: 0, 0.4, 0.8, and 1.2 ppm prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

APPROXIMATE SENSITIVITY: 1 µg/mL gives approximately 0.100 Abs.

7.6. Zinc

METHOD: Flame atomic absorption

INSTRUMENTATION: Atomic absorption spectrophotometer Perkin-Elmer 3110

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength:	213.9 nm
Slit width:	0.7 nm
Lamp:	Zn HCL, 15 mA
Background correction:	Deuterium arc
Peak measurement:	Peak height (absorbance)
Read mode:	Peak

FLAME SETTINGS:

Fuel:	Acetylene
Oxidant:	Air
Flame:	Oxidizing
Burner:	Single slot, 10 cm, parallel

STANDARDS: 0, 0.5, 1.0, and 2.0 ppm prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

APPROXIMATE SENSITIVITY: 1 µg/mL gives approximately 0.190 Abs.

7.7. Silver

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 328.1 nm
Slit width: 0.7 nm
Lamp: Ag HCL, 10 mA
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	50	200	
2	900	10	20	200	
3	100	1	5	200	
4	1400	0	5	0	*
5	2500	1	4	300	

AUTOSAMPLER PROGRAM:

Solution: Volume (µL):
Sample 20
Blank 0
Modifier 1 10
Modifier 2 0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 3, 6, and 9 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) 2.5% ammonium phosphate in 1 M HNO₃; prepared from SPEX ammonium dihydrogen orthophosphate
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.130 A-sec for 20 µL of 3 ng/mL.

7.8. Arsenic

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer 4100-ZL
Graphite furnace: Perkin-Elmer THGA
Autosampler: Perkin-Elmer AS 70
Printer: Okidata Microline 320

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelengths: 193.7 nm
197.0 nm (samples with high Al)
Slit width: 0.7 nm
Lamp: As EDL, 380 W
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	120	1	30	250	
2	150	5	60	250	
3	800	10	20	250	
4	2200	0	5	0	*
5	2600	1	4	250	

AUTOSAMPLER PROGRAM:

Solution: Volume (µL):
Sample 20
Blank 0
Modifier 1 10
Modifier 2 0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 20, 40, and 60 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS (1) 1:1:1 citric acid (2%): Pd (1000 ppm): Ni (4000 ppm)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.100 A-sec for 20 µL of 50 ng/mL at 193.7 nm; and 0.050 A-sec at 197.0 nm.

7.9. Cadmium

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 228.8 nm
Slit width: 0.7 nm
Lamp: Cd EDL, 5 W
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	35	300	
2	350	10	20	300	
3	100	1	5	300	
4	1300	0	5	0	*
5	2300	1	4	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	10
Blank	0
Modifier 1	5
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 1, 2.5, and 4 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) 2% citric acid
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.110 A-sec for 10 μ L of 1.4 ng/mL.

7.10. Chromium

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microliner 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 357.9 nm
Slit width: 0.7 nm
Lamp: Cr HCL, 25 mA
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	50	200	
2	1000	10	20	200	
3	100	1	5	200	
4	2400	0	5	0	*
5	2500	1	4	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	15
Blank	0
Modifier 1	15
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 7, 14, and 21 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) Dilute NH_3 , prepared in quartz-distilled water by isothermal distillation
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.160 A-sec with 15 μL of 7 ng/mL.

7.11. Copper

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 324.8 nm
Slit width: 0.7 nm
Lamp: Cu HCL, 15 mA
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	110	10	35	200	
2	500	10	20	200	
3	100	1	5	200	
4	1600	0	5	0	*
5	2500	1	4	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	10
Blank	0
Modifier 1	5
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 20, 40, and 60 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) Citric acid (2%)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.100 A-sec for 10 μ L of 20 ng/mL.

7.12. Nickel

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 232 nm
Slit width: 0.2 nm
Lamp: Ni HCL, 25 mA
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	40	200	
2	1000	10	20	200	
3	100	1	5	200	
4	2200	0	4	0	*
5	2500	1	5	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	20
Blank	0
Modifier 1	5
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 15, 30, and 45 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) 2:2:1 Pd (1000 ppm) : La (1000 ppm) : MgNO₃ (2%)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.150 A-sec for 20 μL of 30 ng/mL.

7.13. Lead

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 283.3 nm
Slit width: 0.7 nm
Lamp: Pb EDL, 10 W
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	45	200	
2	800	10	15	200	
3	100	1	5	200	
4	1800	0	5	0	*
5	2500	1	5	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	20
Blank	0
Modifier 1	10
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 10, 20, and 30 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) 1:1 ammonium phosphate (4%, prepared from ammonium dihydrogen orthophosphate) and citric acid (2%)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.160 A-sec for 20 μ L of 30 ng/mL.

7.14. Selenium

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 196.0 nm
Slit width: 0.7 nm
Lamp: Se EDL, 6 W
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	50	200	
2	900	10	15	200	
3	100	1	5	200	
4	2300	0	5	0	*
5	2500	1	4	300	

AUTOSAMPLER PROGRAM:

Solution:	Volume (µL):
Sample	20
Blank	0
Modifier 1	10
Modifier 2	0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 25, 50, and 75 ppb prepared from 1000 ppm Ricca standard in 0.2 M HNO₃.

MATRIX MODIFIERS: (1) 5:3:1 Pd (1000 ppm) : Ni (4000 ppm) : hydroxylamine hydrochloride (2%)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.140 A-sec for 20 μ L of 50 ng/mL.

7.15. Tin

METHOD: Graphite furnace atomic absorption

INSTRUMENTATION:

Spectrophotometer: Perkin-Elmer Z3030
Graphite furnace: Perkin-Elmer HGA 600
Autosampler: Perkin-Elmer AS 60
Printer: Okidata Microline 184

ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS:

Wavelength: 286.3 nm
Slit width: 0.7 nm
Lamp: Sn EDL, 8 W
Background correction: Zeeman effect
Peak measurement: Peak area
Read delay: 0 sec
Read time: 5 sec
Output: To printer

GRAPHITE FURNACE SETTINGS:

Tube/platform: L'vov platform, pyrolytically coated
Carrier gas: Argon (high purity)

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)	Read Step
		Ramp	Hold		
1	130	10	50	200	
2	800	10	10	200	
3	100	1	5	200	
4	2100	0	5	0	*
5	2300	1	4	300	

AUTOSAMPLER PROGRAM:

Solution: Volume (µL):
Sample 20
Blank 0
Modifier 1 10
Modifier 2 0

Number of injections: 1
Recalibrate after positions: 16, 28, and 40

STANDARDS: 0, 10, 25, and 50 ppb prepared from 1000 ppm Ricca standard in 0.2 M HCl.

MATRIX MODIFIERS: (1) 1:1 magnesium nitrate (0.2%) and ammonium phosphate (4%, prepared from ammonium dihydrogen orthophosphate)
(2) none

APPROXIMATE SENSITIVITY: Approximately 0.200 A-sec for 20 μ L of 50 ng/mL.

7.16. Aluminum

METHOD: Instrumental neutron activation analysis for Al in sediments

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution germanium detector
Nuclear Data Genie MCA

IRRADIATION CONDITIONS:

Position:	Pneumatics
Nominal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	30 sec
Cooling period:	15 min
Peak measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	2-10 cm
g-ray peak energy:	1779 KeV
Count time:	300 sec

STANDARDS:

Pure element standards:	0.01 g prepared from 1000 ppm Ricca standard.
Matrix standards:	0.1 g of: TAMU HS2 [*] , USGS GXR-5, NIST SRM 1646, NRC BCSS-1, NRC MESS-2.

SAMPLE PREPARATION: Approximately 0.1 g weighed to nearest 0.0001 g into 0.4-dram polyethylene vials.

APPROXIMATE SENSITIVITY: Depends upon background; 100 µg results in approximately 1000 counts with associated counting error of 10%; with typical background and 0.1 g sample size, this corresponds to 0.1% Al.

* TAMU HS2 is a "house" reference sediment standard collected using a box corer in the Mississippi river delta. The sediment was washed several times in distilled water to remove dissolved salts, freeze dried, ground with a mortar and pestle, and homogenized.

7.17. Chromium

METHOD: Instrumental neutron activation analysis for Cr in sediments

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution germanium detector
Nuclear Data Genie MCA

IRRADIATION CONDITIONS:

Position: Rotisserie
Nominal neutron flux: 1×10^{13} neutrons/cm²/sec
Length of irradiation: 14 hr
Cooling period: 10 days
Peak measurement: Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position: 10 cm
g-ray peak energy: 320.1 KeV
Count time: 60 min

STANDARDS:

Pure element standards: 1000 µg prepared from 1000 ppm Ricca standard.
Matrix standards: 0.5 g of: TAMU HS2, USGS GXR-5, NIST SRM 1646, NRC BCSS-1, NRC MESS-2.

SAMPLE PREPARATION: Approximately 0.5 g weighed to nearest 0.0001 g into 0.4-dram polyethylene vials.

APPROXIMATE SENSITIVITY: Depends upon background; 1 µg results in approximately 1000 counts with associated counting error of 15%; with typical background and 0.5 g sample size, this corresponds to 2 ppm Cr.

7.18. Iron

METHOD: Instrumental neutron activation analysis for Fe in sediments

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution germanium detector
Nuclear Data Genie MCA

IRRADIATION CONDITIONS:

Position:	Rotisserie
Nominal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	14 hr
Cooling period:	10 days
Peak measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	10 cm
g-ray peak energy:	1099.2 KeV
Count time:	60 min

STANDARDS:

Pure element standards:	0.01 g prepared from 1000 ppm Ricca standard.
Matrix standards:	0.5 g of: TAMU HS2, USGS GXR-5, NIST SRM 1646, NRC BCSS-1, NRC MESS-2.

SAMPLE PREPARATION: Approximately 0.5 g weighed to nearest 0.0001 g into 0.4-dram polyethylene vials.

APPROXIMATE SENSITIVITY: Depends upon background; 150 µg results in approximately 1000 counts with associated counting error of 10%; with typical background and 0.5 g sample size, this corresponds to 300 ppm Fe.

7.19. Manganese

METHOD: Instrumental neutron activation analysis for Mn in sediments

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution germanium detector
Nuclear Data Genie MCA

IRRADIATION CONDITIONS:

Position: Pneumatics
Nominal neutron flux: 1×10^{13} neutrons/cm²/sec
Length of irradiation: 30 sec
Cooling period: 15 min
Peak measurement: Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position: 2-10 cm
g-ray peak energy: 846.8 KeV
Count time: 300 sec

STANDARDS:

Pure element standards: 1000 µg prepared from 1000 ppm Ricca standard.
Matrix standards: 0.1 g of TAMU HS2, USGS GXR-5, NIST SRM 1646, NRC BCSS-1, NRC MESS-2.

SAMPLE PREPARATION: Approximately 0.1 g weighed to nearest 0.0001 g into 0.4-dram polyethylene vials.

APPROXIMATE SENSITIVITY: Depends upon background: 1 µg results in approximately 1000 counts with associated counting error of 10%; with typical background and 0.1 g sample size, this corresponds to 10 ppm Mn.

7.20. Arsenic

METHOD: Instrumental neutron activation analysis for As in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position:	Rotisserie
Nominal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	14 hr
Cooling period:	7 days
Peak measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	Variable, depending upon sample activity; normally 10-20 cm
Gamma-ray peak energy:	559.1 KeV
Count time	30-45 min, depending on concentration

STANDARDS:

Pure element standards:	6 µg prepared from 1000 ppm Ricca standard
Matrix standards:	0.25 g of: NRC DOLT-2; NRC DORM-2, NIST SRM 1566

SAMPLE PREPARATION: Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into 0.4 dram polyethylene vial

APPROXIMATE SENSITIVITY: Depends upon background; 0.85 µg, results in approximately 1000 counts above background with associated counting error of 10%; with typical background and 0.25 g sample size, this corresponds to 3.4 ppm on a dry weight basis.

7.21. Chromium

METHOD: Instrumental neutron activation analysis for Cr in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position: Rotisserie
Nominal neutron flux: 1×10^{13} neutrons/cm²/sec
Length of irradiation: 14 hr
Cooling period: 10 days
Peak measurement: Net peak area using Nuclear Data peak program

COUNTING CONDITIONS.

Counting position: 1-10 cm
Gamma-ray peak energy: 320.1 KeV
Count time: 75 min

STANDARDS:

Pure element standards: 5 -g prepared from 1000 ppm Ricca standard
Matrix standards: 0.25 g of: NRC DOLT-2; NRC DORM-2, NIST SRM 1566a.

SAMPLE PREPARATION: Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into 0.4 dram polyethylene vial

APPROXIMATE SENSITIVITY: Depends upon background; 0.25 µg, results in approximately 1000 counts above background with associated counting error of 10%; with typical background and 0.25 g sample size, this corresponds to 1.0 ppm Cr on a dry weight basis.

7.22. Iron

METHOD: Instrumental neutron activation analysis for Fe in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position: Rotisserie
Nominal neutron flux: 1×10^{13} neutrons/cm²/sec
Length of irradiation: 14hr
Cooling period: 10 days
Peak measurement: Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position: 1-10 cm
Gamma-ray peak energy: 1099.2 KeV
Count time: 75 min

STANDARDS:

Pure element standards: 333 µg prepared from 1000 ppm Ricca standard
Matrix standards: 0.25 g of: NRC DOLT-2; NRC DORM-2, NIST SRM 1566a

SAMPLE PREPARATION: Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into dram polyethylene vial

APPROXIMATE SENSITIVITY: Depends upon background; 36 µg, results in approximately 1000 counts above background with associated counting error of 10%; with typical background and 0.25 sample size, this corresponds to 145 ppm on a dry weight basis.

7.23. Selenium

METHOD: Instrumental neutron activation analysis for Se in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position:	Rotisserie
Nominal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	14 hr
Cooling period:	10 days
Peak measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	1-10 cm
Gamma-ray peak energy:	264.6 KeV
Count time:	75 min

STANDARDS:

Pure element standards	6 µg prepared from 1000 ppm Ricca standard
Matrix standards:	0.25 g of: NRC DOLT-2; NRC DORM-2, NIST SRM 1566a

SAMPLE PREPARATION: Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into 0.4 dram polyethylene vial

APPROXIMATE SENSITIVITY: Depends upon background; 0.22 µg, results in approximately 1000 counts above background associated counting error of 10%; with typical background and 0.25 g sample size, this corresponds to 0.87 ppm on a dry weight basis.

7.24. Silver

METHOD: Instrumental neutron activation analysis for Ag in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position:	Rotisserie
Nominal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	14 hr
Cooling period:	10 days
Peak measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	1-10 cm
Gamma-ray peak energy:	657.8 KeV
Count time:	75 min

STANDARDS:

Pure element standards:	5 µg prepared from 1000 ppm Ricca standard
Matrix standards:	0.25 g of: NRC DOLT-2; NRC DORM-2, NIST SRM 1566a

SAMPLE PREPARATION:	Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into 0.4 dram polyethylene vial
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APPROXIMATE SENSITIVITY:	Depends upon background; 0.14 µg, results in approximately 1000 counts above background with associated counting error of 10%; with typical background and 0.25 g sample size, this corresponds to 0.55 ppm Ag on a dry weight basis.
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7.25. Zinc

METHOD: Instrumental neutron activation analysis for Zn in tissues

INSTRUMENTATION:

TRIGA 1 MW reactor
Ortec high-resolution, high-purity germanium detector
Nuclear Data Genie MCA implemented on Digital VAX workstation

IRRADIATION CONDITIONS:

Position:	Rotisserie
Normal neutron flux:	1×10^{13} neutrons/cm ² /sec
Length of irradiation:	14 hr
Cooling period:	10 days
Peak Measurement:	Net peak area using Nuclear Data peak program

COUNTING CONDITIONS:

Counting position:	1-10 cm
Gamma-ray peak energy:	1115.6 KeV
Count time:	75 min

STANDARDS:

Pure element standards:	10 µg prepared from 1000 ppm Ricca standard
Matrix standards:	0.25 g of: NRC DOLT-2, NRC-DORM-2, NIST SRM 1566A

SAMPLE PREPARATION:	Approximately 0.25 to 0.5 g tissue weighed to nearest 0.0001 g into 0.4 dram polyethylene vial
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APPROXIMATE SENSITIVITY:	Depends upon background; 2.3 µg, results in approximately 1000 counts above background with associated counting error of 10%; with typical background and 0.25 g sample size, this corresponds to 9.1 ppm Zn on a dry weight basis.
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**Analysis of Marine Sediment and Bivalve Tissue
by X-Ray Fluorescence, Atomic Absorption and Inductively Coupled
Plasma Mass Spectrometry**

E. Crecelius, C. Apts, L. Bingler, J. Brandenberger, M. Deuth, S. Kiesser, and R. Sanders *
Battelle/Marine Sciences Laboratory
1529 West Sequim Bay Rd.
Sequim, Washington

ABSTRACT

Analytical chemistry techniques including atomic absorption spectrophotometry, inductively coupled plasma mass spectrometry (ICP-MS) and energy dispersive x-ray fluorescence (XRF) have been applied to the analysis of 17 elements in aquatic sediment and bivalve tissue in support of the NOAA NS&T Program. Complete acid digestion of samples at elevated pressure and temperature in a sealed Teflon container minimizes contamination and loss of elements. Multi-elemental techniques such as ICP-MS and XRF provide sensitive, accurate, and precise results for a variety of elements at a reasonable cost.

1. INTRODUCTION

Methods used for analysis of 17 metals in estuarine sediments and tissues were developed as part of the Mussel Watch Project of the National Status and Trends (NS&T) Program. The total digestion procedure used for sediments, without loss of volatile elements, was developed by Taylor and Presley (this volume) at Texas A&M University (TAMU). The digestion procedure for tissues was a combination of HCl and HNO₃. Metals were analyzed predominantly by graphite furnace atomic absorption (GFAA) spectrometry and x-ray fluorescence (XRF). Mercury was analyzed by cold vapor atomic absorption (CVAA). Selected metals were determined by inductively-coupled plasma mass spectrometry (ICP-MS). The primary objective in developing each instrumental method was to keep the method as simple and straightforward as possible while producing acceptable accuracy and precision.

It is expected that users of the instrumental methods described below will modify certain parameters to suit their particular instrumentation and equipment due to variations in performance between instruments. Such variations are caused by differences in furnace calibration, lamp intensities, nebulizer characteristics, and other parameters.

2. EQUIPMENT AND SUPPLIES

2.1. Instrumentation

Perkin-Elmer model Z/5000 spectrophotometer. Perkin-Elmer, Norwalk, CT.

Dual lamp EDL power supply
Model 500 graphite furnace atomizer with Zeeman background correction system
Model 3600 data terminal
Model AS40 autosampler

* KLM Analytical, 2000 Logston Blvd., Richland, WA 99352.

Perkin-Elmer model Z/3030 spectrophotometer

Dual lamp EDL power supply
Model 600 graphite furnace atomizer with Zeeman background correction system
Model AS60 autosampler
Model PRS 100 printer

Thermo-Separation Products Fully Automated Mercury Analysis System, MM3200, TSP, Portland, OR.

KEVEX x-ray fluorescence excitation and detection subsystem, 0810Z. Fisons Instruments, San Carlos, CA.

Canberra series 80 multichannel analyzer. Canberra Nuclear Products, Meriden, CT.
Digital PDP-11/34A computer operating system
Digital RL01 floppy disc drive
Digital RL01 hard disc drive
Digital video terminal (model 102)
KEVEX 4620 detector bias supply
KEVEX high voltage generator
PHA/LTC model 8623 amplifier
Versatec printer/plotter. Versatec Inc., Santa Clara, CA.

Perkin-Elmer model 5000 inductively coupled plasma mass spectrometer

2.2. Supplies

Argon, 99.999% purity	Hollow cathode lamps (HCL)
Electrodeless discharge lamps (EDL)	Laboratory press, 3.2-cm diameter, 27,000 kg
Electron multiplier, model 4870V, Galileo channeltron. Galileo Inc., Sturbridge, MA.	Nitrogen, ultra-pure
Graphite tubes, pyrolytically coated, grooved, Perkin-Elmer B0121-092. Perkin-Elmer, Norwalk, CT.	Platforms, pyrolytically coated L'vov, Perkin-Elmer B0121-091
Graphite tubes, pyrolytically coated, non-grooved, Perkin-Elmer B0135-653. Perkin-Elmer, Norwalk, CT.	Quartz torch
Helium	Sample cones, nickel
	Skimmer cones, nickel
	Thin film standards. Micro Matter, Eastsound, WA

2.3. Labware

Balance, Mettler model AC100 and H30, Sartorius MCI LAB LC1200 S	Flask, volumetric, polyethylene, 100-mL
Bomb lids, 120-mL, Teflon perfluoralkoxy (PFA), double-ported, 0104-4-2. Savillex, Minnetonka, MN.	Freeze dryers, 6206-0101. Virtis Co., Gardiner, NY.
Bombs, 60-mL, Teflon perfluoralkoxy (PFA) bomb, 561R2; 120-mL, Teflon perfluoralkoxy (PFA), 577. Savillex, Minnetonka, MN.	Jars, 125-mL, polystyrene, 8002. Spex Industries, Edison, NJ.
Capping station. CEM Corp., Mathews, NC.	Methacrylate balls, 3112. Spex Industries, Edison, NJ.
Cups, 2-mL, polystyrene, B2713-2. Baxter, McGaw Park, IL.	Mixer/Mills, model 8000. Spex Industries, Edison, NJ.
	Oven, stainless steel, Imperial II Radiant Heat Oven. Lab Line Inc., Melrose Park, IL.

Pipettes, macro and micro	Teflon tubing, 1-mm i.d.
Polyethylene tubing, 7420. Clay Adams Co., Parsippany, NJ.	Vials, 20-mL, polyethylene, threaded, with screw caps, Kimble 66022-241
Spatulas	Vials, dry grinding, ceramic, 8003. Spex Industries, Edison, NJ.
Stopwatch or timer	

Polyethylene vials were cleaned by soaking for five days in 5% HNO₃, at room temperature, rinsed with deionized water and dried in Class 100 laminar flow hoods. All other plastic ware was soaked for three days in 10% HNO₃ at room temperature, rinsed with deionized water, and dried as above. Teflon bombs were soaked for two days in 50% concentrated HNO₃.

2.4. Reagents

All reagents are ultrapure grades except where so designated.

Ammonium dihydrogen phosphate (NH ₄ H ₂ PO ₄) [7722-76-1], Ultrex 7-9431. J. T. Baker, Phillipsburg, NJ.	Nickel nitrate [Ni(NO ₃) ₂ · 6H ₂ O] [13478-00-7], solid, Puratonic Grade. Johnson Matthey Chemicals, West Chester, PA.
Ammonium nitrate (NH ₄ NO ₃) [6484-52-2], solid, reagent grade, 729-1. J. T. Baker, Phillipsburg, NJ.	Nitric acid (HNO ₃) [7697-37-2], concentrated (70%), Instra-analyzed, 9598-33. J. T. Baker, Phillipsburg, NJ.
Ascorbic acid (C ₆ H ₈ O ₆) [50-81-7], solid, reagent grade, 581-5. J. T. Baker, Phillipsburg, NJ.	Perchloric acid (HClO ₄) [7601-90-3], concentrated (70%), 230. G. Frederick Smith, Columbus, OH.
Atomic absorption standards, 1000 µg/mL. High-Purity Standards, Inc., Charleston, SC.	Soda lime [variable mixture of NaOH and CaO/Ca(OH) ₂] [8006-28-8], 4-8 mesh, used as acid fume trap in CVAA, 3448-01. J. T. Baker, Phillipsburg, NJ.
Hydrochloric acid (HCl) [7647-01-0], concentrated (37%), Instra-analyzed, 9530-33. J. T. Baker, Phillipsburg, NJ.	Stannous chloride (SnCl ₂ · 2H ₂ O) [10025-69-1], 3980-01. J. T. Baker, Phillipsburg, NJ.
Hydrofluoric acid (HF) [7664-39-3], Instra-analyzed, 9563-01. J. T. Baker, Phillipsburg, NJ.	Sulfuric acid (H ₂ SO ₄) [7664-93-9], concentrated (98%), Instra-analyzed, 9673-33. J. T. Baker, Phillipsburg, NJ.
Magnesium nitrate [Mg(NO ₃) ₂ · 6H ₂ O] [13446-18-9], solid, Puratonic Grade. Johnson Matthey Chemicals, West Chester, PA.	Water, deionized, 10 megohm-cm resistivity.

2.5. Solvents and matrix modifiers

Ammonium phosphate: 2%, monobasic, 2 g of NH₄H₂PO₄ and 0.200 g of Mg(NO₃)₂ per liter of deionized water.

Ammonium phosphate: 4%, monobasic, 4 g NH₄H₂PO₄ and 13 g NH₄NO₃ diluted to 100 mL with deionized water.

Magnesium nitrate solution: 250 mg/L of deionized water.

0.10 M Nickel nitrate: 2.91 g Ni(NO₃)₂ · 6H₂O in 100 mL of deionized water.

Sulfuric acid modifier solution: Dilute 1 mL of concentrated sulfuric acid to 99 mL with deionized water and add 10 µL of 0.1 M Ni(NO₃)₂ · 6H₂O.

3. SAMPLE TREATMENT

3.1. Drying and homogenization

3.1.1. Sediments

Sediments were obtained with a grab sampler that collects the top 2 cm of surface sediment. Sample aliquots were weighed, freeze-dried for approximately 5 days to constant weight in 125-mL polystyrene Spex jars in a freeze dryer, and reweighed to determine percent dry weight. Approximately 3 g were ground for 5 min using a Spex ceramic ball mill. Aliquots of 0.5 g were used for X-ray fluorescence, or digested for atomic absorption or ICP-MS analysis.

3.1.2. Tissues

Bivalves were hand-collected and shucked at the lab. Tissues were freeze-dried in Spex jars and homogenized using methacrylate balls in a Spex mixer/mill.

3.2. Digestion

Battelle used minor modifications of the total digestion techniques for sediments developed by Taylor and Presley (this volume). Tissues were digested with a mixture of HCl and HNO₃ which has been shown to provide much better recovery of Ag than HNO₃ or HNO₃ and HClO₄ (Crecelius and Daskalakis, 1994; Daskalakis *et al.*, 1997).

Two reagent blanks and three standard reference material samples were included in each analytical string of 50 samples. Reagent blanks contained no sediment or tissue and were processed like the samples.

3.2.1. Sediments

Approximately 200 ± 7 mg of homogenized ground dried sediment was weighed into a tared 60-mL Teflon digestion bomb. One mL of 4:1 HNO₃/HClO₄ was added to each bomb and the lid tightened at the capping station.

The bombs were heated in the oven at 130 ± 10 °C for 4 hr and allowed to cool.

Three mL of concentrated HF were added to each bomb, rinsing the walls of the bomb to insure that all solids were washed down into the acid mixture. The lid was tightened at the capping station.

The bombs were heated in the oven at 130 ± 10 °C for 8 hr and allowed to cool.

The bombs were opened and the digestates diluted to approximately 20 mL with deionized water.

The solutions were weighed in the Teflon bombs and the volume calculated using a density factor of 1.05 g/mL.

The contents of each bomb were transferred quantitatively to a 20-mL polyethylene screw-cap vial for storage prior to analysis.

Digestates were analyzed directly by GFAA or CVAA. Before analysis by ICP-MS, a 10-mL aliquot was transferred to a Teflon bomb and dried slowly, uncovered, on a hot plate (203 °C) in a perchloric acid hood to eliminate chloride and fluoride. White fumes were indicative of successful elimination of these elements. The dried digestate was dissolved in 1 mL of 10% HNO₃ and heated again to dryness. The dried digestate was dissolved again with 1 mL of 10% HNO₃ and 9 mL of deionized water.

3.2.2. Tissues

Approximately 500 ± 15 mg of homogenized, dried tissue was weighed into a tared 60-mL Teflon digestion bomb. Five mL of HCl and 3.5 mL of HNO₃ were added to each bomb and the lid screwed on but not tightened.

The bombs were placed in a cold water bath in an acid hood and the water bath was heated slowly to 60 ± 3 °C. The total heating time was 3 to 4 hr.

The bombs were allowed to cool. The lids were tightened at the capping station.

The bombs were heated in the oven at 130 ± 10 °C for 16 hr and allowed to cool.

The bombs were opened and the digestates diluted to approximately 20 mL with deionized water.

The solutions were weighed in the Teflon bombs and the volume calculated using a density factor of 1.09 g/mL.

Digestates were analyzed directly by GFAA and CVAA, or diluted 10:1 for ICP-MS analysis.

4. CALIBRATION

Calibration standards were prepared by serial dilution of commercially available atomic absorption standards using class-A glass pipettes, volumetric flasks, and 10% HNO₃. Some metals, such as Sb and Sn, required use of a different acid diluent. Final working standards were prepared in 1% HNO₃ or the appropriate acid diluent, using micropipettes and glass volumetric flasks. The element concentrations in each standard should be sufficient to cover the appropriate range of sample concentrations and produce good measurement precision and accurately define the slope of the response curve. Concentrations of commercial standards were verified by comparison with National Institute of Standards and Technology (NIST) spectrophotometric standards.

5. SPECTRAL INTERFERENCES

Spectral interferences may be minimized by sample dilution, use of an alternate analyte wavelength, or selective volatilization of the analyte. Non-spectral interferences may be detected and compensated for using the method of standard additions. Matrix modifiers that were used include Mg(NO₃)₂, NH₄H₂PO₄, Ni(NO₃)₂, and H₂SO₄. Their specific use is described in the instrumental analysis section for each element.

6. CALCULATIONS

6.1. Graphite furnace and ICP-MS

Sample concentrations were determined from calibration results and from the dilution factors using the following equation:

$$\text{Concentration } (\mu\text{g/g dry wt}) = \frac{(S_A - B_A) m S_V}{1000 (S_{DW}) (DF)}$$

where S_A was the sample absorbance or intensity value, B_A was the procedural blank absorbance or intensity value, m was the slope of standard addition calibration line, S_V was the sample volume, S_{DW} was the sample dry weight in grams, and DF was the dilution factor.

6.2. Cold vapor atomic absorption

Sample concentrations were determined from calibration results and from the dilution factors involved in instrumental analysis and sample digestion according to the following equation:

$$\text{Concentration } (\mu\text{g/g dry wt}) = \frac{(S_A - B_A) m S_V}{1000 (S_{DW}) (DF)}$$

where S_A was the sample absorbance value, B_A was the procedural blank absorbance value, m was the slope of the calibration line, S_V was the sample volume, S_{DW} was the sample dry weight in grams, and DF was the dilution factor.

6.3. X-ray fluorescence

This procedure used energy dispersive x-ray fluorescence spectroscopy to quantify elemental concentrations in sediment and tissue samples (Nielson *et al.*, 1982). The backscatter/fundamental parameter approach using the SAP3 computer code incorporated thin film standards and scattering ratios to produce matrix corrections (Nielson and Sanders, 1982).

Thin film standards were used for the determination of intensity in count/min/ μg (element)/ cm^2 versus element energy in KeV. Thin film standards produced by vapor deposit of the elements on Mylar or polycarbonate substrate were purchased from Micro Matter of Eastsound, WA. These standards were traceable to the U.S. National Institute of Standards and Technology.

The peak analysis and elemental concentration sections of the computer program (SAP3) used arrays of fundamental physical parameters of x-ray energies, mass adsorption coefficients, cross sections, fluorescence yields, absorption edge, and jump ratios to perform the matrix corrections for relating net peak intensities to element concentrations. These arrays were read into the computer from a disc file when the program was initiated. Each excitation source had its own unique disc file or library. Thin film sensitivities or calibration factors of the spectrometer using the intended excitation source was also part of the library.

7. CONCLUSIONS

A variety of analytical instruments provided the optimum method for the analysis of 17 elements in sediment and bivalve tissue. The advantage of XRF was that the sample did not require digestion but was analyzed as a dry powder. Crustal elements such as Al, Cr, Fe, Ni, and Si, that are difficult to dissolve from sediment, could be quantified by XRF. Also, Se and As can be difficult to quantify in tissue digestates by ICP-MS but were quantified easier using XRF. ICP-MS had the advantage of simultaneous analysis of many elements with detection limits much lower than the XRF and similar to those of GFAA. Elements that were particularly sensitive and relatively interference free by ICP-MS include Al, Cr, Ni, Ag, Cd, Sn, Sb, Pb, and Tl. Cold vapor atomic absorption is a very sensitive and reliable technique for Hg analysis. Care must be taken to avoid leakage at high pressure or Hg can be lost during digestion. With the use of the sealed Teflon digestion vessel, mercury could be analyzed from the same digestion as the other metals (Al, Cr, Ni, Ag, Cd, Sn, Sb, Pb, and Tl).

The advantage of freeze drying both sediment and tissue was that the dry material was easily ground or homogenized. No Hg loss occurred during freeze drying. Sediment digestates received special treatment to remove silicon, fluoride and perchloric acid before analysis by ICP-MS. Analysis by GFAA requires matrix modifiers and standardization of the instrument by method of addition to the sample matrix to provide accurate results.

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9. INSTRUMENTAL ANALYSIS

9.1. Atomic absorption spectrometry

9.1.1. Aluminum

Graphite furnace atomic absorption for tissue

METHOD: Graphite furnace atomic absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 309.3 nm
Lamp: HCL, 25 ma (Perkin-Elmer 0303-6009)
Tube: Non-platform, pyrolytically coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 5 sec
Output: Recorded from spectrophotometer display

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	1600	45	25	300
Atomize	2300	0	4	50
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Addition calibration using 22.2, 43.5, and 83.3 µg/L Al to CRM DOLT-1 tissue digestate. Method of standard addition.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 0.4 µg Al standard.

CALIBRATION: Peak height versus concentration of standards used to compute the slope, intercept, and correlation coefficient of calibration line using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFIER: 5 µL of 2% NH₄H₂PO₄.

9.1.2. Chromium

Graphite furnace atomic absorption for tissue

METHOD: Graphite furnace atomic absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 357.9 nm
Lamp: HCL, 25 ma (Perkin-Elmer 0303-6021)
Tube: Pyro, non-platform
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 5 sec
Output: Recorded from spectrophotometer display

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	1500	20	25	300
Atomize	2500	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Method of standard addition calibration using 20.0, 33.3, and 66.7 µg/L Cr to CRM DOLT-1 tissue digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.200 for 0.165 µg Cr standard.

CALIBRATION: Peak height versus concentration of standards used to compute the slope, intercept, and correlation coefficient of calibration line using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFICATION: 5 µL of 250 mg/L Mg as Mg(NO₃)₂.

9.1.3. Nickel

Graphite furnace atomic absorption for tissues

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 232.0 nm
Lamp: HCL, 35 ma (Perkin-Elmer 0303-6047)
Tube: Pyro, non-platform
Carrier gas: Argon
Slit width: 0.2
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 5 sec
Output: To printer

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	1200	20	20	300
Atomize	2300	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Method of standard addition calibration using 24.39, 47.62, and 90.91 µg/L Ni to CRM DOLT-1 digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 0.6 µg Ni standard.

CALIBRATION: Peak height of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFICATION: 5 µL of 2% NH₄H₂PO₄.

MODIFIER VOLUME: 5 µL

9.1.4. Selenium

Graphite furnace atomic absorption for sediment

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~4% 4:1 HNO₃:HClO₄ + 12% HF by volume.

INSTRUMENT SETTINGS:

Wavelength: 195.9 nm
Lamp: EDL, 6 watts (Perkin-Elmer 0303-6262)
Tube: L'vov, pyro coated
Carrier gas: Argon
Slit width: 2.0
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 5 sec
Output: Recorded from spectrophotometer display

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	1300	20	20	300
Atomize	2100	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Method of standard addition calibration using 47.6, 90.9, and 130.4 µg/L Se to CRM MESS-1 sediment digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 1.0 µg Se standard.

CALIBRATION: Peak height of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 20 µL

MATRIX MODIFICATION: 10 µL of 0.1 M Ni(NO₃)₂.

9.1.5. Silver

9.1.5.1. Graphite furnace atomic absorption for tissue

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 327.9 nm
Lamp: HCL, 12 ma (Perkin-Elmer 0303-6064)
Tube: L'vov, pyro coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 4 sec
Output: To printer

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	800	20	20	300
Atomize	2100	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Addition calibration using 0.99, 1.96, and 4.76 µg/L Ag to CRM DOLT-1 tissue digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 0.03 µg Ag standard.

CALIBRATION: Peak height of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 10 µL

MATRIX MODIFICATION: 5 µL of 2% NH₄H₂PO₄.

Silver

9.1.5.2. Graphite furnace atomic absorption for sediment

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~4% 4:1 HNO₃:HClO₄ + 12% HF by volume.

INSTRUMENT SETTINGS:

Wavelength: 327.9 nm
Lamp: HCL, 12 ma (Perkin-Elmer 0303-6064)
Tube: L'vov, pyro coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 4 sec
Output: To printer

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	800	20	20	300
Atomize	2100	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Addition calibration using 2.44, 4.76, and 9.09 µg/L Ag to SRM 1646 sediment digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.120 for 0.05 µg Ag standard.

CALIBRATION: Peak height of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 10 µL

MATRIX MODIFICATION: 5 µL of 2% NH₄H₂PO₄.

9.1.6. Cadmium

9.1.6.1. Graphite furnace atomic absorption for tissue

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 228.9 nm
Lamp: EDL, 5 watts (Perkin-Elmer 0303-60216)
Tube: L'vov, pyro coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak area
Scale expansion: None
Read time: 4 sec
Output: To printer

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	800	20	20	300
Atomize	1800	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Addition calibration using 0.96, 2.37, and 4.62 µg/L Cd to SRM 1566a tissue digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 0.02 µg Cd standard.

CALIBRATION: Peak area of standard versus concentration of standard. Compute the slope, intercept and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFICATION: 10 µL of 2% NH₄H₂PO₄.

Cadmium

9.1.6.2. Graphite furnace atomic absorption for sediment

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~4% 4:1 HNO₃:HClO₄ + 12% HF by volume.

INSTRUMENT SETTINGS:

Wavelength: 228.8 nm
Lamp: EDL, 5 watts (Perkin-Elmer 0303-6216)
Tube: L'vov, pyro coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak area
Scale expansion: None
Read time: 4 sec
Output: To printer

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	800	20	20	300
Atomize	1800	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Addition calibration using 0.90, 2.22, and 4.35 µg/L Cd to SRM 1646 sediment digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.100 for 0.015 µg Cd standard.

CALIBRATION: Peak area of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFICATION: 10 µL of 2% NH₄H₂PO₄.

9.1.7. Mercury

Cold vapor/gold foil amalgam for tissue or sediment

METHOD: Cold vapor/gold foil amalgam

DIGEST MATRIX: ~25% 5:3.5 HCl and HNO₃ by volume for tissues.
~4% 4:1 HNO₃ and HClO₄ + 12% HF by volume for sediments.

INSTRUMENT SETTINGS:

Reaction volume: 0.020 -1 mL
Diluent: 3% HNO₃
Carrier Gas: Ar
Flow rate: 200 mL/min
Scale expansion: Attenuation - mid-range
Integration time: 5 min
Output: Peak integrated by system software

REDUCTANT: 2% SnCl₂ in 10% HCl

STANDARDS: 0.5, 1.25, 5, 7.5, and 12.5 µg/L made in 3% HCl using High Purity standards.

TYPICAL SENSITIVITY: 0.001 µg/g Hg.

CALIBRATION: Instrument read-out is in peak area and input is in ng Hg, so calibration curve is based on Hg input versus Hg output reading. The slope, intercept, and correlation coefficient are calculated using linear, least-squares regression.

INJECTION VOLUME: 1 to 5 mL

REDUCTANT VOLUME: 2.5 mL

MATRIX MODIFICATION: None

9.1.8. Lead

Graphite furnace atomic absorption for tissue

METHOD: Graphite Furnace Atomic Absorption

DIGEST MATRIX: ~15% 4:1 HNO₃:HClO₄ by volume.

INSTRUMENT SETTINGS:

Wavelength: 283.3 nm
Lamp: EDL, 5 ma (Perkin-Elmer 0303-6039)
Tube: L'vov, coated
Carrier gas: Argon
Slit width: 0.7
Background correction: Zeeman
Signal mode: Peak height
Scale expansion: None
Read time: 5 sec
Output: Recorded from Spectrophotometer display

FURNACE PROGRAM:

Step	T (°C)	Time (sec)		Internal Gas Flow (mL/min)
		Ramp	Hold	
Dry 1	80	9	1	300
Dry 2	140	45	5	300
Dry 3	250	20	5	300
Char	800	20	20	300
Atomize	2100	0	4	0
Cleanout	2650	1	3	300
Cool	20	1	10	300

STANDARDS: Method of standard addition calibration at 9.9, 19.6, and 24.4 µg/L Pb to CRM DOLT-1 tissue digestate.

TYPICAL SENSITIVITY: Absorbance is approximately 0.160 for 0.25 µg Pb standard.

CALIBRATION: Peak height of standard versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

INJECTION VOLUME: 5 µL

MATRIX MODIFICATION: 10 µL of 2% NH₄H₂PO₄.

9.2. Inductively coupled plasma mass spectrometry

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

METHOD:	Inductively coupled plasma mass spectrometry
DIGEST MATRIX:	1 to 10 dilution of digestate with deionized water plus 0.1 mL of 1 ppm In standard.
INSTRUMENT SETTINGS:	Instrument settings change on a daily basis as sensitivity is optimized. This is especially true of lens settings. The following gives general ranges on parameters that remain relatively constant in day-to-day operation:
Power:	1250-1500 watts
Coolant flow:	13-14 L/min.
Auxiliary flow:	0.5-1.0 L/min.
Nebulizer flow:	0.85-1.0 L/min.
EM voltage:	2000-3000 volts
Sample uptake:	0.5-1.5 mL/min.
Integration method:	Constant area
Integration area:	0.8
Background counts:	35
Dead time:	40 nsec
Quad slew factor:	0.10
PROCEDURE:	
Element menu:	tissue2
Mass range:	25.98 to 212.05 amu
Number of channels:	2048
Number of sweeps:	100
Dwell time:	320 μ sec
Collector type:	pulse
Internal standards:	In
Skipped mass regions:	28.00 - 43.00, 63.00 - 105.00, 125.00 - 200
STANDARDS:	Appropriate calibration standards (representative of sample concentration) are prepared from dilutions of NIST single- and/or multi-element standards. Other reference materials (NIST or second party single/multi-element standards) are used as check standards. Method of standard addition can be used to produce instrumental response curve.
TYPICAL SENSITIVITY:	Sensitivity is approximately 5×10^4 million counts per sec per ppm for In ¹¹⁵ .
CALIBRATION:	Constant area integration (using 0.8 of total peak) versus concentration of standard. Compute the slope, intercept, and correlation coefficient using linear, least-squares regression.

9.3. X-Ray fluorescence

Elements in sediment: Al, As, Cr, Cu, Fe, Mn, Ni, Pb, Si, and Zn.
Elements in tissue: As, Cu, Fe, Mn, Se, Si, and Zn.

METHOD: X-ray fluorescence

Sample preparation: 0.5 g dried and homogenized sediment or tissue pressed in a pellet, 2 cm in diameter.

INSTRUMENT SETTINGS:

Gain: PHA/LTC for Series 80 MCA
Secondary target: Zr
Target gain: 25 eV/channel
Resolution: 182 eV at 6.4 KeV
Timing counter: 12 μ sec
Bias: -1000 V
Live time: 1500 sec
Dead time: \leq 40%
Tungsten tube voltage: 40 V
Tube current: 20 milliamps

ORGANIC ANALYSES

Extraction and Clean-Up of Sediments for Trace Organic Analysis

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Measurement of organic contaminants, such as polychlorinated biphenyls, polynuclear aromatic hydrocarbons, and chlorinated pesticides, in soil/sediment require the isolation of these contaminants from the matrices. An aliquot of the homogenized sediment sample was chemically dried with sodium sulfate and extracted with dichloromethane using a Soxhlet apparatus. The extract was concentrated and purified using silica gel/alumina column chromatography before instrumental analysis. Quality control samples were processed with each batch of samples in a manner identical to the samples.

1. INTRODUCTION

Assessment of the environmental concentrations of polynuclear aromatic hydrocarbons (PAHs), chlorinated pesticides and polychlorinated biphenyls (PCBs) requires their measurement in sediments at trace levels (parts per billion to parts per trillion).

Ten to thirty grams of chemically-dried sediment was Soxhlet extracted with dichloromethane. The extract was concentrated and purified using silica gel/alumina column chromatography to remove matrix interferences.

2. SAMPLE COLLECTION, PRESERVATION AND STORAGE

2.1. Sample collection

Sediment was collected and stored in precleaned glass jars and stored frozen (-20 °C).

2.2. Sample preservation and storage

Sediment samples were shipped frozen to the laboratory and stored at -20 °C until analysis. After subsampling, excess sample was archived at -20 °C in the dark. Extracts were stored in the dark at or below 4 °C.

3. INTERFERENCES

Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware, and lead to false positives during instrumental analysis. All materials used in this method were routinely demonstrated to be free from interferences by processing procedural blanks identical to samples (one blank per 20 samples or each batch whichever is more frequent).

Matrix interferences result from co-extraction of compounds other than the analytes of interest. Elemental sulfur and naturally occurring lipids can cause interferences in the analysis

of sediment extracts. Silica gel/alumina cleanup with activated copper was used to remove interfering materials from the sample prior to analysis.

4. APPARATUS AND MATERIALS

4.1. Labware and apparatus

Glassware was cleaned by washing with Micro cleaning solution and rinsing with water. The glassware was then combusted in a muffle furnace at 440 °C for at least 4 hr. Solvent rinses of acetone followed by dichloromethane may be substituted for muffle furnace heating. After drying and cooling, the glassware was sealed and stored in a clean environment to prevent the accumulation of dust or other contaminants. Stored glassware was maintained capped with combusted aluminum foil.

The following labware and equipment is needed to perform the sediment extraction and purification procedure:

- Beaker, 10-mL (for dry weight).
- Glass jars, 250-mL or 500-mL glass jars, or other suitable containers
- Vials, 1-mL to 7-mL glass vials with Teflon-lined caps
- Glass funnels
- Flat bottom flasks, 250- and 500-mL
- Soxhlet extractor flasks, 40 mm i.d. and condenser
- Thimbles, Alundum, medium or coarse, 44-mL round bottom
- Concentrator tube, Kuderna-Danish - 25-mL, graduated. Ground glass stoppers are used to prevent evaporation of extracts
- Snyder column, Kuderna-Danish - 3-ball column
- Micro reaction vessels, 2.0-mL or 1.0-mL autosampler vials with crimp cap septa
- Chromatographic column, 300 mm x 10 mm i.d., with Pyrex glass wool at bottom and Teflon stopcock
- Analytical balance, capable of weighing to 0.0001 mg
- Analytical balance, capable of weighing to 0.1 g
- Water bath, heated to 60 - 70 °C
- Teflon boiling chips, solvent extracted
- Syringes, 10 or 25 µL
- Disposable glass Pasteur pipettes, 1-mL
- Pyrex glass wool, combusted at 400 °C for 4 hr
- Nitrogen gas evaporation unit

Volumetric glassware for sample measurement or introduction of internal standards must be calibrated.

4.2. Reagents

- Water (Reagent water that contains no analytes above the method detection limit.)
- Sand, combusted at 400 °C for 4 hr
- Sodium sulfate (Na_2SO_4) [7757-82-6], granular, anhydrous, J. T. Baker, ACS Reagent grade or equivalent combusted at 440 °C for 4 hr and stored at 120 °C prior to use, cooled to room temperature in a dessicator
- Hexane (C_6H_{14}) [110-54-3], pesticide quality or equivalent
- Dichloromethane (CH_2Cl_2) [75-09-2], pesticide quality or equivalent
- Pentane, Burdick and Jackson pesticide grade or equivalent, lot tested

Alumina, Neutral 80-325 MCB chromatographic grade or equivalent Combusted at 400 °C for 4 hr and stored at 120 °C prior to use
Silica, Grade 923, 100-200 mesh Aldrich 21,447-7 or equivalent stored at 170 °C for 24 hr prior to use
Activated copper turnings, Fisher Scientific, 6575-500, hydrochloric acid washed; water, methanol, and dichloromethane rinsed.
Activated granular copper, J. T. Baker, analytical grade, 1720-05, 20-30 mesh, hydrochloric acid washed, water, methanol, and dichloromethane rinsed.
Surrogate spiking solutions
Matrix spike standard
Internal standard solution

5. PROCEDURE

5.1. Sample Preparation

An aliquot of approximately 1 g of sample was weighed in a clean 10-mL beaker. After oven drying at 63 - 56 °C for at least 24 hr, this aliquot was reweighed and returned to the oven to further dry the samples for at least another 2 hr before the second weighing. If the difference between the two weights of the dried aliquot is less than ± 0.02 g, the second reading of the weight was used to calculate the percent solid of the sample. If the difference was greater than ± 0.02 g, the oven drying and reweighing was continued until a constant weight was obtained.

A weighed aliquot of sample (10 to 30 g) was chemically dried by mixing with anhydrous sodium sulfate (30 - 100 g). The mixture of the sample and the sodium sulfate was stirred continuously with a clean stainless steel spatula until the dried sample was free-flowing. The sample was transferred to an extraction thimble. The thimble was placed into the Soxhlet holder. Three hundred mL of dichloromethane, 1 or 2 boiling chips and 2 g of copper turnings (activated by rinsing with concentrated HCl) were added to the 500-mL flat bottom extraction flask. The Soxhlet holder with sample thimble inside was attached to the flask and the sample in the thimble was wetted with approximately 50 mL of CH_2Cl_2 .

After the sediment samples in the thimble were spiked with surrogates [(and matrix spike solution to the matrix spike (MS) and matrix spike duplicate (MSD)], the samples were extracted in the Soxhlet apparatus on a hot sand bath for 8 hr. Recycling of the solvent in the Soxhlet apparatus was maintained at approximately one cycle for every 4 min (i.e., 15 cycles per hour).

A 3-balled Snyder column was attached to sample extracts in the 500-mL flat bottom flask. The extracts were concentrated down to 10 - 15 mL on a water bath at 60 - 80 °C. If sediment or other particulates were present in the sample extract, the extracts were filtered through a funnel containing glass wool and sodium sulfate. The concentrated sample extract was transferred to a 25-mL concentrator tube. The flat bottom flask was rinsed two to three times with dichloromethane and the rinses transferred to the concentrator tube. The sample extract was then concentrated and solvent changed to about 2 mL of hexane.

5.2. Silica/alumina column cleanup

A glass chromatographic column (30 cm x 10 mm) with a 300-mL reservoir was filled with dichloromethane. A plug of glass wool and 1 cm of combusted sand were placed in the glass chromatographic column. Ten grams of alumina (deactivated with 1% water) in CH_2Cl_2 was slurry-packed into the column and the alumina allowed to settle. Twenty grams of silica gel

(deactivated with 5% water) in CH_2Cl_2 was slurry-packed into the column. The silica gel was allowed to settle. About 1 cm of combusted sand and 1 - 2 cm of activated granular copper was then placed on top of the packed column. The CH_2Cl_2 in the column was drained until the solvent reached the top of the copper. Next 50 mL of pentane were added to the column and the pentane drained to the top of the copper.

The concentrated sample extract in 2 mL of hexane was transferred to the column using a disposable pipette. The sample was drained to the top of the copper and the eluent was collected in a 250-mL flat bottom flask. The concentrator tube that contained the sample extract was rinsed twice with 1 mL of 50/50 pentane/dichloromethane, and the rinses added to the column. The solvent was drained to the copper layer and collected in the flask. Two hundred mL of 50/50 pentane/ CH_2Cl_2 was added to the column and collected in the flask at a flow rate of approximately 1 mL/min. This fraction contained the aromatic and chlorinated hydrocarbons. A 3-balled Snyder column was attached to the 250-mL flat bottom flask and the pentane/ CH_2Cl_2 column fraction and concentrated to 1 mL in hexane on a water bath at 40 - 60 °C.

6. QUALITY CONTROL

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 set, whichever was more frequent. Blank levels were no more than three times the method detection limit (MDL). If blank levels for any component were above three times MDL, samples analyzed in that sample set were re-extracted and reanalyzed. If insufficient sample was available for extraction, the data was reported and appropriately qualified.

Matrix spike/matrix spike duplicate (MS/MSD) samples were run with every 20 samples, or with every sample set, whichever was more frequent.

Surrogate standards were spiked into every sample and quality control sample.

Sediment reference material with certified aromatic and chlorinated hydrocarbons concentrations were analyzed with each sample batch (approximately 20 samples) to demonstrate the method was in a state of control.

7. CONCLUSIONS

This method has proven to be reliable in quantitatively extracting most organic contaminants from sediment samples.

Extraction of Biological Tissues for Trace Organic Analysis

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Measurements of organic contaminants (such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons and chlorinated pesticides) in biological tissues require the isolation of these contaminants from the matrices. An aliquot of the homogenized tissue sample was extracted three times with dichloromethane in the presence of sodium sulfate by maceration with a tissumizer. An aliquot of 20 mL of the extract was quantitatively removed for lipid determination. The remaining extract was concentrated by Kuderna-Danish technique. The concentrated extract was purified using alumina/silica gel column chromatography. Quality control samples were processed with each batch of samples in the identical manner as that of the actual samples.

1. INTRODUCTION

Assessment of the environmental levels of chlorinated hydrocarbons [pesticides and polychlorinated biphenyls (PCBs)] requires their measurement in tissues at trace levels (parts per billion to parts per trillion).

A tissue sample was homogenized and the sample percent moisture was determined. A subsample (0.5 - 15 g wet weight) was extracted in the presence of sodium sulfate with dichloromethane. Prior to concentration, the percent lipids were determined on the extract. The concentrated extract was then purified using silica gel/alumina column chromatography. Tissue samples require further purification by geopermeation chromatography (GPC) before instrumental analysis for pesticides and PCBs.

2. APPARATUS AND MATERIALS

2.1. Apparatus

Balance, top loading with an accuracy of 0.001 g
Balance, Harvard trip dual pan mechanical balance or equivalent
Centrifuge, Beckman Model TJ-6 or equivalent
Electrobalance, Cahn or equivalent, with an accuracy of 0.0001 mg, set on the 250 mg - 1
 μg scale
Micropipettes, 50 μL, 100 μL
Nitrogen gas evaporation unit, prefiltered, dried N₂
Tissumizer, Pro Scientific, Tek-Mar homogenizer or equivalent
Water bath, heated to 60 - 70 °C

2.2. Labware

Beakers, assorted sizes, borosilicate glass
Boiling chips, Teflon, dichloromethane rinsed

Centrifuge bottles, 200-mL, borosilicate glass
Concentrator tubes, Kuderna-Danish, 25-mL, graduated, with ground glass stoppers
Cutting tools (scissors, scalpels, etc.), stainless steel, washed, dried and rinsed with dichloromethane
Flasks, flat-bottomed, 500-mL capacity, borosilicate glass
Funnels, powder, 85 mm, borosilicate glass
Glass wool, borosilicate glass (combusted)
Pasteur pipettes, 1-mL, disposable, glass
Snyder columns, Kuderna-Danish, 3 ball column
Stoppers, ground glass, 24/40 and 19/22, borosilicate glass

All glassware was washed, and then solvent rinsed or combusted at 440 °C for 4 hr.

2.3. Solvents and reagents

Sodium sulfate (Na_2SO_4) [7757-82-6], granular, anhydrous, J. T. Baker ACS reagent grade or equivalent, combusted at 440 °C for 4 hr and stored at 120 °C prior to use, cooled to room temperature in a dessicator
Hexane (C_6H_8) Burdick and Jackson pesticide grade or equivalent (each lot tested for purity)
Methanol (CH_3OH), Burdick and Jackson pesticide grade or equivalent
Dichloromethane (CH_2Cl_2), Burdick and Jackson pesticide grade or equivalent (each lot tested for purity)
Standards
Matrix spike standard solution, appropriate for pesticide/PCB analysis
Surrogate standard solution, appropriate for pesticide/PCB analysis
Water, Burdick and Jackson HPLC grade or equivalent

3. PROCEDURE

Frozen samples were thawed, either in a refrigerator overnight, or on the counter. After defrosting, the samples were kept in the refrigerator when not needed.

Careful, thorough mixing of the thawed tissue homogenate was essential since oily or fatty materials and water tend to migrate to the top of the sample during the freezing/thawing process.

The top-loading balance was calibrated according to the manufacturer's instructions before using.

Unless the client has specified the sample to be used for QC samples, the amount of the homogenized sample was evaluated for the best selection of the QC samples. matrix spike/matrix spike duplicate (MS/MSD) samples require three times the normal subsample weight. Duplicates require two times the normal subsample weight.

An aliquot of 0.5 - 15 g (depending on tissue type and availability) was weighed into a 200-mL centrifuge bottle on the top loading balance. The method blank was prepared for this extraction procedure contained all solvents, reagents and surrogate spikes and was processed in the same manner as the samples in the extraction batch. Analytical results for a method blank assess the presence of contamination introduced during the extraction procedure.

An appropriate amount of standard reference material (SRM) was prepared according to the supplier's instructions. Matrix spike, matrix spike duplicate and duplicate samples were prepared by weighing sub-aliquot of the tissue sample from the same sample. A laboratory spike sample (LBS) was prepared by spiking a known volume of a certified spiking standard solution into a centrifuge bottle. One hundred mL of dichloromethane was added to each centrifuge bottle containing these samples.

Appropriate amounts of pesticide/PCB surrogate(s) and pesticide/PCB spike solution(s) were added directly to every sample, blank, SRM or labeled compound spiking solution (LCSS), matrix spike, matrix spike duplicate, and/or duplicate. The MS/MSD and LBS samples were processed with each sample batch to determine if the matrix affects analytes recoveries.

Approximately 20 - 50 g of anhydrous sodium sulfate was added to each sample, depending on the amount of sample used and upon the moisture content of the samples. In general, for small sample weights (<5 g), 20 - 30 g of sodium sulfate was added. For larger sample weights (5 - 15 g), 50 g of sodium sulfate was added. Similar amounts of sodium sulfate were added to the method blank.

Tissumizer probes were cleaned with micro-soap and rinsed with tap water. Before using, the probes were rinsed with water, methanol and dichloromethane in that order. The tissue samples and QC samples were macerated for 3 min with the tissumizer.

The sample extract was decanted and filtered through sodium sulfate/glass wool funnel. To filter the samples, the stem of an 85 mm glass powder funnel was lightly packed with glass wool. Approximately 10 g of sodium sulfate was added to the funnel. The funnel was placed on a labeled 500-mL flask and the sodium sulfate was pre-wetted with dichloromethane.

The extraction was repeated two more times with 100-mL aliquots of dichloromethane, and the extracts combined. The sample bottles were rinsed 3 times with small amounts of dichloromethane after the third extraction step and the rinses poured through the filter into the 500-mL flask.

The total extract volume was marked on the 500-mL flask with a permanent marker. An aliquot of 20 mL was removed to determine the lipid content.

A few clean boiling chips were added to the 500-mL flat bottom flask and a 3-ball Snyder column was attached to the flask. The apparatus was placed in a hot water bath (60 - 70 °C) and the sample was concentrated to about 10 mL.

The apparatus was removed from the water bath and the Snyder column was rinsed with a few milliliters of dichloromethane into the 500-mL flask. The concentrated extract was quantitatively transferred to a 25-mL concentrator tube. The 500-mL flat bottom flask was rinsed at least twice with dichloromethane. The total volume of the extract was determined by filling the empty 500-mL flasks to the marker with tap water and volume of the water measured with a 500-mL graduate cylinder.

The sample extract volume in the concentrate tube was further reduced to approximately 2 - 3 mL in a water bath (60 - 70 °C). Then small volumes of hexane were continuously added until the final volume in the concentrator tube was approximately 2 mL and contained only hexane (the solution stopped boiling).

4. ALUMINA/SILICA GEL CHROMATOGRAPHY

The concentrated extracts in 2 mL of hexane were purified with alumina/silica column chromatography. The glass columns (30 cm x 1.1 cm i.d.) with 250-mL reservoir and Teflon stopcock were washed with detergent and rinsed with tap water.

After setting up the columns on the column rack located in a hood, the columns were rinsed three times with approximately 5 mL methanol followed by three rinses of approximately 5 mL of dichloromethane. The solvent completely covers the inside wall of the column.

A plug of combusted glass wool was inserted into the bottom of the column with a clean glass rod. The column was filled with approximately 30 mL of dichloromethane. About 2 cm of combusted sand was added to the column. Deactivation of absorbent was carried out by adding appropriate amount of water to the activated alumina and/or silica gel and then shaking for at least 1 hr to thoroughly homogenize the absorbent with water. Ten grams of alumina deactivated with 1% water was poured into the column while the column was gently tapped. Twenty grams of silica gel deactivated with 5% water was then slurry packed into each column on top of the alumina. Approximately 2 cm of anhydrous sodium sulfate was added into the column on top of the silica gel. The solvent was drained to the top of sodium. The solvent in the column was changed to pentane by adding about 50 mL of pentane into the column and draining the pentane to the surface of the sodium sulfate.

The concentrated sample extract was transferred onto the column using a disposable Pasteur pipette. The solvent was drained to the top of the sodium sulfate and the eluent was collected in a clean flask. The concentrator tube that contained the sample extract was rinsed with 1 mL of pentane/dichloromethane (50/50) twice and the rinses transferred to the column. The solvent was drained and collected in the flask. Approximately 200 mL of pentane/dichloromethane (50/50) was then added to the column. The sample was collected in the flask at a flow rate of about 2 mL/min.

After all the solvent passed through the column and was collected in the flask, boiling chips were added to each flask and a 3-ball Snyder column was attached to the flask. The samples were then concentrated on a hot water bath (60 - 70°C) to approximately 10 mL. The concentrated extract was transferred to a concentration tube and the sample further concentrated to about 0.5 mL.

The concentrated sample was transferred into a tear-shaped (tapered bottom) autosampler vial. The concentration tube was rinsed with small amounts of dichloromethane twice and the rinses were transferred into the vial. The final volume in the autosampler vial was less than 1 mL. If more than 1 mL was in the vial, the sample in the vial was concentrated to about 1 mL using a gentle stream of N₂. The sample extract was then ready for further purification using gel permeation chromatography.

5. QUALITY CONTROL

All quality control samples were processed in a manner identical to actual samples. Quality control samples included a method blank, a standard reference material, a matrix spike, and a matrix spike duplicate with every sample set.

6. CONCLUSIONS

This method provides quantitative extraction of most organic contaminants from tissue samples, including those with high lipid contents.

Procedures for the Extraction of Tissues and Purification of Extracts for Analysis of Polychlorinated Dibenzo-*p*-dioxins and Polychlorinated Dibenzofurans

P. Gardinali, L. Chambers, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Dioxin and furans are compounds of environmental concern because of their high toxicity at low concentrations. Measurements of these contaminants are made at low parts per trillion concentration levels. This requires extensive sample clean-up followed by analysis by high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS).

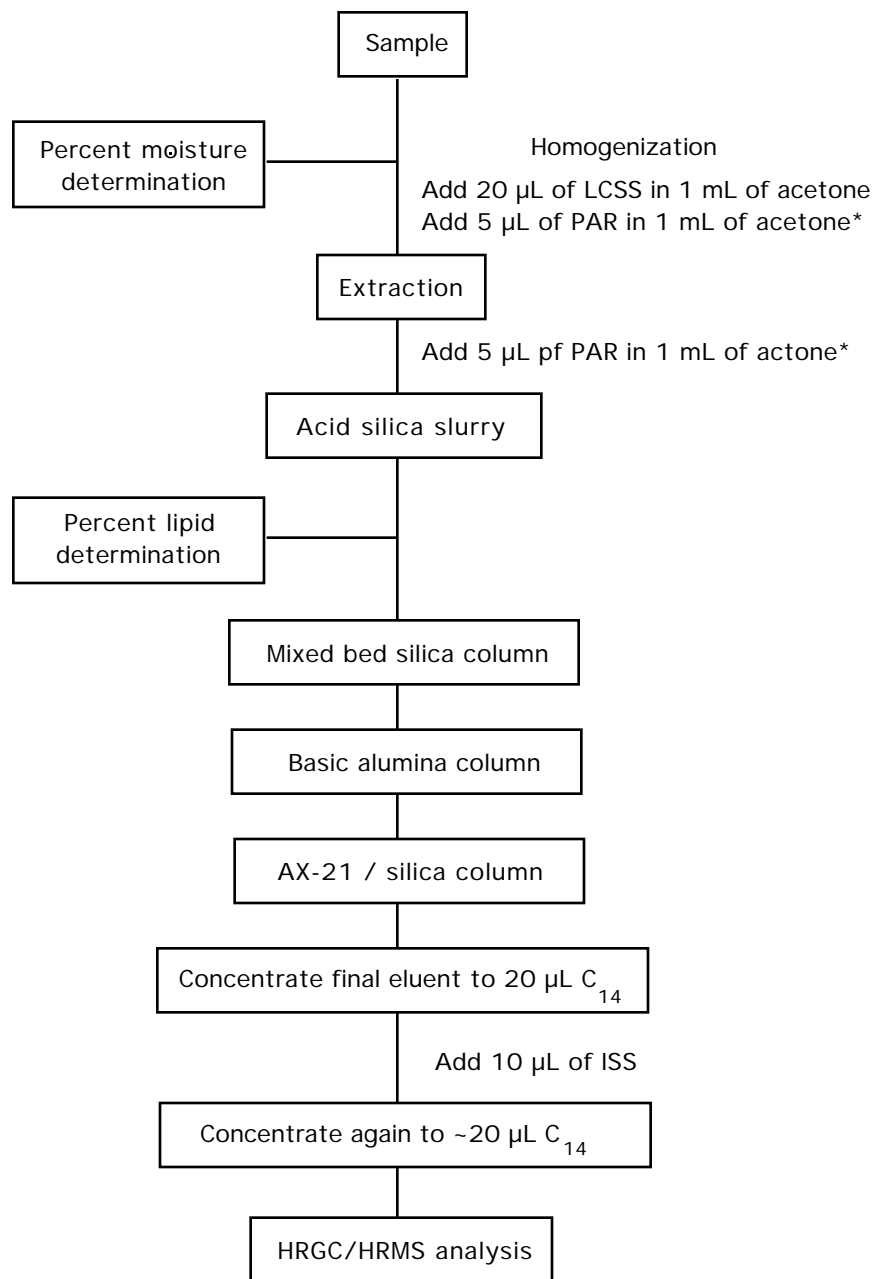
1. PURPOSE

This procedure describes the protocols for the extraction of biological tissue samples and purification of the extracts for the measurement of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs).

This procedure uses matrix-specific extraction and analyte-specific purification steps to allow the determination of the 2,3,7,8-substituted PCDD and PCDF isomers using high resolution gas chromatography/high resolution mass spectrometry. The method provides selective cleanup procedures to aid in the elimination of interfering contaminants. The method is outlined in Figure 1.

Samples were spiked with labeled compound spiking solution (LCSS) containing specified amounts of isotopically $^{13}\text{C}_{12}$ -labeled 2,3,7,8-substituted PCDDs and PCDFs, and homogenized in a 200-mL centrifuge tube. Tissue samples were dried with 50 g of sodium sulfate (Na_2SO_4) and extracted by maceration with a tissumizer three times in 100-mL aliquots of dichloromethane (CH_2Cl_2). After extraction, the samples were spiked with a cleanup recovery standard (CRS), $^{37}\text{Cl}_4$ -2,3,7,8-TCDD, to monitor losses through the extract purification steps. An aliquot for percent lipid determination was then removed. The extract was dried with sodium sulfate, concentrated and subjected to a bulk purification involving a silica gel/sulfuric acid slurry. The extract was then processed through three column chromatographic procedures to remove co-extracted matrix interferences: a mixed bed silica gel column, a basic alumina column, and an activated charcoal column. All concentration steps were performed using tetradecane. Due to the high boiling point of tetradecane compared to other solvents, there was less chances of analyte losses due to volatilization when using this solvent. The final concentration step reduces the extract to approximately 10 μL tetradecane. An internal standard solution (ISS) containing selected $^{13}\text{C}_{12}$ -labeled PCDD was added to all final extracts before analysis bringing the final volume of the extract to 20 μL of tetradecane.

The sample extracts were then ready for identification and determination of the 2,3,7,8-substituted PCDDs and PCDF isomers.



* Precision and recovery spiking solution should be added to MS, MSD, LBS and OPR samples only.

Figure 1. Flow chart for sample processing

Interferences in the matrix, solvents, reagents, glassware and other sample processing hardware may yield discrete artifacts and/or other elevated baselines that cause misinterpretation of chromatographic data. All materials used during the cleanup procedure were demonstrated to be free from interferences by analyzing laboratory method blanks at a frequency of one blank per 20 samples or one with each batch if the number of samples is less than 20.

The minimum level (ML) for each analyte was defined as the level at which the entire system must give a recognizable signal and acceptable calibration point. It was equivalent to the concentration of the lowest calibration standard, assuming that all method specific sample weights, volumes, and procedures were employed. The MLs vary with degree of chlorination. Based on 10 g (dry weight) of tissue extracted, the MLs are listed below (refer to EPA method 1613) .

Chlorination (Number of Cl)	Concentration (ML)
Tetras (4)	1.0 pg/g
Pentas (5)	5.0 pg/g
Hexas (6)	5.0 pg/g
Heptas (7)	5.0 pg/g
Octas (8)	10.0 pg/g

Minimum Levels were reported for all analytes in all samples.

2. QUALITY CONTROL REQUIREMENTS

2.1. Method blank

A method blank was used to demonstrate that the analytical method was free of contaminating interferences. The blank was analyzed by executing all of the specified extraction and purification steps except for the introduction of a sample. The blank was spiked with the labeled compound spiking solution, the clean-up recovery standard, and the internal standard solution (ISS) at the appropriate stages of the preparation. A blank was analyzed with each set of 20 or fewer samples.

2.2. Laboratory blank spike

A laboratory blank spike (LBS) was used to demonstrate analytical precision of the method. It was prepared by executing all of the specified extraction and extraction purification steps except for the introduction of a sample. The LBS was spiked with the precision and recovery spiking solution (PAR), the labeled compound spiking solution (LCSS), the clean-up recovery standard (CRS) and the internal standard solution (ISS) at the appropriate stages of the preparation. A LBS was analyzed with each set of 20 or fewer sample.

2.3. Ongoing precision and recovery

An ongoing precision and recovery (OPR) sample was used to demonstrate analytical precision in the presence of a clean matrix. An OPR was prepared by executing all of the specified extraction and extract purification steps, using 5 mL of corn oil spiked with the PAR, LCSS, CRS, and ISS solutions at the appropriate stage of preparation.

2.4. Matrix spike

A matrix spike (MS) was used to demonstrate analytical precision in the presence of a representative matrix. A MS was prepared by executing all of the specified extraction and purification steps on a selected sample. The MS was spiked with the Precision and Recovery spiking solution (PAR), the LCSS, CRS, and ISS solutions at the appropriate stages of the preparation. The MS was analyzed with each set of 20 or fewer samples. A matrix spike duplicate (MSD) was also analyzed with each set of 20 or fewer samples if required.

2.5. Duplicate

A sample duplicate (DUP) was used to demonstrate matrix homogeneity and analytical precision in the presence of a representative matrix. A duplicate was prepared by executing all of the specified extraction and purification steps on replicate portions of a selected sample. The DUP was spiked with the LCSS, CRS and ISS solutions at the appropriate stages of the preparation. A duplicate was analyzed with each set of 20 or fewer samples.

2.6. Reference materials

NIST SRM 1944 (for sediment) and NRC CARP-1 (for tissues) were used to demonstrate analytical accuracy on a certified reference matrix from an independent source. All of the specified extraction and purification steps were performed on the RM. The RM was spiked with the LCSS, CRS and ISS solutions at the appropriate stages of the preparation. An RM was analyzed with each set of 20 or fewer samples. The SRM represented, as closely as possible, the matrix being analyzed.

2.7. Labeled compound recovery

The percent recovery of the labeled compounds was used to monitor method performance on the sample matrix. All samples were spiked with LCSS, extracted, purified and analyzed according to this method.

3. APPARATUS AND MATERIALS

3.1. Glassware and hardware

The following laboratory glassware and hardware was needed to perform the tissue extraction and purification procedure:

Balance: Top loading with an accuracy of 0.001 g

Chromatography columns: 30 cm x 13 mm (i.d.) with 250-mL reservoir and Teflon stopcock

Desiccator: Stainless steel construction, no plastic components

Erlenmeyer Flask: 500-mL, with Teflon lined PVC flexible cap

Flat Bottom Flasks: 125-, 250- and 500-mL capacity

Funnels: Glass, 100 mm O.D. wide stem

Glass centrifuge tubes: 200-mL capacity

Graduated cylinders: 10-, 25-, 50-, 100- and 4000-mL capacity

Magnetic stirring plate: Nine-Place, independent stirring speeds for all vessels

Micro-liter syringes, Micro-pettor pipettes and Disposable micro-capillary pipettes: 1000-, 250-, 100-, 50-, 25-, 20-, 10-, 5-, 4-, 3-, 2- and 1-mL capacity

Microreaction Vessels: 1.0- and 2.0-mL capacity vials with screw cap and Teflon lined septa

Nitrogen blowdown apparatus: Dry heat source and filtered nitrogen stream
Pipettes, disposable, Pasteur: 150 mm x 5 mm (i.d.)
Pipettes, disposable, Serological: 10 mL x 8 mm (i.d.) for preparation of the carbon column
Rotary-evaporator trap: 100 mL
Rotary-evaporator: Buchi, model R144 or equivalent and a low temperature water recirculator to assure an adequate recovery of the solvents being used.
Stainless steel forceps
Stainless steel knife or shears: for dissecting tissue samples
Tissumizers: Teckmar Polytron homogenizer or equivalent
Vials and Teflon lined caps: 40 mL capacity
Water bath: heated to 60 - 70 °C

No grease, oil, or any other lubricants were used on the desiccator or the rotavap junctions to avoid contamination.

3.2. Reagents and consumable materials

1 N Sodium hydroxide (NaOH), ACS reagent grade or better from J. T. Baker, Cat. # 3728-01 or equivalent; prepare a 1 N solution in purified water
Acetone (C₃H₆O): Burdick and Jackson; Cat. # 010-4; Grade: High Purity Solvent
Cyclohexane (C₆H₆) [110-82-7], high purity solvent, cat. 053-4. Burdick and Jackson, Muskegon, MI.
Hexane (C₆H₁₄) [110-54-3], capillary GC/GC-MS solvent grade, cat. GC60393-4. Burdick and Jackson, Muskegon, MI.
Methanol (CH₃OH) [67-56-1], high purity solvent, cat. 230-4. Burdick and Jackson, Muskegon, MI.
Dichloromethane (CCl₂H₂): Burdick and Jackson; Cat. # 300-4; Grade: High Purity
Purified water: HPLC Grade or better
Sulfuric acid (H₂SO₄), concentrated J. T. Baker; Cat. # JT9673-00 or equivalent; 95.0-98.0%; suitable for trace metal analysis.
Tetradecane (C₁₀H₂₂): Fluca Chemical Co.; Cat. # 87140; Highest Grade Available
Toluene (C₇H₈): Burdick and Jackson; Cat. # 347-4; Grade: High Purity Solvent
Prepurified nitrogen Gas: Nitrogen gas used in final evaporation is purified through an activated carbon trap. No rubber or plastic is used in the nitrogen delivery system.
Silica gel: Desiccating 8 mesh indication
Anhydrous sodium sulfate (NaSO₄): J. T. Baker; Cat. #3891-05 or equivalent; Reagent Grade: Combusted at 400 °C for 4 hr and stored covered with aluminum foil at 130 °C
Silica gel for column chromatography: E. M. Science; Cat. #7734-5 or equivalent; Silica Gel 60, 70-230 mesh. Activated by heating for more than 16 hr at 170 °C. Stored covered with aluminum foil at 170 °C.
Basic alumina: E. M. Science; Cat. #AX0612-3 or equivalent; Aluminum Oxide, basic, chromatographic grade, 80-200 mesh, Alcoa Type GC-20. Combusted at 600 °C for more than 16 hr. Stored covered with aluminum foil at 130 °C. Used within 3 days, then reactivated or discarded after that. Recoveries of the targeted analytes were strongly influenced by the degree of activation of the alumina. Extreme caution must be taken to avoid use of the adsorbent after 3 days from the original activation.
Activated carbon: AX-21 Carbon (Anderson Development Co.): Wash 100 g of AX-21 carbon powder (as received) by suspending in 300 mL methanol and subsequently vacuum filtering through a pre-cleaned glass fiber filter fitted in a 350-mL Buchner funnel Rinse two times with 100 mL methanol and vacuum dry. Keep the washed AX-21 carbon at 130 °C for a minimum of 72 hr. Then store in the same oven covered with aluminum foil at 130 °C.

AX-21 carbon/silica gel: Combine 5 g of prepared AX-21 Carbon with 95 g of prepared Silica Gel in a 500-mL Erlenmeyer with a Teflon lined PVC flexible screw cap (do not put the cap into the oven). Blend by shaking until a uniform color is achieved. Activate the mixture at 130 °C for a minimum of 24 hr and store covered with aluminum foil in the same oven at 130 °C.

H₂SO₄/silica gel: Prepare by mixing 100 g of concentrated H₂SO₄ with 150 g of activated silica gel. Shake and roll on a roller table for a minimum 2 hr. Store in 500-mL Erlenmeyer flask with Teflon lined PVC flexible screw cap at room temperature.

NaOH/silica gel: Prepare by mixing 33 mL of 1 N NaOH solution and 67 g of activated silica gel. Store in 500-mL Erlenmeyer flask with Teflon-lined PVC flexible screw cap at room temperature.

4.2. Analytical standards

Analytical standards were purchased as solutions with certificates of purity, concentration and authenticity. The LCSS, CRS, PAR and ISS solutions were used as received from the manufacturer without further treatment. When not being used, standards were stored in the dark at 4 ± 2 °C in amber glass screw-capped vials with PTFE-lined caps.

4.2.1. Labeled compound spiking solution

The LCSS contained the fifteen ¹³C₁₂-labeled PCDD and PCDF quantitation standards in nonane at the nominal concentrations listed in Table 1. Twenty µL of the LCSS were diluted in 1 mL of acetone and spiked into each tissue sample prior to extraction.

4.2.2. Cleanup recovery standard

The CRS solution contained the cleanup recovery standard contained 40 pg/µL of ³⁷Cl₄-2,3,7,8-TCDD. Five µL of this solution was spiked into each sample extract immediately after the extraction and before any cleanup procedures were initiated.

4.2.3. Precision and recovery standard

This solution contained the seventeen 2,3,7,8-substituted native PCDD and PCDF isomers at the nominal concentrations listed on Table 2. Five µL of the precision and recovery standard (PAR) solution were diluted in 1 mL of acetone and spiked into the selected LBS, MS and MSD prior to extraction.

Table 1. Composition of the labeled compound spiking solution (pg/ μ L of nonane).

Analyte	Concentration (pg/ μ L)
$^{13}\text{C}_{12}$ - 2,3,7,8-TCDD	100
$^{13}\text{C}_{12}$ - 2,3,7,8-TCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,7,8-PeCDD	100
$^{13}\text{C}_{12}$ - 1,2,3,7,8-PeCDF	100
$^{13}\text{C}_{12}$ - 2,3,4,7,8-PeCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,4,7,8-HxCDD	100
$^{13}\text{C}_{12}$ - 1,2,3,6,7,8-HxCDD	100
$^{13}\text{C}_{12}$ - 1,2,3,4,7,8-HxCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,6,7,8-HxCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,7,8,9-HxCDF	100
$^{13}\text{C}_{12}$ - 2,3,4,6,7,8-HxCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,4,6,7,8-HpCDD	100
$^{13}\text{C}_{12}$ - 1,2,3,4,6,7,8-HpCDF	100
$^{13}\text{C}_{12}$ - 1,2,3,4,7,8,9-HpCDF	100
$^{13}\text{C}_{12}$ - OCDD	200

Table 2. Composition of the precision and recovery standard solution (pg/ μ L of nonane).

Analyte	Concentration (pg/ μ L)
2,3,7,8-TCDD	40
2,3,7,8-TCDF	40
1,2,3,7,8-PeCDD	200
1,2,3,7,8-PeCDF	200
2,3,4,7,8-PeCDF	200
1,2,3,4,7,8-HxCDD	200
1,2,3,6,7,8-HxCDD	200
1,2,3,7,8,9-HxCDD	200
1,2,3,4,7,8-HxCDF	200
1,2,3,6,7,8-HxCDF	200
1,2,3,7,8,9-HxCDF	200
2,3,4,6,7,8-HxCDF	200
1,2,3,4,6,7,8-HpCDD	200
1,2,3,4,6,7,8-HpCDF	200
1,2,3,4,7,8,9-HpCDF	200
OCDD	400
OCDF	400

Table 3. Composition of the internal standard solution (pg/ μ L of nonane).

Analyte	Concentration (pg/ μ L)
$^{13}\text{C}_{12}$ -1,2,3,4-TCDD	200
$^{13}\text{C}_{12}$ -1,2,3,7,8,9-HxCDD	200

4.2.4. Internal standard solution

The ISS contained two $^{13}\text{C}_{12}$ -labeled PCDD isomers at the nominal concentrations listed in Table 3. Ten μ L of the ISS were added to the final sample extract before HRGC/HRMS analyses to determine the percent recoveries for the $^{13}\text{C}_{12}$ -labeled compounds.

4.3. Reference materials

4.3.1. CARP-1

CARP-1 is a ground whole carp reference material for organochlorine compounds which contains nine of the seventeen target analytes with certified concentrations. This reference material is available from the National Research Council of Canada.

4.3.2. EDF-2524

EDF-2524 is a clean, natural matrix reference material (fish) gathered in clean waters with a history of sustaining relatively untainted fish. One analyte, 2,3,7,8-TCDF, is present at a certified concentration. This reference material is available from Cambridge Isotope Laboratories.

4.3.3. EDF-2525

EDF-2525 is a contaminated natural matrix reference material (fish) gathered from a polluted Great Lakes region which contains selected target analytes at certified concentrations within the calibration range for this method. This reference material is available from Cambridge Isotope Laboratories.

4.3.4. EDF-2526

EDF-2526 is a fortified natural matrix reference material (fish) which contains all seventeen target analytes at certified concentrations within the calibration range for this method. This reference material is available from Cambridge Isotope Laboratories.

4.4. Miscellaneous materials.

Boiling chips: Teflon, solvent rinsed with methane and dichloromethane prior to use.

Glass wool: Combusted at 400 °C for at least 4 hr.

Stirring bars: Teflon coated.

Glass fiber filter paper: Gelman Type A/E or equivalent, Whatmann GF/F or equivalent

5. EXTRACTION AND CLEANUP PROCEDURES

5.1. Sample Preparation

An aliquot of the macerated tissue to be extracted was weighed into a centrifuge tube. The tissue sample size was 10 - 15 g (wet weight), although smaller amounts may be used depending on the matrix and sample availability.

All glassware was rinsed with methanol, acetone, dichloromethane, toluene, and hexane prior to use in this extraction procedure.

5.2. Extraction procedure

The tissumizer probe was washed with soap (Micro), rinsed with tap water, and then rinsed with methanol, acetone, dichloromethane, toluene, and hexane prior to use.

The appropriate amount of tissue was weighed in a solvent-rinsed, labeled 200-mL centrifuge bottle.

The dioxin LCSS (20 μ L) was added to all the samples. The LCSS was first added to 1 mL acetone in a test tube. This acetone solution was then quantitatively transferred to the appropriate sample or blank. The test tube was rinsed three times with acetone, and the rinse solutions were also transferred to the corresponding sample. The PAR standard was added to the LCSS/acetone mixture which was used for the LBS, MS, and MSD as required. This acetone solution was then quantitatively transferred to the appropriate sample or blank. The test tube was rinsed three times with acetone, and the rinse solutions were also transferred to the corresponding sample. Dichloromethane (100 mL) was added to each sample.

Combusted sodium sulfate (40 mL) was added to each sample immediately before tissumizing. The tissue was macerated with a tissumizer for three min. A large funnel was placed on top of a labeled 500-mL flat bottom flask. The stem was plugged with glass wool, and about 2 inches of sodium sulfate was added. The sodium sulfate was wetted with dichloromethane. The dichloromethane extract was decanted through the funnel into the labeled 500-mL flat bottom flask. Another 100 mL of dichloromethane solution was added to the centrifuge bottle and the extraction step was repeated two more times (a total of three dichloromethane extractions).

5.3. Addition of cleanup recovery standard

The CRS (5 μ L) was added to each extract before proceeding to next step.

5.4. Solvent exchange to hexane.

Solvent-cleaned Teflon boiling chips were added to the remaining sample extract in the 500-mL flask. The Teflon boiling chips were cleaned by rinsing repeatedly with methanol and dichloromethane (6 - 7 times) and stored in a closed container before use. A three-ball Snyder column was placed on top of the 500-mL flask. The flask was placed on a hot water bath (60 - 70 °C) and the contents of the flask reduced to about 10 mL. The flask was removed from the water bath and allowed to cool. The Snyder column was rinsed with hexane. Hexane (120 mL) was added from a graduated cylinder and the flask was placed back on the bath. The extract was boiled until the top of the Snyder column became opaque, indicating that the dichloromethane was gone. After the dichloromethane had completely boiled off, the flask was removed from the water bath allowed to cool. The Snyder column was rinsed with hexane and removed. The sample flask was capped with a glass stopper.

5.5. Sulfuric acid/silica gel slurry

The H₂SO₄/silica gel slurry was prepared as described in Section 3.2.1. The labeled flasks containing the hexane sample extracts were placed on a magnetic stirring plate. A Teflon-coated magnetic stirring bar was added to each flask. Prior to use, the magnetic stirring bars were rinsed with solvents in the following order: methanol, acetone, dichloromethane, toluene, and hexane. Metal forceps were rinsed with hexane and used to place the stirring bar in the sample. The forceps were rinsed with hexane after picking up each stirring bar. The magnetic stirring plate was turned on and set at medium, making sure the sample did not splash against the side of the flask. A 50-mL beaker with approximately 40 g of the H₂SO₄/silica gel slurry was added to each flask. The samples were stirred for a minimum of 2 hr. The samples were filtered using a large funnel plugged with combusted glass wool containing combusted sodium sulfate. The entire contents of the 500-mL flask were poured into the funnel and the flask rinsed with hexane repeatedly until all the material in the original 500-mL flask was transferred. After the liquid completely drained through the funnel, the sodium sulfate was rinsed in the funnel three times with 5 mL of hexane. The 250-mL sample flask was capped with a glass stopper.

5.6. Rotary evaporation

Fifty μ L of tetradecane was added to each sample extract using a 50 μ L micropipette. The rotavap (Rotavapor, Buchi, Brinkman RE121) was set to 250 mbars and the hexane evaporated. The rotavap trap was rinsed with methanol, acetone, dichloromethane, toluene, and hexane before the sample flask was attached. The samples were rotavapped to a final volume 50 μ L of tetradecane.

5.7. Mixed bed silica columns

A 300 mm x 13 mm (i.d.) column with a 250-mL reservoir was clamped upside down and rinsed with methanol, acetone, dichloromethane, toluene, and hexane making sure that the solvents completely coated and rinsed the inside walls of the column.

The column was turned right-side-up and packed with the following materials added first (bottom) to last (top):

- Combusted glass wool plug
- 1 scoop of combusted sand (about 1 cm)
- 1 g activated silica gel
- 4 g NaOH/silica gel (see Section 3.2.1)
- 1 g activated silica gel
- 8 g H₂SO₄/Silica Gel acid slurry
- 2 g activated silica gel
- 1 scoop combusted sodium sulfate (about 1 cm)

A waste jar was placed under the completed column and 25 mL hexane was added as a pre-rinse. The stopcock was open. The stopcock was closed when the level of hexane was 1 cm above the sodium sulfate. The bottom tip of the column was rinsed with hexane. The waste jar was replaced with a 250-mL flat bottom flask. The stopcock was opened and the sample added to the column using a clean, combusted disposable pipette. The 250-mL flask was rinsed three times with 2 mL hexane and the rinsate was added to the column using the same pipette for sample transfer and rinsates. The solvent was drained to the sodium sulfate level. One hundred and 20 mL of hexane was added to the column with the stopcock open and the hexane eluted at a

rate of 1 to 2 drops per second. The column was covered with a piece of aluminum foil. After the hexane had completely passed through the column, 50 μ L tetradecane was added to the collection flask. The tetradecane was evaporated using a rotavap to a final volume of 50 μ L.

5.8. Basic alumina column

A 250 mm x 13 mm (i.d.) column with a 200-mL reservoir was clamped upside down and rinsed with methanol, acetone, dichloromethane, toluene, and hexane making sure the solvents completely coated and rinsed the inside walls of the column. The column was turned right-side-up and packed with the following materials added first (bottom) to last (top):

- Combusted glass wool
- One scoop combusted sand (about 1 cm)
- 6 g activated alumina (see Section 3.2.1)
- 1 scoop combusted sodium sulfate (about 1 cm)

A waste jar was placed under the column and the column rinsed with 25 mL of hexane. The column stopcock was open. The stopcock was closed when the level of the solvent was 1 cm above the sodium sulfate. The bottom tip of the column was rinsed with hexane. A labeled 125-mL flat bottom flask was placed underneath the column. With the stopcock closed, the sample extract was added to the column using a combusted disposable pipette with a glass wool plug. The flask was rinsed three times with 2 mL hexane and the rinsate added to the column using the same disposable pipette for the sample and rinsate transfer. The solvent was drained to 1 cm above the surface of the sodium sulfate. Hexane (60 mL) was added to the reservoir and drained to the sodium sulfate level and the stopcock closed. This fraction was kept until sample analysis was completed. The waste jar was replaced with a labeled 125-mL flat bottom flask.

A solvent mix (40 mL) of hexane/dichloromethane (70:30) was added to the top of the column.

The 70:30 solvent was made by adding 300 mL dichloromethane to 700 mL hexane in a 1 liter graduated cylinder. The 70:30 solution was transferred to a 1000-mL flat bottom flask and mixed thoroughly before each use. The solvent mixture was used for one day and was remade for each set of samples.

The stopcock was adjusted so the flow was one drip per second and the column was capped with a piece of solvent rinsed aluminum foil. After the 70:30 solvent completely drained to the sodium sulfate level, 50 μ L of tetradecane was added to the 125-mL sample flask. The solution was evaporated with a rotavap to a final volume of 50 μ L tetradecane.

5.9. Charcoal column

A glass fiber filter paper was placed in a 350-mL Buchner funnel and 100 g of AX-21 carbon powder was placed on top of the filter paper. Methanol (300 mL) was added to the carbon and vacuum filtered. The process was repeated two more times with methanol, then vacuum was used to dry the carbon for 10 sec. The washed carbon was placed in an oven at 130 °C for a minimum of 72 hr before use.

Activated, room temperature silica gel (95 g) was placed in a 500-mL Erlenmeyer flask. Washed and cooled AX-21 carbon (5 g) was added to the flask. The flask was capped with a piece of aluminum foil and shaken by hand until a uniform black color was achieved.

Charcoal columns were made by cutting off both ends of a clean disposable serological pipette. The pipettes were placed in a 4-L beaker and combusted for 4 hr at 440 °C.

The column was packed with combusted glass wool approximately 4 cm from the end. The column was placed on the rack so the glass wool plug was at the bottom. With a small funnel and a spatula, 1 cm of activated silica gel was added to the top of the glass wool plug. One gram of 5% AX-21 carbon/silica gel was added being careful not to shake or pack the column. The open end was plugged with combusted glass wool. The glass wool touched the charcoal but did not compress it down.

Outline of charcoal column (Section 5.9):

Amount	Materials
(TOP)	Glass wool plug
1.0 g	5% AX-21 carbon/silica gel
1 cm	Activated silica gel
(BOTTOM)	Glass wool plug

Twenty-two mL of 50:50 dichloromethane/cyclohexane was added to a labeled 25-mL concentrator tube. The 50:50 solvent was made by adding equal volume of dichloromethane and cyclohexane. The 50:50 solvent was used on the day it was prepared and the remaining solvent was discarded. Fresh solution was prepared for each new set of samples. A waste jar was placed under the column. The column was pre-rinsed with 5 mL of 50:50 solvent and the solvent allowed to drain to the glass wool level. The column was turned over and rinsed with another 5 mL of 50:50 solvent, collecting the solvent in the waste jar. The solvent was drained to the glass wool level. The combusted disposable pipette with a glass wool plug was used to add concentrated sample extract from the 125-mL flask (Section 6.10) to the top of the column. The flask was rinsed with 2 mL of 50:50 solvent three times, using the same pipette for the removal of the sample and its rinsate, and adding the rinsate to the column. After the solvent and sample drained to the glass wool level, the remaining 50:50 solvent was added to the column.

Twenty mL of 75:20:5 solvent mixture was added to the concentrator tubes.

The 75:20:5 solvent mixture was made by mixing 750 mL dichloromethane with 200 mL of methanol and 50 mL of toluene in a 1000-mL graduated cylinder. This mixture was only good for one day and was discarded after use. The 20 mL of 75:20:5 was pipetted to the charcoal column and drained into the waste jar.

When the solvent completely drained down to the glass wool level and stopped dripping, the column was turned over. A labeled 125-mL flat bottom flask was switched with the waste jar. The long side (silica gel at the bottom) of the charcoal column was upward. The column was eluted with 50 mL of toluene. After the toluene completely drained into the 125-mL flask, 20 μ L tetradecane was added to the sample extract and rotovapped to a final volume of 20 μ L tetradecane.

5.10. Final evaporation

A 2-mL tear-drop vial was prepared by adding ten (10) μ L of tetradecane to each vial. The samples from the 125-mL flask were transferred to the vials using a disposable, combusted Pasteur pipette with a glass wool plug. The vials were filled to three quarters of their volume. New combusted disposable glass Pasteur pipettes were placed in the nitrogen blowdown apparatus. The Teflon nitrogen hoses were inserted into the pipettes. The 2-mL tear-drop vials containing the sample were placed on the nitrogen blowdown apparatus and the heat turned on. The pipette was inserted into the vials, making sure the tip of the pipette did not touch the sample. The individual controls adjusted until a slight movement of the sample surface was

seen. As the volume of the vial was reduced, rinsate from the 125-mL flask was added to the tear-drop vial. Each 125-mL flask was rinsed three times with about 1 mL of toluene and the rinsate added to the tear-drop vial. The sample was reduced to a final volume of 20 μ L tetradecane. The vials were removed and placed on a vial tray, leaving the vials uncapped. Ten μ L of ISS was added to each sample using a separate disposable micropipette for each sample. The vials were replaced on the blowdown apparatus making sure they were in the same position as before. The sample volumes were reduced to a final volume of 20 μ L of tetradecane. The samples were capped.

6. CONCLUSIONS

This method is an adaptation of EPA regulatory methods that have proven to be reliable extractions of dioxin and furans at trace (μ g/g) concentrations.

Extraction of Sediments for Butyltin Analysis

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Measurements of organotin compounds in soil/sediment require the isolation of these contaminants from the matrices. An aliquot of the freeze-dried sediment sample was extracted four times with 0.2% tropolone in dichloromethane. The extract was concentrated by a Kuderna-Danish technique and the solvent exchanged to hexane. Organotin compounds were hexylated with Grignard reagent and the derivatized extract purified using silica gel/alumina column chromatography prior to instrumental analysis. Quality control samples were processed with each batch of samples in a manner identical to that used for the actual samples.

1. INTRODUCTION

Assessment of the environmental impact of butyltins (i.e., tetra-, tri-, di-, and mono-butyltins) requires their measurement in sediments at trace levels (parts per billion to parts per trillion).

Freeze-dried samples were serially extracted with 0.2% tropolone in dichloromethane. The extract was then hexylated with a Grignard reagent (see Section 5.3). The hexylated extract was dried and concentrated. A silica gel/alumina column cleanup step was used prior to instrumental analysis to remove matrix interferences.

2. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

Sediment samples were collected and placed in pre-cleaned mason jars. Sediment samples were stored in the dark at or below -20 °C. Sample extracts were stored in the dark at 4 °C.

3. INTERFERENCES

Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing labware that lead to false positive detections. All materials used in this method were routinely demonstrated to be free from interferences by processing procedural blanks using the same procedure as that used for the samples (one blank per 20 samples or each batch whichever was more frequent).

Matrix interferences result from the co-extraction of compounds other than the analytes of interest. Previous analyses of sediments indicate that matrix interferences were generally low.

4. APPARATUS AND MATERIALS

4.1. Labware and apparatus

Glassware was cleaned by washing with detergent and water and rinsing with water. The glassware was then combusted in a muffle furnace at 440 °C for at least 4 hr. Solvent rinses of methanol to dry the glassware followed by dichloromethane may be substituted for the muffle furnace combustion. After drying and cooling, the glassware was sealed and stored in a clean environment to prevent the accumulation of dust or other contaminants. Stored glassware was capped with combusted aluminum foil.

The following glassware was needed to perform the sediment extraction and purification procedures:

- Centrifuge tubes: Corex 50-mL with Teflon-lined screw caps
- Chromatographic column: 300 mm x 10 mm i.d., with Pyrex glass wool at bottom and Teflon stopcock
- Concentrator tube: Kuderna-Danish - 25-mL, graduated. Ground glass stoppers are used to prevent evaporation of extracts.
- Disposable glass Pasteur pipettes: 1- and 3 mL
- Drying column: 20 mm i.d. Pyrex chromatographic column with glass wool at bottom and Teflon Stopcock, or Pyrex glass funnel
- Evaporative flask: Kuderna-Danish - 250- and 500-mL flat bottom flask
- Gas evaporation unit: Nitrogen
- Graduated cylinder: 1 or 2 L
- Micro reaction vessels: 1.0-mL or 2.0-mL autosampler vials with crimp cap septa
- Snyder column: Kuderna-Danish - 3 ball column
- Syringes: 10 or 25 μ L
- Teflon boiling chips: Solvent extracted
- Vials: 1-mL to 7-mL glass vials with Teflon-lined caps
- Water bath: heated to 60-70 °C

Volumetric glassware for sample measurement or introduction of internal standards was calibrated.

4.2. Reagents

6 N HCl, 36.5-38%

Alumina oxide: Basic Brockmann 1, standard grade 150 mesh Aldrich 19, 744-3 or equivalent. Combust 4 hr at 440 °C. Store at 120 °C prior to use.

Hexylmagnesium bromide: 0.5 M in diethyl ether

Internal standard solution

Matrix spike standard

Reagent water: Water containing no analytes above the method detection limit (i.e., HPLC water).

Silica gel: Grade 923, 100-200 mesh Aldrich 21,447-7 or equivalent. Store at 170 °C before use.

Sodium sulfate: (ACS) Granular, anhydrous (purified by heating at 440 °C for 4 hr in a shallow tray, or other suitable method).

Solvents: Methanol (for rinsing), dichloromethane, hexane, pentane (pesticide quality or equivalent).

Surrogate spiking solutions

Tropolone: Aldrich T8, 970-2, purity 98%

5. PROCEDURE

5.1. Sample extraction

An aliquot of about 15 g of freeze dried sediment was weighed into a 50-mL Corex tube. Appropriate amounts of surrogate standards (approximately 10 times the MDL) were added to all samples, spikes, and blanks. Appropriate amounts of spiking standards were added to the matrix spike/matrix spike duplicate (MS/MSD) and/or laboratory blank spike (LBS).

Approximately 30 - 40 mL of 0.2% tropolone in dichloromethane was added to the Corex tube. The Cortex tube was then sealed with a Teflon screw cap and was shaken on a wrist action shaker for 1 hr. The sample was centrifuged and the extract decanted into a 500-mL flat bottom flask. Another 30 mL of 0.2% tropolone in dichloromethane was added into the Cortex tube and the extraction was repeated three more times.

The sample extract was concentrated in a water bath. A few clean boiling chips were added to the 500-mL flat bottom flask and a 3-ball Snyder column attached to the flask. The apparatus was placed in a hot water bath (60 - 70 °C) and the sample concentrated to about 20 mL. The concentrated extract was transferred to a 50-mL centrifuge tube and further concentrated to 4 - 10 mL. The solvent was exchanged to hexane by adding about 30 mL of hexane to the extract and continuing the concentration to a final volume of about 10 - 20 mL at which point only hexane remained.

5.2. Hexylation

Extreme care was taken when handling Grignard reagents because it can react violently with water. The test tube that contained sample extract was purged with nitrogen and 2 mL of hexylmagnesium bromide (2 M; Grignard reagent, see below) was added to the sample extract container. The test tube was then sealed with a Teflon lined screw cap and placed in a 70 °C water bath for 30 min to hexylate the sample. After cooling, the excessive Grignard reagent was neutralized with 5 mL of 6 N HCl. The hexylated sample was shaken vigorously before the organic phase was transferred with a pasture pipette into a 125-mL flat bottom flask. About 15 mL of pentane:CH₂Cl₂ (3/1, v/v) was added to the aqueous phase in the 50-mL centrifuge tube and the sample shaken vigorously. The organic phase was transferred to the 125-mL flat bottom flask. The pentane:CH₂Cl₂ extraction was repeated one more time.

The combined hexylated extract in the 125-mL flask was dried by adding 10 - 20 g of anhydrous Na₂SO₄ and then concentrated to 1 - 10 mL in a water bath (60 - 70 °C). The concentrated extract was transferred into a concentrator tube and further concentrated to about 2 mL.

Grignard reagent was made from magnesium and bromohexane in anhydrous ether. The apparatus used for making Grignard reagent consisted of a 1-L round bottom flask, a Y-shaped connector, a condenser, a graduated addition cylinder (250-mL) with a stopcock, and a desiccating tube filled with desiccant. The condenser and the graduate addition cylinder were connected to the flask via the Y-shaped connector. The desiccating tube was placed on top of the condenser so that water vapor could not react with the Grignard reagent being made. All the glassware was cleaned by rinsing with methanol followed by dichloromethane. After the glassware was dried in an oven for a few min, it was placed in a dessicator before use.

Approximately 15 g of magnesium turning (98% purity, Aldrich) was weighed into the 1-L flask. Approximately 100 mL of anhydrous ethyl ether was added immediately to the flask to

completely cover the magnesium turnings. The condenser and the addition cylinder were connected to the flask via the Y-shaped connector. The apparatus was flushed with a stream of N₂ for a few min to remove any water vapor. It was then sealed with a desiccating tube on top of the condenser and by turning off the stopcock at the bottom of the addition cylinder. It was critical to maintain a moisture-free environment inside the apparatus because the Grignard reagent reacts violently with water. A pre-mixed solution of 150 mL of anhydrous ethyl ether and 90 mL of bromohexane was added to the addition cylinder. The stopcock at the bottom of the addition cylinder was then opened to let the ether/bromohexane slowly drip into the flask. The flask was warmed on the water bath (60 - 65 °C) until the solution the flask started to boil. The apparatus was then removed from the water bath and gently swirled. The process of heating on the water bath and swirling of the apparatus was repeated until the solution in the flask started to react with the magnesium turnings continuously without heating, or nearly all of the bromohexane/ether was in the flask. When the reaction became violent, the flask was chilled in an ice water bath. When most of the ether/bromohexane had been added to the flask, the stopcock at the bottom of the addition cylinder was closed. The apparatus was then placed in an ice water bath for about one hour. The Grignard reagent in the flask was then transferred into an amber bottle. An aliquot of reagent was tested for its reactivity by testing using with butyltin standards. Once the satisfactory reactivity of the reagent (as determined by the recoveries of the derivatized butyltin compounds by GC-FPD) was achieved, the reagent was used for hexylation of actual samples.

5.3. Silica gel/alumina column cleanup

Glass chromatographic columns (30 cm x 1 cm with 300-mL of reservoir) were filled with 30 mL of pentane. Approximately 10 g of silica gel was poured into the column while the column was gently tapped to evenly distribute the silica gel. Alternatively, a slurry of silica gel in pentane may be used to pack the column. About 10 g of alumina was then added to the top of the silica gel. The solvent in the column was drained to the top surface of the absorbent. The concentrated hexylated sample extract in 2 mL of hexane was transferred onto the column. The extract container was rinsed twice with 1 mL of pentane and the rinses were added to the column. The eluent from the column was collected in a 250-mL flat bottom flask. Another 50 mL of pentane was added to the column and the sample was eluted at a flow rate of approximately 2 mL/min. The eluent was collected in the 250-mL flat bottom flask. The collected sample fraction was concentrated to 0.5 to 1 mL on a water bath.

6. QUALITY CONTROL

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 set, whichever was more frequent. Analyte levels in the Blank were less than three times the method detection limit (MDL). If blank levels for any component were above three times MDL, the whole sample set was re-extracted and reanalyzed. If insufficient sample was available for re-extraction, the data was reported and appropriately qualified.

Matrix spike/matrix spike duplicate (MS/MSD) samples were run with every 20 samples, or with every sample set, whichever was more frequent. The appropriate spiking level was ten times the MDL.

Reference materials were extracted with each set of sample and were analyzed when available. NRC PACS-1 was a sediment reference material certified for butyltins but it is not longer available. A replacement is currently under preparation.

7. CONCLUSIONS

The method of extraction of dried sediment with a shaker and the complexing agent tropolone followed by hexylation provides reliable extraction and hexylation of tributyltin as well as other organotins.

Extraction of Tissues for Butyltin Analysis

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Measurements of organotin compounds in biological tissues require the isolation of these contaminants from the matrices. An aliquot of the homogenized tissue sample was extracted three times by maceration with 0.05% tropolone in dichloromethane in the presence of sodium sulfate. The extract was concentrated by Kuderna-Danish technique and the solvent was exchanged to 10 mL of hexane. Organotin compounds were hexylated with Grignard reagent and the derivatized extract was purified using silica gel/alumina chromatography before instrumental analysis. Quality control samples were processed with each bath of samples in a manner identical to that of the actual samples.

1. INTRODUCTION

Assessment of the environmental impact of butyltins, i.e., tetra-, tri-, di-, and monobutyltins, requires their measurement in tissues at trace levels (parts per billion to parts per trillion).

Tissue samples were serially extracted with 0.05% tropolone in dichloromethane using a Tissumizer. The extracts were then hexylated with Grignard reagent (see Section 5.3). The hexylated extract was dried and concentrated. A silica gel/alumina column cleanup step was used before the instrumental analysis to remove matrix interferences. The extract was then ready for analysis of butyltins.

2. SAMPLE COLLECTION, PRESERVATION AND STORAGE

Tissue samples were collected and placed in combusted glass jars and were frozen at -20 °C as soon as possible. The samples were stored at -20 °C in the dark. Sample extracts were stored in the dark at or below 4 °C.

3. INTERFERENCES

Method interferences may be caused by contaminants in solvents, reagent, glassware, and other sample processing hardware that lead to false positive detections. All materials used in this method were routinely demonstrated to be free from interferences by processing procedural blanks in a manner identical to that used for the samples (one blank per 20 samples or each batch, whichever was more frequent).

Matrix interferences may be caused by compounds other than the analytes of interest that were coextracted from the sample. Biogenic materials that cause interferences in the analysis of tissue extracts were removed prior to GC/FPD analysis by silica gel/alumina chromatography.

4. APPARATUS AND MATERIALS

4.1. Labware and apparatus

Glassware was cleaned by washing with detergent (micro cleaning solution) and rinsing with water. The glassware was then combusted in a muffle furnace at 440 °C for at least 4 hr. Solvent rinses of methanol to dry followed by dichloromethane may be substituted for the muffle furnace heating. After drying and cooling, glassware was sealed and stored in a clean environment to prevent the accumulation of dust or other contaminants.

The following labware was needed to perform the tissue digestion and purification procedure:

Balance: Top loading with an accuracy of 0.01 g
Boiling chips: Solvent extracted, Teflon
Chromatographic column: 300 mm x 10 mm, with Pyrex glass wool plug at bottom and Teflon stopcock.
Concentrator tubes: Kuderna-Danish 25-mL, graduated with ground glass stoppers.
Disposable glass Pasteur pipettes: 1- and 3-mL
Electrobalance: Cahn or equivalent, with an accuracy of 0.0001 mg
Erlenmeyer flask: Various sizes
Flat bottom flasks: 125-mL and 500-mL capacity
Gas evaporation unit: Nitrogen
Glass centrifuge bottles: 500-mL capacity
Mason jars
Microliter syringes: 1000-, 500-, 100-, 50- and 10- μ L capacity
Microreaction vessels: 2.0-mL or 1.0-mL autosampler vials with crimp cap septa
Pyrex glass wool: Combusted to 400 °C
Snyder column: Kuderna-Danish, 3-ball column
Stainless steel knife or shears: For dissecting animals
Test tubes: Corex 50-mL with Teflon-lined screw caps
Tissumizers: Tekmar; Polytron homogenizer, or equivalent
Vials and Teflon lined caps: 1-mL to 7-mL capacity
Water bath: Heated to 60 - 70 °C

Volumetric glassware used for sample measurement or introduction of internal standards was calibrated.

4.2. Solvents and reagents

6N HCl
Alumina Oxide: Basic Brockmann 1, standard grade 150 mesh Aldrich 19,744-3 or equivalent. Combust 4 hr at 440 °C. Store at 120 °C prior to use.
Hexylmagnesium bromide: 2M in diethyl ether
Internal standard solution
Magnesium turnings (98% purity, Aldrich)
Matrix spike standard
Reagent water: Water containing no analytes above the method detection limit (i.e., HPLC water).
Silica gel: Grade 923, 100-200 mesh Aldrich 21,447-7 or equivalent. Store at 170 °C for at least 16 hr before use.
Sodium sulfate: (ACS) Granular, anhydrous (purified by heating at 440°C for 4 hr in a shallow tray or other suitable method).
Solvents: Dichloromethane, hexane, pentane (pesticide quality or equivalent)

Surrogate spiking solutions
Tropolone: Aldrich T8, 970-2, purity 98%

5. PROCEDURES

5.1. Preparation of samples

The bivalves were shucked with a stainless steel knife and the tissue was removed from shell. The tissue was macerated using a Tissumizer or Polytron blender. An aliquot of the macerated tissue was weighed into a 200-mL centrifuge bottle (2 - 15 g wet weight).

An aliquot of 1 g of macerated tissue was removed and placed in a combusted 10-mL beaker. This aliquot was dried at 63 - 65 °C. The difference in the weight of the wet sample and the dried sample was used to determine the percent dry weight.

5.2. Digestion and extraction

Approximately 100 mL 0.05% tropolone in CH_2Cl_2 and 50 g anhydrous Na_2SO_4 were added to the weighed tissue sample (2 - 15 g) in the 200-mL centrifuge bottle. Appropriate amounts of surrogate standards were added to each sample, and spiking standards to the matrix spike and matrix spike duplicates. The tissue sample was then macerated for 3 min with the Tissumizer. The extract was decanted into a 500-mL flat bottom flask (centrifuged at 2000 rpm, if necessary) through a funnel containing sodium sulfate. The extraction was repeated two more times with 100 mL of 0.05% tropolone in CH_2Cl_2 each time.

After the extraction, a 3-ball Snyder column was attached to the 500-mL flat bottom flask. A few clean boiling chips were added to sample extract. The sample extract was concentrated to 10 - 20 mL on a hot water bath (60 - 70 °C). The sample was then transferred to a 50-mL test tube. The 500-mL flat bottom flask was rinsed with 5 - 10 mL of hexane and the rinse was added to the test tube. The sample extract in the test tube was further concentrated on the water bath and the solvent was exchanged to about 10 mL of hexane by adding hexane while the dichloromethane was evaporated.

5.3. Hexylation

Extreme care was taken when handling Grignard reagents because it can react violently with water. After the test tube that contained the sample extract in 10 mL of hexane was purged with nitrogen, 2 mL of hexylmagnesium bromide (2 M; Grignard reagent) was slowly added into the sample. The test tube was sealed with a Teflon-lined screw cap and shaken. The hexylation process occurred while warming the sample in 70 °C in a hot water bath for at least 30 min. At the end of the 30 min, the samples was taken out of the water bath and allowed to cool to room temperature. The excess Grignard reagent was neutralized by adding 5 mL of 6 N HCl into the sample container. The sample was shaken vigorously. The organic phase was transferred with a pasture pipette into a 125-mL flat bottom flask. The aqueous phase was extracted twice more with 15 mL of CH_2Cl_2 , and the CH_2Cl_2 was transferred to the 125-mL flat bottom flask. The sample was then dried by adding 10 - 20 g sodium sulfate to the 125-mL flask. The solution was transferred to a concentrator tube and concentrated to about 2 mL.

Grignard reagent was made from magnesium and bromohexane in anhydrous ether. The apparatus used for making Grignard reagent consists of a 1-L round bottom flask, a Y-shaped connector, a condenser, a graduated addition cylinder (250-mL) with a stopcock, and a desiccating tube filled with desiccant. The condenser and the graduate addition cylinder were

connected to the flask via the Y-shaped connector. The desiccating tube was placed on top of the condenser so that water vapor could not react with the Grignard reagent being made. All the glassware was cleaned by rinsing with methanol followed by dichloromethane. After the glassware was dried in an oven for a few minutes, it was stored in a dessicator prior to use.

Approximately 15 g of magnesium turnings was weighed into the 1-L flask. Approximately 100 mL of anhydrous ethyl ether was added immediately to the flask to completely cover the magnesium turnings. The condenser and the addition cylinder were connected to the flask via the Y-shaped connector. The apparatus was flushed with a stream of N₂ for a few minutes to remove any water vapor. It was then sealed with a desiccating tube on top of the condenser and by turning off the stopcock at the bottom of the addition cylinder. It was critical to maintain a moisture-free environment inside the apparatus because the Grignard reagent reacts violently with water. A pre-mixed solution of 150 mL of anhydrous ethyl ether and 90 mL of bromohexane was added to the addition cylinder. The stopcock at the bottom of the addition cylinder was then opened to let the ether/bromohexane slowly drip into the flask. The flask was warmed on the water bath (60 - 65 °C) until the solution the flask started to boil. The apparatus was then removed from the water bath and gently swirled. The processing of heating on the water bath and swirling of the apparatus was repeated until the solution in the flask started to react with the magnesium turnings continuously without heating, or nearly all of the bromohexane/ether was in the flask. When the reaction became violent, the flask was chilled in an ice water bath. When most of the ether/bromohexane was added to the flask, the stopcock at the bottom of the addition cylinder was closed. The apparatus was then placed in an ice water bath for about one hour. The Grignard reagent in the flask was then transferred into an amber bottle. An aliquot of reagent was tested for its reactivity by reacting with butyltin standards. Once the satisfactory reactivity of the reagent (as determined by the recoveries of the derivatized butyltin compounds by GC-FPD) was achieved, the reagent was used for hexylation of actual samples.

5.4. Silica gel/alumina column cleanup

Glass chromatographic columns (30 cm x 10 mm with 300-mL reservoir) were filled with about 30 mL of pentane. Approximately 10.0 g of silica gel was added into the column. The column was tapped gently to evenly distribute the silica gel. Alternatively, a slurry of silica gel in pentane may be used to pack the column. Then 10 g of alumina was added into the column on the top of the silica gel. The pentane was drained through the column until the surface of the liquid in the column was just above the top of the alumina.

The hexylated and concentrated sample extract in 2 mL of hexane was transferred onto the column. The sample was allowed to drain to the top of the alumina. The concentrator tube was then rinsed twice with 1 mL pentane and the rinses were added to the column. Another 50 mL of pentane was added to the column and the sample was eluted at a flow rate of approximately 2 mL/min. The eluent was collected in a 250-mL flat bottom flask.

The collected sample fraction was concentrated using a three-ball Snyder column and solvent was exchanged to 0.5 to 1 mL of hexane on the hot water bath (60 - 70 °C). The samples are then ready for the butyltin instrumental analyses.

6. QUALITY CONTROL

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 set, whichever was more frequent. The analyte levels in the Blank was required to be less than three times the method detection limit (MDL). If blank levels for any component were above three times the MDL, samples analyzed in that sample set were re-extracted and reanalyzed. If insufficient sample was available for re-extraction, the data was reported and appropriately qualified.

Matrix spike/matrix spike duplicate (MS/MSD) samples were run with every 20 samples, or with every sample set, whichever was more frequent. The appropriate spiking level was ten times the MDL.

Reference materials were extracted and analyzed when available.

7. CONCLUSIONS

This tissue extraction was adapted from the standard NS&T organic tissue extraction technique with the addition of the complexing agent tropolone. The method provides reliable extraction and hexylation of tributyltin as well as other organotins.

Purification of Biological Tissue Samples by Gel Permeation Chromatography for Organic Analyses

Y. Qian, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Gel permeation chromatography is an extract purification step which uses a chromatographic column to separate components in a complex mixture based upon their molecular size or shape. The size exclusion column used for this procedure separated lipids and high molecular weight components from target chlorinated and aromatic hydrocarbons in tissue extracts. An automated gel permeation chromatographic technique using a high performance liquid chromatography system consisting of an autosampler, a ultraviolet detector, and an isocratic pump was used to purify sample extracts. Extracts were processed through a guard column and two size exclusion columns connected in series and the desired fraction was collected with a fraction collector. The collected fraction was then concentrated and analyzed for polycyclic aromatic hydrocarbons, pesticides, and polychlorinated biphenyls using the appropriate instrumental procedures.

1. INTRODUCTION

Sample extracts, particularly those from biological tissue samples, contain a large amount of lipids and high molecular weight components. These non-target components may interfere with the instrumental analyses of polycyclic aromatic hydrocarbons (PAHs), pesticides, and polychlorinated biphenyls (PCBs). Purification of the sample extracts by alumina/silica gel column chromatography helps alleviate this problem. However, a large amount of neutral lipids and high molecular weight components from tissue samples are eluted together with the target analytes. Further purification of the sample extracts is required in order to analyze the PAHs, pesticides, and PCBs at parts per billion level. Size exclusion chromatography can separate the target analytes from the high molecular weight lipids and other components.

2. APPARATUS AND MATERIALS

2.1. Equipment

Autosampler: Thermo Separation Products model AS100 or equivalent.
HPLC isocratic pump: Thermo Separation Products model P1000 or equivalent.
Fraction collector: Isco model Foxy 200 or equivalent.
Size Exclusion column: two connected in series, Phenomenex 250 mm X 22.5 mm Phenogel 100 Å columns or equivalent.
Phenogel 100 Å guard column: Phenomenex 50 mm x 7.8 mm.
UV absorbance detector: Waters 440 or equivalent, 254 nm.
Hewlett-Packard 3396A integrator
Electrobalance: Cahn or equivalent, with an accuracy of 0.001 mg
Water bath: heated to 60 - 65 °C.

2.2. Materials

Boiling chips, Teflon, dichloromethane rinsed.

Concentrator tubes, glass, 25-mL.

Fraction collector vials, 50-mL, 25 x 100 mm.

Autosampler vials, tear-shaped (tapered bottom), 1.2 mL Rainin or equivalent.

Pasteur pipettes: 1-mL, glass, disposable.

Autosampler vials, 2-mL, amber borosilicate glass with Teflon liner caps.

Biphenyl, 99% purity, Aldrich Cat. # B3,465-6 or equivalent.

4,4'-Dibromooctafluorobiphenyl (DBOBF), 99% purity, Aldrich Cat. # 10,199-0 or equivalent.

Perylene, 99% purity, Aldrich Cat. # P1,129-4 or equivalent.

Dichloromethane, Burdick and Jackson Cat. #300-4, pesticide grade or equivalent, Lot tested.

Helium, 99.99% purity.

2.3. HPLC calibration standard

Stock solutions

Biphenyl (0.25 mg/mL): 6.25 mg of biphenyl was weighed into a 25-mL volumetric flask using a Cahn electrobalance. The biphenyl was dissolved in dichloromethane and the solvent was filled to the 25 mL mark. After the biphenyl was completely dissolved, the solution was transferred to a clean, labeled bottle with a Teflon-lined cap.

Perylene (0.125 mg/mL): 3.125 mg of perylene was weighed into a 25-mL volumetric flask and the flask was filled with dichloromethane to the mark. After the perylene was dissolved, the solution was transferred to a clean, labeled bottle with a Teflon-lined cap.

4,4'-Dibromooctafluorobiphenyl (DBOBF) (0.125 mg/mL): 3.125 mg of DBOBF was weighed into a 25-mL volumetric flask and the flask was filled to the mark with dichloromethane. After the DBOBF was dissolved, the solution was transferred to a clean labeled bottle with a Teflon-lined cap.

2.4. GPC/HPLC calibration standard

The GPC/HPLC calibration standards was prepared by adding 1 mL of each of the stock solutions to a 250-mL volumetric flask and filling to the mark with dichloromethane. Concentrations of biphenyl, perylene, and DBOBF should be 1 µg/mL, 0.5 µg/mL, and 0.5 µg/mL, respectively.

When not being used, all solid, stock, and diluted standards were stored in the dark at 20 °C in the freezer. The solutions were brought to room temperature prior to use. With proper storage, standard solutions were stable for 12 months.

2.5. GPC/HPLC Calibration

Prior to processing analytical extracts through the GPC/HPLC, the system performance was checked and the GPC/HPLC was calibrated. The calibration of GPC/HPLC verified the instrument performance based upon the retention time and area of each of the calibration standards. The time to start collecting the fractions for actual samples was 1 min before the elution of DBOBF. For this test, three GPC/HPLC calibration solution and a blank were used to verify the

performance criteria. Adjustment and/or re-calibration was performed until the criteria were met.

The GPC/HPLC calibration standard was brought to room temperature prior to use. Approximately 1 mL of the GPC/HPLC calibration standard solution was transferred into the Rainin tapered autosampler vials using a disposable Pasteur pipette. The vials were sealed with Teflon-lined septa that were pre-rinsed with dichloromethane. The GPC/HPLC calibration standards were used during instrument calibration and in the sample extract purification sequence.

Solvent blanks were prepared by filling the autosampler vials with dichloromethane. Blanks were used during instrument calibration and in the sample extract purification sequence.

The HPLC was set at a flow rate of 7 mL/min. The normal operation pressure of the HPLC system was from 700 to 900 psi at this flow rate. If the pressure was outside this range, the source of the pressure variation was identified and corrected.

Three calibration standard vials and one blank vial were placed in the sample tray of the autosampler. The autosampler was programmed to start the sample sequence from the first position and inject 1 mL of the sample. The cycle time (the total running time per sample) was set at 25 min. After placing four collection vials in the fraction collector tray, the sample sequence was started.

The separation of the three standards was checked on the chromatogram. The three compounds should be completely separated. DBOFB should elute at approximately 15 min, followed by biphenyl at about 16 min. Perylene should elute at approximately 18 min. The retention time of the three peaks should not vary more than 5%, or approximately 0.6 min, among the three chromatograms. The peak area of DBOFB should be at least 1,000,000 integrator units. The blank should have no peaks. If the calibration is verified, the system performance is acceptable and the analysis of samples may begin. If the calibration failed, the GPC/HPLC system must be checked and the problem must be corrected.

Before starting the sample analysis, the collection time of the fraction collector should be checked and verified, making sure the collection time corresponds to the elution time of the calibration standards. The starting time of collection was 1 min before the elution of DBOFB. The total collection time was 7 min and 30 sec after starting the collection. For example, if the DBOFB elutes at 15:10 min, the starting collection time is set at 14:10 min and the end of collection time is set at 21:40 min.

2.6. Preparation of sample extracts for GPC/HPLC

After the extraction of the tissue samples and purification of the extracts using column chromatography, the sample extracts were concentrated to a final volume of 0.5 mL in dichloromethane and transferred to tear-drop (tapered bottom) autosampler vials. The final volume in the autosampler vial should not exceed 1 mL.

For extremely viscous extracts or extracts with high lipid content (greater than 500 mg), the sample extracts can be diluted and divided into multiple autosampler vials. In this case, fractions collected from the HPLC are concentrated and combined prior to the addition of the GC internal standards.

A sample sequence was setup in the autosampler sample tray starting from the first position using the following sequence: three initial calibration standards, one blank, five samples, one

blank, five samples, one blank, one continuing calibration standard (CCS), five samples, one blank, five samples.

For every five samples an instrument solvent blank was run. For every ten samples an instrument solvent blank and a CCS was added. Each GPC/HPLC sample sequence must end with a blank and CCS.

Appropriate number of collection vials was placed in the sample tray of the fraction collector, following the correct sample sequence in the autosampler tray. The number of the collection vials should be equal or greater than the number of samples plus the instrument blanks and calibration standards. The autosampler was programmed to run the sample sequence. The injection volume was set to inject all of the sample (1 mL) into the column. The cycle time was set at 25 min. The needle height of the autosampler was adjusted so that the entire sample extract was injected.

After the sample sequence was completed, the correct number of samples collected by the fraction collector was verified. The runs of calibration standards were verified to meet the QC criteria. The instrument blank was verified to contain no peaks. The autosampler vials were also checked to ensure that the entire extract had been injected. The vials should be empty. If there is extract remaining in the autosampler vials, the volume of the extract may be brought up to 1 mL with dichloromethane and reinjected into the HPLC system. The reinjected fraction collected was then combined with the original collected fraction.

The collected sample vials were placed in a vial rack and 1-2 pieces of boiling chips were added. The sample was then concentrated to approximately 5-10 mL on the 60-65 °C water bath. The concentrated sample was transferred to a 25-mL concentrator tube. The collection vials were rinsed at least three times with dichloromethane and the rinses were added to the concentrator tube. The transferred sample extract was concentrated and the solvent was exchanged to 1 mL hexane. After adding appropriate amount of required GC internal standards, the sample was transferred to a labeled 2-mL brown vial. The vial was capped with a Teflon lined screw cap and was stored at 0 °C in the dark. The purified sample extract was ready for instrumental analysis.

3. CONCLUSIONS

This HPLC gel permeation technique allows for the separation of most biological lipids from the PAHs, pesticides, and PCBs measured. With the lipids removed, reliable quantitations can be achieved.

Quantitative Determination of Polynuclear Aromatic Hydrocarbons by Gas Chromatography/Mass Spectrometry (GC/MS) - Selected Ion Monitoring (SIM) Mode

G. J. Denoux, P. Gardinali, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Polycyclic aromatic hydrocarbons can be determined at low concentrations by mass spectrometry due to their strong molecular ion response. Therefore, GC/MS in the selected ion monitoring mode provides unambiguous and sensitive detection for PAHs.

1. INTRODUCTION

The quantitative method described in this document was used to determine polycyclic aromatic hydrocarbons (PAH) and their alkylated homologues in extracts of water, sediments and biological tissues. Quantitation was performed by gas chromatography mass spectrometry (GC/MS) in the selected ion monitoring (SIM) mode. Target analytes are listed in Table 1.

2. APPARATUS AND MATERIALS

The analytical system included a temperature programmable gas chromatograph (Hewlett-Packard 5890A, or equivalent) equipped with a split-splitless injection port routinely operated in the splitless mode. A 30-m long x 0.32-mm i.d., 0.25 μm phase thickness DB-5MS fused silica capillary column was used for the analytical determination. Helium was used as the carrier gas and the samples were handle by an autosampler capable of making 1 - 4 μL injections.

The mass spectrometer (HP5970/5972 MSD) was operated at 70 eV electron energy in the electron impact ionization mode and was tuned to maximize the sensitivity of the instrument according to the manufacturer's specifications. The GC capillary column was directly interfaced with the ion source of the mass spectrometer.

3. REAGENTS

3.1. Surrogate spiking solution

A surrogate spiking solution was made by weighing appropriate aliquots of pure compounds into a volumetric flask and diluting to volume with dichloromethane [or by using a commercially available certified standard (NIST or equivalent)]. Surrogates were added to all samples prior to extraction at a concentration of approximately ten times the MDL.

The compounds in the surrogate solution were naphthalene- d_8 , acenaphthene- d_{10} , phenanthrene- d_{10} , chrysene- d_{12} and perylene- d_{12} . The surrogate solution provides a spike where approximately 100 μL of solution gave a concentration of 40 ng/mL in the final extract

regardless of matrix. All sample analyte concentrations were quantitated based on the applicable surrogate (Table 1).

3.2. Internal standard solutions

A solution containing each internal standard (fluorene-d₁₀, and benzo[a]pyrene-d₁₂) at 4 µg/mL was prepared by weighing a certified standard (NIST or equivalent) into a volumetric flask and diluting to volume with dichloromethane. The GC conditions were set so that the internal standards were resolved, but elute in close proximity to, the analytes of interest. Sufficient solution was added to the extract prior to analysis to give a final concentration of the internal standard of 40 ng/mL.

3.3. Matrix recovery standard spiking solution

A solution containing 2- to 5-ring PAH compounds was used to fortify matrix spike samples (Table 2). A certified solution (NIST SRM 2260) was purchased and diluted to the appropriate working concentration. Dibenzothiophene was not present in the SRM and was added to the solution by weighing the neat material to make a concentration of 1.00 µg/µL. The spiking solution was used to fortify samples to a final concentration of approximately ten times the MDL.

3.4. Reference oil solution

A solution of a laboratory reference oil was analyzed as an instrument reference solution with each analytical batch. The concentration of oil was approximately 0.8 mg/mL. The oil was weighed into a volumetric and brought to volume with dichloromethane.

4. GC/MS CALIBRATIONS

A five-point calibration curve was established to demonstrate the linear range of the detector. The recommended standard concentrations used were 20, 100, 250, 500 and 1000 ng/mL. Standard solutions were made by diluting NIST SRM 1491 to the appropriate concentration. Dibenzothiophene was not present in the SRM and was added to the calibration solution by weighing the neat material. When response factors were used, the averaged response factors for the targeted analytes were kept within a ±15% RSD. Linear regression was used for the calibration curve evaluation. The control values of the R² term were set to be greater than 0.99.

After every 8 - 10 samples, the mass spectrometer response for each PAH relative to the internal standard was determined using check standards at concentrations of 250 ng/mL. Daily response factors for each compound were compared to the initial calibration curve. If the average daily response factors for all analytes were within ±15% of the calibration value, analyses proceeded. If, for any analyte, the daily response factor exceeded ±25% of calibration value, a five point calibration was repeated.

Qualitative identification of target compounds was based on relative retention time (RRT) criteria. Table 3 contains example RRT data for unsubstituted PAHs. RRT windows for alkyl homologues were based on analysis of the reference oil.

Table 1. Target compounds.

Compounds	IS reference	Surrogate reference	Compounds	IS reference	Surrogate reference
Naphthalene	A	1	Benz[a]anthracene	B	4
C ₁ -Naphthalenes	A	1			
C ₂ -Naphthalenes	A	2	Chrysene	B	4
C ₃ -Naphthalenes	A	2	C ₁ -Chrysene *	B	4
C ₄ -Naphthalenes *	A	2	C ₂ -Chrysene *	B	4
			C ₃ -Chrysene *	B	4
Biphenyl	A	2	C ₄ -Chrysene *	B	4
Acenaphthylene	A	2			
			Benzo[b]fluoranthene	B	4
Acenaphthene	A	2	Benzo[k]fluoranthene	B	4
			Benzo[e]pyrene	B	4
Fluorene	A	2	Benzo[a]pyrene	B	4
C ₁ -Fluorenes *	A	2	Perylene	B	5
C ₂ -Fluorenes *	A	2	Indeno[1,2,3- <i>cd</i>]pyrene	B	4
C ₃ -Fluorenes *	A	2	Dibenz[<i>a, h</i>]anthracene	B	4
			Benzo[<i>ghi</i>]perylene	B	4
Dibenzothiophene	A	3			
C ₁ -Dibenzothiophenes *	A	3	<u>Internal standards</u>		
C ₂ -Dibenzothiophenes *	A	3	1-Methylnaphthalene	A	1
C ₃ -Dibenzothiophenes *	A	3	2-Methylnaphthalene	A	1
			2,6-Dimethylnaphthalene	A	2
Phenanthrene	A	3	1,6,7-Trimethylnaphthalene	A	2
C ₁ -Phenanthrenes	A	3	1-Methylphenanthrene	A	3
C ₂ -Phenanthrenes *	A	3			
C ₃ -Phenanthrenes *	A	3	<u>Internal Standards</u>		
C ₄ -Phenanthrenes *	A	3	Fluorene-d ₁₀	(A)	
			Benzo[a]pyrene-d ₁₂	(B)	
Anthracene	A	3			
C ₁ -Anthracenes *	A	3	<u>Surrogates</u>		
C ₂ -Anthracenes *	A	3	Naphthalene-d ₈	(1)	
C ₃ -Anthracenes *	A	3	Acenaphthene-d ₁₀	(2)	
C ₄ -Anthracenes *	A	3	Phenanthrene-d ₁₀	(3)	
Fluoranthene	B	3	Chrysene-d ₁₂	(4)	
C ₁ -Fluoranthenes *	B	3	Perylene-d ₁₂	(5)	
Pyrene	B	3			
C ₁ -Pyrene	B	3			

*Alkylated homologues not included in the calibration solution.

NOTE: Alkylated phenanthrenes and anthracenes, and alkylated fluoranthenes and pyrenes were quantified together as total alkylated (Cx) phenanthrene/anthracenes and total alkylated (Cx) fluoranthenes/pyrenes. Only the parent compounds and specific isomers were reported as individual compounds.

Table 2. Final concentration of PAH matrix spike solution (in CH₂Cl₂).

Compound	Spiking solution (µg/mL)
1-Methylnaphthalene	1.31
1-Methylphenanthrene	1.30
2-Methylnaphthalene	1.27
2,6-Dimethylnaphthalene	1.31
2,3,5-Trimethylnaphthalene	1.17
Acenaphthene	1.36
Acenaphthylene	1.26
Anthracene	0.10
Benzo[<i>a</i>]pyrene	1.19
Benzo[<i>b</i>]fluoranthene	1.31
Benzo[<i>e</i>]pyrene	1.31
Benzo[<i>ghi</i>]perylene	1.17
Benzo[<i>k</i>]fluoranthene	1.31
Benz[<i>a</i>]anthracene	1.14
Biphenyl	1.32
Chrysene	1.32
Dibenzothiophene	1.01
Dibenz[<i>a, h</i>]anthracene	0.99
Fluoranthene	1.32
Fluorene	1.01
Indeno[1,2,3- <i>cd</i>]pyrene	1.17
Naphthalene	1.32
Perylene	0.99
Phenanthrene	1.31
Pyrene	1.32

Table 3. Relative retention times and confidence intervals.*

	Absolute retention time (min)	Average RRT	Percent SD	95% confidence RSD	Limits
Naphthalene-d ₈ (Surr.)	6:46	1.01	0.02	2.29	0.98-1.04
Naphthalene	6:52	1.01	0.02	2.29	0.97-1.04
2-Methylnaphthalene	8:10	0.87	0.02	2.08	0.84-0.91
1-Methylnaphthalene	8:36	1.20	0.02	2.05	1.16-1.23
Acenaphthylene	10:25	0.96	0.02	1.82	0.93-1.0
Acenaphthene	10:72	0.99	0.02	1.85	0.95-1.02
Fluorene-d ₁₀ (IS)	11:50	0.87	0.01	1.73	0.84-0.90
Fluorene	11:96	0.87	0.01	1.74	0.84-0.90
Dibenzothiophene	14:15	0.97	0.02	1.82	0.94-1.01
Phenanthrene	14:35	0.99	0.02	1.59	0.95-1.02
Anthracene	14:45	0.99	0.02	1.60	0.96-1.03
Fluoranthene	17:33	1.13	0.02	1.46	1.10-1.16
Pyrene	17:87	1.16	0.02	1.44	1.12-1.19
Benz[<i>a</i>]anthracene	20:96	0.87	0.01	1.32	0.85-0.90
Chrysene-d ₁₂ (Surr.)	20:99	0.90	0.01	1.32	0.87-0.92
Chrysene	21:04	0.88	0.01	1.32	0.85-0.90
Benzofluoranthenes	23:52	0.96	0.01	1.50	0.93-0.99
Benzo[<i>e</i>]pyrene	24:08	0.98	0.02	1.59	0.95-1.02
Benzo[<i>a</i>]pyrene	24:19	0.99	0.02	1.61	0.96-1.02
Perylene	24:38	1.00	0.02	1.84	0.96-1.03
Indeno [1,2, 3- <i>cd</i>] pyrene	26:99	1.112	0.02	2.27	1.06-1.16
Dibenz[<i>a, h</i>]anthracene	27:08	1.11	0.03	2.74	1.05-1.17
Benzo[<i>ghi</i>] perylene	27:71	1.15	0.03	2.42	1.09-1.20

* This table is to serve as an example. Absolute retention times may vary depending on the length and condition of the GC column.

5. DAILY GC/MS PERFORMANCE TESTS

The mass spectrometer performance was checked daily using perfluorotributylamine (PFTBA) according to manufacturer's tuning procedures. These procedures included the optimization of peak widths, relative abundances, and mass axis calibration for the PFTBA fragments at *m/z* 69, 219, and 502 according to the recommended criteria. Isotope abundances were also checked according to the manufacturer's criteria.

6. GAS CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSES

Just prior to analysis, an aliquot of internal standard solution was added to the sample producing a final internal standard concentration of approximately 40 ng/mL. Representative aliquots were injected into the capillary column of the gas chromatograph using the following conditions:

Injector temp:	300 °C
Transfer line temp:	280 °C
Initial oven temp:	40 °C
Initial hold time:	0 min.
Ramp rate:	10 °C
Final temperature:	300 °C
Final hold time:	4 min.

The effluent from the GC capillary column was routed directly into the ion source of the mass spectrometer (MS). The MS was operated in the selected ion monitoring (SIM) mode using appropriate windows to include the quantitation and confirmation masses for the PAHs listed in Table 4. For all compounds detected at a concentration above the MDL the confirmation ion was checked to confirm its presence.

Compounds identified and quantified below the MDL were appropriately flagged and reported. If the concentration of any target compound in a sample exceeded the linear range defined by the standards above, the extract was diluted and reinjected.

7. CALCULATIONS

7.1. Qualitative identification

The extracted ion current profiles of the primary m/z ion for each analyte met the following criteria:

The retention time fell within ± 0.2 min of the retention time of the authentic compound or alkyl homologue grouping determined by analysis of reference materials.

The alkylated PAH homologue groupings (e.g. C₃-naphthalene) appeared as clusters of isomers. The pattern of each cluster and the retention time window for the cluster was established by analysis of reference oil solution. The cluster was integrated in its entirety and the total response was used for quantification.

The relative peak heights of the primary ion compared to the confirmation or secondary ion masses should be within $\pm 30\%$ of the relative intensities of these masses in a reference mass spectrum if the compound was correctly identified. The reference mass spectrum was obtained from reference standards. A compound that does not meet secondary ion confirmation criteria may indicate interference. The confirmation ion was checked only where interference was suspected.

7.2. Quantitation

The following formula was used to calculate the response factors of the surrogate relative to each of the calibration standards.

$$RF = \frac{A_s C_{su}}{A_{su} C_s}$$

Table 4. Quantitations, confirmation ions, and relative abundance.

Analyte	Quant. ion	Conf. ions	Percent abundance*
d ₈ -Naphthalene	136	134	15
Naphthalene	128	127	15
C ₁ -Naphthalenes (including isomers)	142	141	80
C ₂ -Naphthalenes	156	141	ND
C ₃ -Naphthalenes	170	155	ND
C ₄ -Naphthalenes	184	169, 141	ND
d ₁₀ -Acenaphthene	164	162	95
Acenaphthylene	152	153	15
Biphenyl	154	152	30
Acenaphthene	154	153	98
d ₁₀ -Fluorene	176	174	85
Fluorene	166	165	95
C ₁ -Fluorenes	180	165	100
C ₂ -Fluorenes	194	179	25
C ₃ -Fluorenes	208	193	ND
d ₁₀ -Phenanthrene	188	184	ND
Phenanthrene	178	176	20
Anthracene	178	176	20
C ₁ -Phenanthrenes/anthracenes	192	191	60
C ₂ -Phenanthrenes/anthracenes	206	191	ND
C ₃ -Phenanthrenes/anthracenes	220	205	ND
C ₄ -Phenanthrenes/anthracenes	234	219, 191	ND
Dibenzothiophene	184	152, 139	15
C ₁ -Dibenzothiophenes	198	184, 197	25
C ₂ -Dibenzothiophenes	212	197	ND
C ₃ -Dibenzothiophenes	226	211	ND
Fluoranthene	202	101	15
d ₁₂ -Chrysene	240	236	ND
Pyrene	202	101	15
C ₁ -Fluoranthenes/pyrenes	216	215	60
Benzo[<i>a</i>] anthracene	228	226	20
Chrysene	228	226	30
C ₁ -Chrysenes	242	241	ND
C ₂ -Chrysenes	256	241	ND
C ₃ -Chrysenes	270	255	ND
C ₄ -Chrysenes	284	269, 241	ND
d ₁₂ -Benz[<i>a</i>]pyrene	264	260	20
Benzo[<i>b</i>]fluoranthene	252	253, 125	30, 10
Benzo[<i>k</i>]fluoranthene	252	253, 125	30, 10
Benzo[<i>e</i>]pyrene	252	253	30, 10
Perylene	264	253	20
d ₁₂ -Perylene	264	260	ND
Benzo[<i>a</i>]pyrene	252	253, 125	30, 10
Indeno[1,2,3- <i>cd</i>]pyrene	276	277, 138	25, 30
Dibenzo[<i>a,h</i>]anthracene	278	279, 139	25, 20
Benzo[<i>ghi</i>]perylene	276	277, 138	25, 20

ND - Not determined

where A_s was the area of the characteristic ion for the parameter to be measured, A_{su} was the area of the characteristic ion for the surrogate, C_{su} was the concentration of the surrogate (ng/mL), and C_s was the concentration of the target analyte to be measured (ng/mL).

The response factor of alkyl homologues was assumed to be equal to that of respective unsubstituted parent compounds. Based on these response factors, sample extract concentrations for each PAH and alkyl homologue grouping was calculated using the following formula:

$$C_e = \frac{A_s C_{su}}{A_{su} RF}$$

where C_e was the sample extract concentration (ng), A_s was the area of the characteristic quantitation ion for the target analyte to be measured, A_{su} was the area of the characteristic quantitation ion for the surrogate, and C_{su} was the amount of surrogate added to each extract (ng).

The actual sample concentration (C) for each compound was calculated using the following formula:

$$C = \frac{C_e}{SA}$$

where C was the concentration in sample (ng) and SA was the sample amount (in grams).

8. QUALITY CONTROL/QUALITY ASSURANCE (QA/QC) REQUIREMENTS

8.1. GC/MS tuning

The GC/MS was tuned using PFTBA according to manufacturer's specifications prior to the analysis of each QC batch.

8.2. GC/MS initial calibration and continuing calibration checks

A five-point response factor calibration curve was established demonstrating that the analyte average response factors were within the $\pm 15\%$ RSD criteria (and an R^2 of 0.99 or better for the linear regression). After every 8 - 10 sample analyses, the mass spectrometer response factor (RF) for each PAH of interest (Table 1) relative to the internal standard was determined. The daily response factors for each compound were compared to the initial calibration curve. The percent difference was calculated using the following equation:

$$\text{Percent difference} = \frac{(RFI - RFC)}{RFI} 100$$

where RFI was the average response factor from initial calibration and RFC was the response factor from current verification check standard. If the average daily response factors for all analytes were within $\pm 15\%$ of the calibration value, analyses proceeded. If, for any analyte, the daily response factor exceeded $\pm 25\%$ percent of the calibration value, a five point calibration was repeated prior to analysis.

8.3. Standard reference oil

The standard reference oil was analyzed with all analytical batches. The reference concentration was defined as the average of all previous analyses plus or minus one standard deviation. The measured concentration was within $\pm 25\%$ of the laboratory value on average for all analytes greater than the MDL and did not exceed $\pm 35\%$ for any individual analyte.

8.4. Method blank analysis

An acceptable method blank did not contain the analytes of interest at concentrations above three times greater than the MDL. If the method blank exceeds this criteria, the analytical procedure was out of control and the source of the contamination was investigated and corrective measures were taken and documented before further sample analysis proceeded. The first corrective action was to re-inject the blank to confirm the out-of-control event. If the blank still exceeded the criteria, all samples in that batch were re-extracted and re-analyzed.

8.5. Surrogate compound analysis

All samples, including quality control samples, were spiked with deuterated PAH surrogate compounds. The surrogate compounds were spiked into the sample prior to extraction to determine sample matrix effects associated with sample preparation and analysis. Surrogates included naphthalene-d₈, acenaphthene-d₁₀, phenanthrene-d₁₀, chrysene-d₁₂ and perylene-d₁₂. Just prior to analysis, the extract was spiked with a solution containing the GC Internal Standards. These compounds were fluorene-d₁₀ and benzo[a]pyrene-d₁₂. The recovery of the surrogates were monitored in each sample using the relative response factor of the Surrogate to the Internal Standard.

$$\text{Percent SUR recovery} = \frac{A_{\text{SUR}} C_{\text{IS}}}{C_{\text{SUR}} A_{\text{IS}} \text{RF}_{\text{SUR}}}$$

where A_{IS} was the area of the characteristic ion for the appropriate internal standard, A_{SUR} was the area of the characteristic ion for the surrogate, C_{SUR} was the amount in ng of deuterated surrogate added to the sample, C_{IS} was the amount in ng of deuterated internal standard added to the sample extract and RF_{SUR} was the response factor for the surrogate.

The laboratory took corrective action whenever the recovery for any surrogates, except perylene-d₁₂, was less than 40% or greater than 120%.

The following corrective actions were taken:

- a. The calculations were checked to assure there were no errors.
- b. The internal standard and surrogate solutions were checked for degradation, contamination, etc., and the instrument performance was checked.
- c. If the surrogate recovery was outside the control limits, the secondary ion was used to check the quantitation of the surrogate. If the secondary ion was within the control limits, this recovery was appropriately annotated.
- d. If the upper control limit was exceeded for only one surrogate, and the instrument calibration and surrogate standard concentration were in control, it was concluded that an interference specific to the surrogate was present that resulted in high recovery and that

this interference did not affect the quantitation of other target compounds. The presence of this type of interference was confirmed by evaluation of chromatographic peak shapes. To correct for the underestimation of the analyte concentration based on this surrogate, the analytes were quantitated using an alternate surrogate that was chromatographically closest to the surrogate exhibiting interference.

e. If the surrogate could not be measured because the amount and nature of the hydrocarbon in the sample, the analytes based on that surrogate will be quantitated based on the closest surrogate. The surrogate recovery was appropriately qualified.

f. If the native concentration of hydrocarbons were high and required that a dilution for quantitation purposes be made, a known aliquot of the extract was sampled and diluted. One hundred (100) μL of surrogate and 100 μL of internal standard were added and the volume brought to 1.0 mL. The appropriate dilution factor was used in the quantitation software for the sample and surrogate recoveries were assumed to be 100% for the re-analyses. The recovery in these cases and the surrogate recoveries were not reported but qualified with a "D" to denote the dilution.

g. If the steps above failed to reveal a problem, the sample or extract was then re-extracted. If reanalysis of the new extract yielded surrogate recoveries within the stated limits, then the reanalysis data was reported. If reanalysis did not yield acceptable recoveries, the data was listed as out of control because of matrix effects.

8.6. Matrix spike analysis

The laboratory analyzed a matrix spike and matrix spike duplicate (MS/MSD) with every 20 samples or with every sample set, whichever was more frequent. A sample randomly chosen was split into three subsamples and two subsamples were fortified with the matrix spiking solution containing the compounds listed in Table 2. The acceptable matrix spike recovery criteria was a recovery for all 25 compounds between 50 and 120% for at least 80% of the analytes.

If the matrix spike criteria were not met, the matrix spike analysis was repeated. If the subsequent matrix spike analysis met the criteria, then the reanalysis data was reported. If the matrix spike criteria were not met on re-injection, the sample set was re-extracted.

8.7. Standard Reference Material

When available, a standard reference material was extracted and analyzed with each batch of samples. Target concentrations were defined as the range of the certified value plus or minus the 95% confidence intervals found in the SRM certification. The measured concentration was within $\pm 30\%$ of the target concentration on average for all analytes either certified or non-certified with concentrations greater than 10 times the MDL.

8.8. Method detection limit

The actual analytical method detection limit (MDL) was determined following procedures outlined in Federal Register (1984), Vol. 49, No. 209: 198-199.

9. CONCLUSIONS

The PAH quantitation method is very rigorous because PAHs have very strong molecular ion peaks under the mass spectrometric conditions used. Also the availability of labeled surrogates internal standards of many of the analytes makes very accurate determinations of analyte concentrations possible.

Quantitative Determination of Tetra- Through Octa-Polychlorinated Dibenzo- *p*-dioxins and Dibenzofurans by Isotope Dilution High Resolution Gas Chromatography/High Resolution Mass Spectrometry

L. Chambers, P. Gardinali, J. L. Sericano, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

This method was used for the determination of tetra- through octa-chlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in sediment and tissue samples by high resolution gas chromatography/high resolution mass spectrometry. The seventeen 2,3,7,8-substituted PCDDs/PCDFs were determined by this method at pg/g levels. The minimum levels reported for the target compounds are the levels at which the PCDDs/PCDFs can be determined with no interferences present. The procedures described here were largely based on the protocols described in EPA Method 1613 and EPA Method 8290 and designed to meet or exceed the quality control criteria outlined in those methods. The analyst is permitted to modify the method provided that all performance criteria in this method are met.

1. PURPOSE AND SUMMARY

This procedure was used to provide quantitative determination of tetra- through octa-polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in sample extracts using isotope dilution high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS).

The instrumental protocols described in this procedure were applied to the quantitative analysis of extracts from sediment and bivalve tissues.

The chlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs and PCDFs) determined by this method and their Chemical Abstract Service (CAS) Registry numbers are listed in Table 1. The retention time references, quantitation references, relative retention times and minimum levels for determination of PCDDs and PCDFs using this method are listed in Table 2. The minimum level (ML) for each analyte was defined as the level at which the entire system must give a recognizable signal and an acceptable calibration point. It was equivalent to the concentration of the lowest calibration standard, assuming that all method specific sample weights, volumes, and procedures were employed. The ML varied with the degree of chlorination. The sample-specific estimated detection limit (EDL) was the concentration of an analyte required to produce a signal with a peak height at least three times the background signal level. An EDL was calculated for each 2,3,7,8-substituted isomer that was not detected. The quantitation software (OPUSQuan) supplied by the instrument manufacturer calculated a sample specific-EDL using an algorithm based on the criteria outlined in EPA Method 8290, Section 7.9.5.1.1. The analyte concentrations of the calibration standards (CS1-CS5) used for instrument calibration are listed in Table 3.

Table 1. Chlorinated dibenzo-*p*-dioxins and dibenzofurans determined by isotope dilution high resolution gas Chromatography (HRGC)/high resolution mass spectrometry (HRMS).

CDDs/CDFs *	CAS Registry	Labeled analog	CAS Registry
2,3,7,8-TCDD	1746-01-6	¹³ C ₁₂ -2,3,7,8-TCDD	76523-40-5
		³⁷ Cl ₄ -2,3,7,8-TCDD	85508-50-5
Total TCDD	41903-57-5	----	----
2,3,7,8-TCDF	51207-31-9	¹³ C ₁₂ -2,3,7,8-TCDF	89059-46-1
Total-TCDF	55722-27-5	----	----
1,2,3,7,8-PeCDD	40321-76-4	¹³ C ₁₂ -1,2,3,7,8-PeCDD	109719-79-1
Total-PeCDD	36088-22-9	----	----
1,2,3,7,8-PeCDF	57117-41-6	¹³ C ₁₂ -1,2,3,7,8-PeCDF	109719-77-9
2,3,4,7,8-PeCDF	57117-31-4	¹³ C ₁₂ -2,3,4,7,8-PeCDF	116843-02-8
Total-PeCDF	30402-15-4	----	----
1,2,3,4,7,8-HxCDD	39227-28-6	¹³ C ₁₂ -1,2,3,4,7,8-HxCDD	109719-80-4
1,2,3,6,7,8-HxCDD	57653-85-7	¹³ C ₁₂ -1,2,3,6,7,8-HxCDD	109719-81-5
1,2,3,7,8,9-HxCDD	19408-74-3	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	109719-82-6
Total HxCDD	34465-46-8	----	----
1,2,3,4,7,8-HxCDF	70648-26-9	¹³ C ₁₂ -1,2,3,4,7,8-HxCDF	114423-98-2
1,2,3,6,7,8-HxCDF	57117-44-9	¹³ C ₁₂ -1,2,3,6,7,8-HxCDF	116843-03-9
1,2,3,7,8,9-HxCDF	72918-21-9	¹³ C ₁₂ -1,2,3,7,8,9-HxCDF	116843-04-0
2,3,4,6,7,8-HxCDF	60851-34-5	¹³ C ₁₂ -2,3,4,6,7,8-HxCDF	116843-05-1
Total-HxCDF	55684-94-1	----	----
1,2,3,4,6,7,8-HpCDD	35822-46-9	¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDD	109719-83-7
Total-HpCDD	37871-00-4	----	----
1,2,3,4,6,7,8-HpCDF	67562-39-4	¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDF	109719-84-8
1,2,3,4,7,8,9-HpCDF	55673-89-7	¹³ C ₁₂ -1,2,3,4,7,8,9-HpCDF	109719-94-0
Total-HpCDF	38998-75-3	----	----
OCDD	3268-87-9	¹³ C ₁₂ -OCDD	114423-97-1
OCDF	39001-02-0	not used	

* Chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans

TCDD	=	Tetrachlorodibenzo- <i>p</i> -dioxin	TCDF	=	Tetrachlorodibenzofuran
PeCDD	=	Pentachlorodibenzo- <i>p</i> -dioxin	PeCDF	=	pentachlorodibenzofuran
HxCDD	=	Hexachlorodibenzo- <i>p</i> -dioxin	HxCDF	=	Hexachlorodibenzofuran
HpCDD	=	Heptachlorodibenzo- <i>p</i> -dioxin	HpCDF	=	Heptachlorodibenzofuran
OCDD	=	Octachlorodibenzo- <i>p</i> -dioxin	OCDF	=	Octachlorodibenzofuran

Table 2. Retention time references, quantitation references, relative retention times, and minimum levels for CDDs and CDFs.

PCDD/PCDF	Retention time andquantitation reference.....		Minimum level*.....		
			Water (pg/L)	Solid (pg/g)	Extract (pg/μL)
Compounds using ¹³ C ₁₂ -1-2,3,4-TCDD as the injection internal standard					
2,3,7,8-TCDF	¹³ C ₁₂ -2,3,7,8-TCDF	0.999-1.003	10	1	0.5
2,3,7,8-TCDD	¹³ C ₁₂ -2,3,7,8-TCDD	0.999-1.002	10	1	0.5
1,2,3,7,8-PeCDF	¹³ C ₁₂ -1,2,3,7,8-PeCDF	0.999-1.002	50	5	2.5
2,3,4,7,8-PeCDF	¹³ C ₁₂ -2,3,4,7,8-PeCDF	0.999-1.002	50	5	2.5
1,2,3,7,8-PeCDD	¹³ C ₁₂ -1,2,3,7,8-PeCDD	0.999-1.002	50	5	2.5
¹³ C ₁₂ -2,3,7,8-TCDF	¹³ C ₁₂ -1,2,3,4-TCDD	0.923-1.103	NA	NA	NA
¹³ C ₁₂ -2,3,7,8-TCDD	¹³ C ₁₂ -1,2,3,4-TCDD	0.976-1.043	NA	NA	NA
³⁷ Cl ₄ -2,3,7,8-TCDD	¹³ C ₁₂ -1,2,3,4-TCDD	0.989-1.052	NA	NA	NA
¹³ C ₁₂ -1,2,3,7,8-PeCDF	¹³ C ₁₂ -1,2,3,4-TCDD	1.000-1.425	NA	NA	NA
¹³ C ₁₂ -2,3,4,7,8-PeCDF	¹³ C ₁₂ -1,2,3,4-TCDD	1.011-1.526	NA	NA	NA
¹³ C ₁₂ -1,2,3,7,8-PeCDD	¹³ C ₁₂ -1,2,3,4-TCDD	1.000-1.567	NA	NA	NA
Compounds using ¹³ C ₁₂ -1,2,3,7,8,9-HxCDD as the injection internal standard					
1,2,3,4,7,8-HxCDF	¹³ C ₁₂ -1,2,3,4,7,8-HxCDF	0.999-1.001	50	5	2.5
1,2,3,6,7,8-HxCDF	¹³ C ₁₂ -1,2,3,6,7,8-HxCDF	0.997-1.005	50	5	2.5
1,2,3,7,8,9-HxCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDF	0.999-1.001	50	5	2.5
2,3,4,6,7,8-HxCDF	¹³ C ₁₂ -2,3,4,6,7,8-HxCDF	0.999-1.001	50	5	2.5
1,2,3,4,7,8-HxCDD	¹³ C ₁₂ -1,2,3,4,7,8-HxCDD	0.999-1.001	50	5	2.5
1,2,3,6,7,8-HxCDD	¹³ C ₁₂ -1,2,3,6,7,8-HxCDD	0.998-1.004	50	5	2.5
1,2,3,7,8,9-HxCDD		1.000-1.019	50	5	2.5
1,2,3,4,6,7,8-HpCDF	¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDF	0.999-1.001	50	5	2.5
1,2,3,4,7,8,9-HpCDF	¹³ C ₁₂ -1,2,3,4,7,8,9-HpCDF	0.999-1.001	50	5	2.5
1,2,3,4,6,7,8-HpCDD	¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDD	0.999-1.001	50	5	2.5
OCDF	¹³ C ₁₂ -OCDD	0.999-1.008	100	10	5.0
OCDD	¹³ C ₁₂ -OCDD	0.999-1.001	100	10	5.0
¹³ C ₁₂ -1,2,3,4,7,8-HxCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.944-0.970	NA	NA	NA
¹³ C ₁₂ -1,2,3,6,7,8-HxCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.949-0.975	NA	NA	NA
¹³ C ₁₂ -1,2,3,7,8,9-HxCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.977-1.047	NA	NA	NA
¹³ C ₁₂ -2,3,4,6,7,8-HxCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.959-1.021	NA	NA	NA
¹³ C ₁₂ -1,2,3,4,7,8-HxCDD	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.977-1.000	NA	NA	NA
¹³ C ₁₂ -1,2,3,6,7,8-HxCDD	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	0.981-1.003	NA	NA	NA
¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	1.043-1.085	NA	NA	NA
¹³ C ₁₂ -1,2,3,4,7,8,9-HpCDF	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	1.057-1.151	NA	NA	NA
¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDD	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	1.086-1.110	NA	NA	NA
¹³ C ₁₂ -OCDD	¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	1.032-1.311	NA	NA	NA

Relative retention time criteria apply to reporting for EPA Method 1613. Unless EPA Method 1613 is specifically required, the -1 to + 3 sec RT criteria are used (Section 3.2.1.1).

*The Minimum Level (ML) for each analyte is defined as the level at which the entire analytical system must give a recognizable signal and acceptable calibration point. It is equivalent to the concentration of the lowest calibration standard, assuming that all method-specific sample weights, volumes, and cleanup procedures have been employed.

The retention time reference for 1,2,3,7,8,9-HxCDD is ¹³C₁₂-1,2,3,6,7,8-HxCDD, and 1,2,3,7,8,9-HxCDD is quantified using the averaged responses for ¹³C₁₂-1,2,3,4,7,8-HxCDD and ¹³C₁₂-1,2,3,6,7,8-HxCDD.

Table 3. Concentration of PCDDs and PCDFs in calibration (CS) and calibration verification (VER CS) solutions.

CDD/CDF	CS1 (pg/ μ L)	CS2 (pg/ μ L)	VER CS3 (pg/ μ L)	CS4 (pg/ μ L)	CS5 (pg/ μ L)
2,3,7,8-TCDD	0.5	2	10	40	200
2,3,7,8-TCDF	0.5	2	10	40	200
1,2,3,7,8-PeCDD	2.5	10	50	200	1000
1,2,3,7,8-PeCDF	2.5	10	50	200	1000
2,3,4,7,8-PeCDF	2.5	10	50	200	1000
1,2,3,4,7,8-HxCDD	2.5	10	50	200	1000
1,2,3,6,7,8-HxCDD	2.5	10	50	200	1000
1,2,3,7,8,9-HxCDD	2.5	10	50	200	1000
1,2,3,4,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,6,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,7,8,9-HxCDF	2.5	10	50	200	1000
2,3,4,6,7,8-HxCDF	2.5	10	50	200	1000
1,2,3,4,6,7,8-HpCDD	2.5	10	50	200	1000
1,2,3,4,6,7,8-HpCDF	2.5	10	50	200	1000
1,2,3,4,7,8,9-HpCDF	2.5	10	50	200	1000
OCDD	5.0	20	100	400	2000
OCDF	5.0	20	100	400	2000
¹³ C ₁₂ -2,3,7,8-TCDD	100	100	100	100	100
¹³ C ₁₂ -2,3,7,8-TCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,7,8-PeCDD	100	100	100	100	100
¹³ C ₁₂ -1,2,3,7,8-PeCDF	100	100	100	100	100
¹³ C ₁₂ -2,3,4,7,8-PeCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,4,7,8-HxCDD	100	100	100	100	100
¹³ C ₁₂ -1,2,3,6,7,8-HxCDD	100	100	100	100	100
¹³ C ₁₂ -1,2,3,4,7,8-HxCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,6,7,8-HxCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,7,8,9-HxCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDD	100	100	100	100	100
¹³ C ₁₂ -1,2,3,4,6,7,8-HpCDF	100	100	100	100	100
¹³ C ₁₂ -1,2,3,4,7,8,9-HpCDF	100	100	100	100	100
¹³ C ₁₂ -OCDD	200	200	200	200	200
Cleanup standard					
³⁷ Cl ₄ -2,3,7,8-TCDD	0.5	2	10	40	200
Internal standards					
¹³ C ₁₂ -1,2,3,4-TCDD	100	100	100	100	100
¹³ C ₁₂ -1,2,3,7,8,9-HxCDD	100	100	100	100	100

2. QUALITY CONTROL

2.1. Instrument criteria

2.1.1. Mass spectrometer performance

The mass spectrometer was operated in the electron ionization mode. A static resolving power of at least 10,000 (10% valley definition) was demonstrated at appropriate masses before any analyses were performed. Static resolving power checks were performed at the beginning of each 12 hr period of operation. Corrective action was implemented whenever the resolving power does not meet the requirement.

Analysis time for PCDDs and PCDFs exceed the long term mass stability of the mass spectrometer. Because the instrument was operated in high-resolution mode, mass drifts of a few parts per million can have serious adverse effects on instrument performance. Therefore, a mass drift correction was mandatory. A lock-mass ion was selected from the reference compound perfluorokerosene (PFK) used for tuning the mass spectrometer. The selection of the lock-mass ion was dependent on the masses of the ions monitored within each descriptor (see Table 4). Any acceptable lock-mass ion at any mass between the lightest and heaviest ion in each descriptor can be used to monitor and correct mass drifts.

The level of the reference compound (PFK) metered into the ion chamber during HRGC/HRMS analyses was adjusted so that the amplitude of the most intense selected lock-mass ion signal did not exceed 10% of the full scale deflection for a given set of detector parameters. Suggested lock-mass ions are listed in Table 4.

Documentation of the instrument resolving power was accomplished by recording the peak profile of the reference compound peaks within each descriptor. The format of the peak profile representation allowed manual determination of the resolution, i.e., the horizontal axis was a calibrated mass scale. The result of the peak width measurement performed at the 10% valley definition did not exceed 100 ppm for all reference compound peaks recorded.

Total cycle time (dwell times plus switching times) for all ions within a single descriptor were one second or less.

2.1.2. GC column performance

Using the GC column performance check solution (CPSM/WDM, see Table 5), the chromatographic separation between 2,3,7,8-TCDD and the peaks representing any other unlabeled TCDD isomers were resolved with a valley of $\leq 25\%$, where:

$$\text{Eq. 1 Valley percent} = \frac{x}{y} 100$$

x is height of the valley between 2,3,7,8-TCDD and the closest eluting isomer, and y is the peak height of 2,3,7,8-TCDD.

When confirmation analysis of the 2,3,7,8-TCDF isomer was required, chromatographic separation between 2,3,7,8-TCDF and the peaks representing any other unlabeled TCDF isomers was resolved with a valley of ≤ 25 percent using Eq. 1, replacing TCDD with TCDF.

Table 4. Descriptors, exact m/z's, m/z types, and elemental compositions of the CDDs and CDFs.

Descriptor	Exact m/z*	m/z type	Elemental composition	Substance	
1	292.9825	Lock	C ₇ F ₁₁	PFK	
	303.9016	M	C ₁₂ H ₄ ³⁵ Cl ₄ O	TCDF	
	305.8987	M+2	C ₁₂ H ₄ ³⁵ Cl ₃ ³⁷ ClO	TCDF	
	315.9419	M	¹³ C ₁₂ H ₄ ³⁵ Cl ₄ O	TCDF	
	317.9389	M+2	¹³ C ₁₂ H ₄ ³⁵ Cl ₄ ³⁷ Cl ₃ O	TCDF	
	319.8965	M	C ₁₂ H ₄ ³⁵ Cl ₄ O ₂	TCDD	
	321.8936	M+2	C ₁₂ H ₄ ³⁵ Cl ₃ O ₂	TCDD	
	327.8847	M	C ₁₂ H ₄ ³⁷ Cl ₄ O ₂	TCDD	
	330.9792	QC	C ₇ F ₁₃	PFK	
	331.9368	M	¹³ C ₁₂ H ₄ ³⁵ Cl ₄ O ₂	TCDD	
	333.9339	M+2	¹³ C ₁₂ H ₄ ³⁵ Cl ₃ ³⁷ ClO ₂	TCDD	
	375.8364	M+2	C ₁₂ H ₄ ³⁵ Cl ₅ ³⁷ ClO	HxCDFE	
	2	339.8597	M+2	C ₁₂ H ₃ ³⁵ Cl ₄ ³⁷ ClO	PeCDF
		341.8567	M+4	C ₁₂ H ₃ ³⁵ Cl ₃ ³⁷ Cl ₂ O	PeCDF
351.9000		M+2	¹³ C ₁₂ H ₃ ³⁵ Cl ₄ ³⁷ ClO	PeCDF	
353.8970		M+4	¹³ C ₁₂ H ₃ ³⁵ Cl ₃ ³⁷ Cl ₂ O	PeCDF	
354.9792		Lock	C ₉ F ₁₃	PFK	
355.8546		M+2	C ₁₂ H ₃ ³⁵ Cl ₄ ³⁷ ClO ₂	PeCDD	
357.8516		M+4	C ₁₂ H ₃ ³⁵ Cl ₃ ³⁷ Cl ₂ O ₂	PECDD	
367.8949		M+2	¹³ C ₁₂ H ₃ ³⁵ Cl ₄ ³⁷ ClO ₂	PeCDD	
369.8919		M+4	¹³ C ₁₂ H ₃ ³⁵ Cl ₃ ³⁷ Cl ₂ O ₂	PeCDD	
409.7974		M+2	C ₁₂ H ₃ ³⁵ Cl ₆ ³⁷ ClO	HpCDFE	
3	373.8208	M+2	C ₁₂ H ₂ ³⁵ Cl ₅ ³⁷ ClO	HxCDF	
	375.8178	M+4	C ₁₂ H ₂ ³⁵ Cl ₄ ³⁷ Cl ₂ O	HxCDF	
	383.8639	M	¹³ C ₁₂ H ₂ ³⁵ Cl ₆ O	HxDDF	
	385.8610	M+2	¹³ C ₁₂ H ₂ ³⁵ Cl ₅ ³⁷ ClO	HxCDF	
	389.8157	M+2	C ₁₂ H ₂ ³⁵ Cl ₅ ³⁷ ClO ₂	HxCDD	
	391.8127	M+4	C ₁₂ H ₂ ³⁵ Cl ₄ ³⁷ Cl ₂ O ₂	HxCDD	
	392.9760	Lock	C ₉ F ₁₅	PFK	
	401.8559	M+2	¹³ C ₁₂ H ₂ ³⁵ Cl ₅ ³⁷ ClO ₂	HxCDD	
	403.8529	M+4	¹³ C ₁₂ H ₂ ³⁵ Cl ₄ ³⁷ Cl ₂ O ₂	HxCDD	
	430.9729	QC	C ₉ F ₁₇	PFK	
445.7555	M+4	C ₁₂ H ₂ ³⁵ Cl ₆ ³⁷ Cl ₂ O	OCDPE		

Table 4. Descriptors, exact m/z's, m/z types, and elemental compositions of the CDDs and CDFs (cont.).

Descriptor	Exact m/z*	m/z type	Elemental composition	Substance
4	407.7818	M+2	C ₁₂ H ³⁵ Cl ₆ ³⁷ ClO	HpCDF
	409.7789	M+4	C ₁₂ H ³⁵ Cl ₅ ³⁷ Cl ₂ O	HpCDF
	417.8253	M	¹³ C ₁₂ H ³⁵ Cl ₇ O	HpCDF
	419.8220	M+2	¹³ C ₁₂ H ³⁵ Cl ₆ ³⁷ ClO	HpCDF
	423.7766	M+2	C ₁₂ H ³⁵ Cl ₆ ³⁷ ClO ₂	HpCDD
	425.7737	M+4	C ₁₂ H ³⁵ Cl ₅ ³⁷ Cl ₂ O ₂	HpCDD
	430.9729	Lock	C ₉ F ₁₇	PFK
	435.8169	M+2	¹³ C ₁₂ H ³⁵ Cl ₆ ³⁷ ClO ₂	HpCDD
	437.8140	M+4	¹³ C ₁₂ H ³⁵ Cl ₅ ³⁷ Cl ₂ O ₂	HpCDD
	479.7165	M+4	C ₁₂ H ³⁵ Cl ₇ ³⁷ Cl ₂ O	NCDPE
5	441.7428	M+2	C ₁₂ ³⁵ Cl ₇ ³⁷ ClO	OCDF
	442.9728	Lock	C ₁₀ F ₁₇	PFK
	443.7399	M+4	C ₁₂ ³⁵ Cl ₆ ³⁷ Cl ₂ O	OCDF
	457.7377	M+2	C ₁₂ ³⁵ Cl ₇ ³⁷ ClO ₂	OCDD
	459.7348	M+4	C ₁₂ ³⁵ Cl ₆ ³⁷ Cl ₂ O ₂	OCDD
	469.7779	M+2	¹³ C ₁₂ ³⁵ Cl ₇ ³⁷ ClO ₂	OCDD
	471.7750	M+4	¹³ C ₁₂ ³⁵ Cl ₆ ³⁷ Cl ₂ O ₂	OCDD
	513.6775	M+4	C ₁₂ ³⁵ Cl ₈ ³⁷ Cl ₂ O	DCDPE

* Nuclidic masses used:

H = 1.007825
 C = 12.0000
¹³C = 13.003355
 F = 18.9984
 O = 15.994915
³⁵Cl = 34.968853
³⁷Cl = 36.965903

DCDPE	=	Decachlorodiphenyl ether	OCDD	=	Octachlorodibenzo- <i>p</i> -dioxin
HpCDD	=	Heptachlorodibenzo- <i>p</i> -dioxin	OCDF	=	Octachlorodibenzofuran
HpCDF	=	Heptachlorodibenzofuran	OCDFE	=	Octachlorodiphenyl ether
HpCDPE	=	Heptachlorodibenzofuran	PeCDD	=	Pentachlorodibenzo- <i>p</i> -dioxin
HxCDD	=	Hexachlorodibenzo- <i>p</i> -dioxin	PeCDF	=	Pentachlorodibenzofuran
HxCDF	=	Hexachlorodibenzofuran	PFK	=	Perfluorokerosene
HxCDPE	=	Hexachlorodiphenyl ether	TCDD	=	Tetrachlorodibenzo- <i>p</i> -dioxin
NCDPE	=	Nonachlorodiphenyl ether	TCDF	=	Tetrachlorodibenzofuran

Labeled compound.

There is only one m/z for ³⁷Cl₄-2,3,7,8-TCDD (cleanup standard)

Table 5. GC retention time window defining solution and isomer specificity test standard (Section 3.1.2).

DB-5 column GC retention-time window defining solution

CDD/CDF	First eluted	Last eluted
TCDF	1,3,6,8-	1,2,8,9-
TCDD	1,3,6,8-	1,2,8,9-
PeCDF	1,3,4,6,8-	1,2,3,8,9-
PeCDD	1,2,4,7,9-	1,2,3,8,9-
HxCDF	1,2,3,4,6,8-	1,2,3,4,8,9
HxCDD	1,2,4,6,7,9-	1,2,3,4,6,7
HpCDF	1,2,3,4,6,7,8-	1,2,3,4,7,8,9
HpCDD	1,2,3,4,6,7,9-	1,2,3,4,6,7,8-

DB-5 column TCDD specificity test standard

1,2,3,7- and 1,2,3,8-TCDD
2,3,7,8-TCDD
1,2,3,9-TCDD

DB-225 column TCDF isomer specificity test standard

2,3,4,7-TCDF
2,3,7,8-TCDF
1,2,3,9-TCDF

The GC column performance check solution (CPSM/WDM, see Table 5) also contained the known first and last PCDD/PCDF eluters under the conditions specified in this method. Their retention times were used to determine the eight homologue retention time windows that were used for qualitative and quantitative purposes. The retention times for the switching of ions characteristic of one homologous series to the next higher homologous series was indicated in the selected ion current profile (SICP). Accurate switching at the appropriate times was absolutely necessary for accurate monitoring of these compounds.

2.2. Analyte identification criteria

2.2.1. Retention times

For 2,3,7,8-substituted congeners with an isotopically labeled quantitation or internal standard present in the sample extract the retention time at maximum peak height of the sample components (i.e., the two ions used for quantitation purposes listed in Table 4) were within -1 to +3 sec of the isotopically labeled standard.

For 2,3,7,8-substituted compounds that did not have an isotopically labeled quantitation or internal standard present in the sample extract, the retention time fell within 0.005 retention time units of the relative retention times measured in the routine calibration. Identification of

OCDF was based on its retention time relative to $^{13}\text{C}_{12}$ -OCDD as determined from the daily routine calibration results.

The ion current responses for both ions used for quantitative purposes reach their maxima within ± 2 sec of each other.

The ion current responses for both ions used for the labeled standards reach their maxima within ± 2 sec of each other.

2.2.2. Ion abundance ratios

The integrated ion current profiles for the two ions used for quantitation purposes had a ratio between the lower and upper limits established for the homologous series to which the peak was assigned. See Table 6 for the required theoretical ion ratios and their QC acceptance limits.

2.2.3. Signal-to-noise ratio

All ion current intensities were greater than or equal to 3 times the noise level to be considered positive identification of a PCDD or PCDF compound or a group of coeluting isomers.

The peaks representing the PCDDs and PCDFs and labeled compounds in the CS1 calibration solution had signal-to-noise (S/N) ratios greater than or equal to 10.

2.2.4. Polychlorinated diphenylether interferences

In addition to the above criteria, the identification of a GC peak as a PCDF was only made if no signal having a S/N greater than 3 was detected at the same retention time (± 2 sec) in the corresponding polychlorinated diphenylether (PCDPE, see Table 4) channel.

2.3. Calibration criteria

2.3.1. Initial calibration

All five calibration solutions listed in Table 3 (CS1-CS5) were used for the initial calibration.

The theoretical ion abundance ratios for all 17 native analytes and all 16 labeled compounds were within the QC control limits specified in Table 6. The signal-to-noise ratio for the GC signals present in each SICP (including the ones for the labeled standards) were greater than or equal to 10. The percent relative standard deviations for the mean relative response factors from the 17 unlabeled standards did not exceed $\pm 20\%$, and those for the fifteen labeled reference compounds did not exceed $\pm 30\%$.

2.3.2. Continuing calibration (VER for EPA Method 1613)

Continuing calibration was performed at the beginning of each 12 hr period after successful mass resolution and GC resolution performance checks were completed. A routine calibration was also required at the end of a 12 hr run.

Table 6. Theoretical ion abundance ratios and QC limits*.

Number of chlorine atoms	m/z's forming ratio	Theoretical ratio	Lower	Upper
4	M/(M+2)	0.77	0.65	0.89
5	(M+2)/(M+4)	1.55	1.32	1.78
6	(M+2)/(M+4)	1.24	1.05	1.43
6	M/(M+2)	0.51	0.43	0.59
7	(M+2)/(M+4)	1.05	0.88	1.20
7 •	M/(M+2)	0.44	0.37	0.51
8	(M+2)/(M+4)	0.89	0.76	1.02

* QC limits represent $\pm 15\%$ windows around the theoretical ion abundance ratios.

Does not apply to $^{37}\text{Cl}_4$ -2,3,7,8-TCDD (cleanup standard).

Used for $^{13}\text{C}_{12}$ -HxCDF only.

• Used for $^{13}\text{C}_{12}$ -HpCDF only.

The isotopic ratios for all 17 native analytes and all 16 labeled compounds were within the control limits for the theoretical ion abundance ratios specified in Table 6.

The signal-to-noise ratio for the GC signals present in each SICP (including the ones for the labeled standards) were greater than 10.

The QC acceptance criteria for the calculated relative response factors for the unlabeled standards were within $\pm 20\%$ of the mean values established during the initial calibration. QC acceptance criteria for the calculated relative response factors for the labeled standards were within $\pm 30\%$ of the mean values established during the initial calibration.

2.4. Criteria for QC samples in an analytical batch

2.4.1. Method blank

A Method Blank was used to demonstrate freedom from contamination in the analytical procedure, and was required for each set of 20 or fewer samples.

If any of the 2,3,7,8-substituted PCDDs or PCDFs were found in the blank at greater than the minimum level (Table 2), or if any potentially interfering compound is found in the blank at the minimum level for each level of chlorination given in Table 2, re-extraction of the analytical batch was indicated.

Analyst discretion was used when contamination was present which did not adversely effect the analytical results.

If any of the 2,3,7,8-substituted PCDDs or PCDFs were found in the blank at greater than the minimum level, but were not detected in the analytical samples above this level, no action was required.

When target analytes were present in the method blank and in the analytical samples at concentrations above the Minimum Level, analytical data for those analytes in the samples were flagged.

2.4.2. Laboratory blank spike

A Laboratory Blank Spike (LBS) was used to demonstrate analytical precision of the method, and was required with each set of 20 or fewer samples.

QC acceptance criteria for analyte recoveries was 70 to 130% of the spiked amount.

If six or more of the seventeen target analytes were outside the acceptance criteria corrective action was taken. Corrective action may have included recalculation, reanalysis, instrument maintenance, recalibration, or re-extraction of the analytical batch.

2.4.3. Matrix spike and matrix spike duplicate

A matrix spike (MS) and a matrix spike duplicate (MSD) sample were used to demonstrate analytical accuracy and precision in the presence of a representative matrix and was required for each set of 20 or fewer samples.

QC acceptance criteria for analyte recoveries in the MS and the MSD was 70 to 130% of the spiked amount.

The results obtained from the MS and MSD samples should agree within a relative standard deviation (RSD) of 20%.

The MS and MSD acceptance criteria were advisory. However, if six or more of the seventeen target analytes were outside the QC acceptance criteria corrective action was taken. Corrective action may have included recalculation, reanalysis of the MS and MSD, instrument maintenance, or recalibration.

2.4.4. Duplicate

A sample duplicate (DUP) was used to demonstrate matrix homogeneity and analytical precision in the presence of a representative matrix, and was required with each set of 20 or fewer samples.

QC acceptance criteria for analyte concentrations greater than ten times the minimum level was a RSD of 20% or less.

The DUP acceptance criteria were advisory. However, if six or more of the seventeen target analytes were outside the QC acceptance criteria corrective action was taken. Corrective action may have included recalculation, reanalysis, instrument maintenance, or recalibration.

2.4.5. Reference material

A Reference Material (RM) was used to demonstrate analytical accuracy on a material from an independent source and was required with each set of 20 or fewer samples.

QC acceptance criteria for analyte concentrations less than ten times the ML were $\pm 35\%$ of the true value.

The QC acceptance criteria was invalid when the analyte concentration falls below the minimum level listed in Table 2.

The SRM acceptance criteria were advisory. However, if more than 35% of the analytes fall outside the QC acceptance criteria, corrective action was taken. Corrective action included recalculation and/or reanalysis of the SRM, instrument maintenance and/or recalibration.

2.4.6. Labeled compound recovery

All samples were spiked with the Labeled Compound Spiking Solution (LCSS) to monitor method performance on the sample matrix. QC acceptance criteria for labeled compound was 40% to 135% recovery. If a labeled compound recovery fell below the acceptance criteria, but the sum of the areas of the affected peaks was 10% or greater than the area of the corresponding peaks in the continuing calibration standard, the analytical data were accepted. If six or more of the fifteen labeled compounds were outside the acceptance criteria corrective action was taken. Corrective action may have included recalculation, reanalysis, instrument maintenance, recalibration, or re-extraction of the sample aliquot.

3. CHROMATOGRAPHIC CONDITIONS

3.1. Gas chromatograph

The gas chromatograph (GC) used for this analytical method was an HP5890 Series II. The GC had an injection port designed for capillary columns and splitless injections, was capable of temperature ramp programming with an isothermal hold, and of meeting all of the performance specifications outlined in Section 2.1.2. A 2 μ L splitless injection was used for all extracts, blanks, calibration solutions and the performance check samples.

All injections were made with a CTC-2000S programmable autosampler.

The GC/MS interface components withstand 350 °C, and were designed so that the separation of 2,3,7,8-TCDD from the other TCDD isomers achieved in the gas chromatographic column was not appreciably degraded. The GC column was fitted directly into the mass spectrometer ion source without being exposed to the ionizing electron beam.

3.2. GC columns

The GC column used for analysis of PCDDs and PCDFs and for isomer specificity of 2,3,7,8-TCDD was a 60 meter J&W DB5 or DB5MS column with a 0.25 mm i.d. and a 0.25 μ m film thickness.

The GC column used for isomer specificity and confirmation of 2,3,7,8-TCDF was a J&W DB225 column with 0.25 mm i.d. and 0.25 μ m film thickness.

3.3. Operating conditions

Operating conditions known to produce acceptable results with the recommended analytical column (DB5MS) are shown below:

Inject at 190 °C, hold for 1 min
Ramp at 20 °C per min to 240 °C
Ramp at 1 °C per min to 295 °C, hold for 2 min

Operating conditions known to produce acceptable results with the recommended confirmation column (DB225) are shown below:

Inject at 200 °C, hold for 1 min
Ramp at 12 °C per min to 300 °C, hold for 10 min

4. DETECTOR AND DATA SYSTEM CRITERIA

The detector used for these analyses was a VG AutoSpec Ultima utilizing 35 eV electron impact ionization, capable of repetitively selectively monitoring a minimum of 12 exact m/z's at high resolution ($\geq 10,000$) during a period of approximately 1 sec or less, and meeting all of the performance specifications outlined in Section 3.2.1.

A dedicated data system was employed to control the rapid multiple-ion monitoring process and to acquire the data. Quantitation data (peak areas and/or peak heights) and Selected Ion Monitoring (SIM) traces are acquired during the analyses and stored. The data system was capable of acquiring data at a minimum of 12 ions in a single scan. It was capable of switching to different sets of ions (descriptors) at specified times during an acquisition, and of providing hard copies of individual ion chromatograms for selected gas chromatographic time intervals. It was capable of acquiring mass spectral peak profiles and providing hard copies of peak profiles to demonstrate the required resolving power. The data system permitted the measurement of noise on the base line.

5. INSTRUMENT CALIBRATION PROCEDURE

5.1. Initial calibration

Initial calibration was required before any samples were analyzed for PCDDs and PCDFs. Initial calibration was also required if any continuing calibration verification did not meet the required criteria listed in Section 3.3.2. All five high resolution concentration calibration solutions listed in Table 3 were used for the initial calibration.

The instrument was tuned with PFK as described in Section 2.1.1.

The CPSM/WDM (Table 5) was injected and the SIM mass spectral data were acquired as described in Section 2.1.1. The criteria listed in Section 2.2.2 were met.

Using the same GC and MS conditions that produced acceptable results with the CPSM/WDM, a 2 μ L portion of each of the five calibration solutions (CS1 - CS5) were analyzed and the following conditions were met.

The ratio of integrated ion currents for the homologous series quantitation ions appearing in Table 6 were within the indicated control limits set for each homologous series.

The ratio of integrated ion currents for the ions belonging to the $^{13}\text{C}_{12}$ -labeled quantitation and internal standards were within the control limits stipulated in Table 6.

All ratios were within the specified control limits simultaneously in one run. Otherwise, corrective action was necessary.

For each SICP and for each GC signal corresponding to the elution of a target analyte and of its $^{13}\text{C}_{12}$ -labeled standard(s), the signal-to-noise (S/N) ratio was greater than or equal to 10.

Referring to Table 2, the 17 relative response factors (RRF) for unlabeled target analytes [RRF(n); n = 1 to 17] relative to their appropriate quantitation standards, and the RRFs for the $^{13}\text{C}_{12}$ -labeled quantitation standards [RRF(m); m = 1 to 15] relative to the two internal standards were calculated according to the following equations.

$$\text{Eq. 2} \quad \text{RRF}(n) = \frac{A_x C_{qs}}{A_{qs} C_x}$$

$$\text{Eq. 3} \quad \text{RRF}(m) = \frac{A_{qs} C_{is}}{A_{is} C_{qs}}$$

where A_x was the sum of the integrated ion abundances of the quantitation ions for unlabeled PCDDs and PCDFs, A_{qs} was the sum of the integrated ion abundances of the quantitation ions for the labeled quantitation standards, A_{is} was the sum of the integrated ion abundances of the quantitation ions for the labeled internal standards, C_x was the concentration of the unlabeled PCDD and/or PCDF analyte in the calibration solution (pg/ μL), C_{qs} was the concentration of the $^{13}\text{C}_{12}$ -labeled quantitation standard in the calibration solution (100 pg/ μL), and C_{is} was the concentration of the $^{13}\text{C}_{12}$ -labeled internal standard in calibration solution (100 pg/ μL). The RRF(n) and RRF(m) are dimensionless quantities. The units used to express C_x , C_{qs} and C_{is} were the same.

The native OCDF was quantitated against labeled OCDD. The concentration of OCDF was corrected for the recovery of the $^{13}\text{C}_{12}$ -OCDD.

The 1,2,3,7,8,9-HxCDD isomer was quantitated using the averaged response of the labeled analogs of the other two 2,3,7,8-substituted HxCDDs. As a result, the concentration of 1,2,3,7,8,9-HxCDD was corrected for the average recovery of the other two $^{13}\text{C}_{12}$ -HxCDDs.

The mean RRFs, $\overline{\text{RRF}(n)}$, for the five calibration solutions were calculated using the following equations.

$$\text{Eq. 4} \quad \overline{\text{RRF}(n)} = \frac{1}{5} \sum_{j=1}^5 \text{RRF}_j(n)$$

$$\text{Eq. 5} \quad \overline{\text{RRF}(m)} = \frac{1}{5} \sum_{j=1}^5 \text{RRF}_j(m)$$

where n was a particular 2,3,7,8-substituted PCDD/PCDF unlabeled target analyte (n = 1 to 17), m was a particular $^{13}\text{C}_{12}$ -labeled quantitation standard (m = 1 to 15), and j was the injection number or calibration solution number (j = 1 to 5).

The percent relative standard deviations (%RSD) for each calibration mixture was determined. It was verified that the initial calibration met all of the acceptance criteria outlined in Section 2.3.1.

5.2. Continuing calibration verification

Routine calibration was performed at the beginning of a 12 hr period after successful MS resolution and GC resolution performance checks. A routine calibration was also required at the end of a 12 hr shift.

Using the same GC and MS conditions that were used for the CPSM/WDM and the initial calibration, a 2 μL portion of the midpoint calibration solution (CS3) was analyzed with the following mass spectrometer operating parameters.

The ratio of integrated ion currents for the homologous series quantitation ions appearing in Table 6 were within the indicated control limits set for each homologous series.

The ratio of integrated ion currents for the ions belonging to the $^{13}\text{C}_{12}$ -labeled quantitation and internal standards were within the control limits stipulated in Table 6.

All ratios were within the specified control limits simultaneously in one run. Otherwise, corrective action was necessary.

For each SICP and for each GC signal corresponding to the elution of a target analyte and of its $^{13}\text{C}_{12}$ -labeled standard(s), the signal-to-noise (S/N) ratio was greater than or equal to 10.

Referring to Table 2, the concentrations of the 17 unlabeled target analytes, and the concentration and percent recovery of the fifteen $^{13}\text{C}_{12}$ -labeled quantitation standards in the CS3 standard were calculated using the following equations.

$$\text{Eq. 6} \quad C_x = \frac{A_x C_{qs}}{A_{qs} \text{RRF}(n)}$$

$$\text{Eq. 7} \quad \text{Percent recovery} = \frac{A_x C_{is}}{A_{is} C_{qs} \text{RRF}(m)} 100$$

where A_x was the sum of the integrated ion abundances of the quantitation ions for unlabeled PCDDs and PCDFs, A_{qs} was the sum of the integrated ion abundances of the quantitation ions for the $^{13}\text{C}_{12}$ -labeled quantitation standards, A_{is} was the sum of the integrated ion abundances of the quantitation ions for the $^{13}\text{C}_{12}$ -labeled internal standards, C_x was the concentration of the unlabeled PCDDs and PCDFs isomers in $\text{pg}/\mu\text{L}$, C_{qs} was the concentration of the $^{13}\text{C}_{12}$ -labeled quantitation standard in the calibration solution (100 $\text{pg}/\mu\text{L}$), C_{is} = concentration of the $^{13}\text{C}_{12}$ -labeled internal standard in the calibration solution (100 $\text{pg}/\mu\text{L}$), $\text{RRF}(n)$ was the mean relative response factor for the unlabeled target analyte relative to its $^{13}\text{C}_{12}$ -labeled quantitation standard [$\text{RRF}(n)$, with $n = 1$ to 17], and $\text{RRF}(m)$ was the mean relative response factor for $^{13}\text{C}_{12}$ -labeled quantitation standard relative to its $^{13}\text{C}_{12}$ -labeled internal standard [$\text{RRF}(m)$, with $m = 1$ to 15].

Referring to Table 2, the 17 relative response factors (RRF) for unlabeled target analytes ($\text{RRF}(n)$; $n = 1$ to 17] relative to their appropriate quantitation standards (Eq. 2, Section 6.1.4), and the RRFs for the $^{13}\text{C}_{12}$ -labeled quantitation standards [$\text{RRF}(m)$; $m = 1$ to 15] relative to the two internal standards (Eq. 3, Section 5.1.4) were calculated.

6. ANALYTICAL STANDARDS

Analytical standards were purchased as solutions with certified purity, concentration and authenticity. The labeled compound spiking solution (LCSS), the cleanup recovery standard (CRS), the precision and recovery standard (PAR), the internal standard (IS) and the instrument calibration solutions (CS1 - CS5) were used as received from the manufacturer without further treatment. When not being used, standards were stored in the dark at 40 °C in screw-capped vials with PTFE-lined caps.

6.1. Labeled compound spiking solution

The labeled compound spiking solution (LCSS) solution contained the fifteen $^{13}\text{C}_{12}$ -labeled PCDD and PCDF quantitation standards in nonane at the nominal concentrations listed in Table 7. The LCSS (20 μL) was diluted in an appropriate solvent and spiked into each sample prior to extraction.

6.2. Precision and recovery standard

The precision and recovery standard (PAR) solution contained the seventeen 2,3,7,8-substituted native PCDD and PCDF isomers at the nominal concentrations listed in Table 7. This solution (5 μL) was diluted in an appropriate solvent and spiked into the selected matrix spike (MS), matrix spike duplicate (MSD), and/or laboratory blank spike (LBS) samples prior to extraction.

6.3. Internal standard

This solution contained two $^{13}\text{C}_{12}$ -labeled PCDD isomers. The internal standard (IS) (10 μL) was added to the final sample extract before HRGC/HRMS analysis to determine the percent recoveries for the LCSS and CRS compounds.

6.4. Instrument calibration standards

These solutions contained the seventeen 2,3,7,8-substituted native PCDDs and PCDFs and the sixteen $^{13}\text{C}_{12}$ -labeled quantitation and internal standards at the nominal concentrations listed in Table 3. The analyte concentrations were homologue dependent, with the lowest concentrations for the tetrachlorinated dioxin and furan and the highest concentrations for the octachlorinated congeners. These solutions permitted the relative response factors to be measured as a function of concentration. The CS3 standard was also used as the continuing calibration verification (VER).

6.5. Column performance and window defining mix

This solution contained the first and last eluting isomers for each homologous series from tetra- through heptachlorinated congeners. The solution also contained a series of other TCDD isomers for the purpose of documenting the chromatographic resolution of 2,3,7,8-TCDD. The $^{13}\text{C}_{12}$ -2378-TCDD was present for positive identification of the 2,3,7,8-TCDD isomer (see Table 5).

6.6. 2,3,7,8-TCDF CPSM

This solution contained a series of three TCDF isomers for the purpose of documenting the chromatographic resolution of 2,3,7,8-TCDF on a DB225 analytical column (see Table 5).

7. SAMPLE ANALYSIS

The instrument was tuned with PFK as described in Section 2.1.1.

The CPSM/WDM (Table 5) was injected and the SIM mass spectral data was acquired as described in Section 2.1.1.

The CS3 Calibration Verification solution (Table 3) was injected and the SIM mass spectral data were acquired as described in Section 2.1.1. The acceptance criteria listed in Section 2.3.2 were met.

Toluene (2 μ L) was injected and the SIM mass spectral data was acquired to demonstrate that the analytical system was free of interfering contamination.

The sample extract (2 μ L) was injected and the SIM mass spectral data acquired under these same conditions.

7.1. Qualitative identification

For a gas chromatographic peak to be identified as a PCDD or PCDF isomer, it met all of the criteria specified in Section 3.2.

7.2. Quantitative determination

For gas chromatographic peaks that met all the qualitative identification criteria, the concentration of the PCDD or PCDF isomers were calculated using the following equation:

$$\text{Eq. 8} \quad C_x = \frac{A_x C_{qs}}{A_{qs} W \text{ RRF}(n)}$$

where C_x was the concentration of the unlabeled PCDD and PCDF isomer in pg/g (ppt), A_x was the sum of the integrated ion abundances of the quantitation ions for unlabeled PCDDs and PCDFs, A_{qs} was the sum of the integrated ion abundances of the quantitation ions for the $^{13}\text{C}_{12}$ -labeled quantitation standards, Q_{qs} was the amount in pg of the $^{13}\text{C}_{12}$ -labeled quantitation standard added to the sample immediately prior to extraction, $\text{RRF}(n)$ was the mean relative response factor for the unlabeled target analyte relative to its $^{13}\text{C}_{12}$ -labeled quantitation standard [$\text{RRF}(n)$, with $n = 1$ to 17], and W was the weight, in g, of the sample.

The percent recovery of the fifteen quantitation standards in the sample extract was calculated using the following equation:

$$\text{Eq. 9} \quad \text{Percent recovery} = \frac{A_{qs} Q_{is}}{A_{is} Q_{qs} \text{ RRF}(m)} 100$$

where A_{qs} was the sum of the integrated ion abundances of the quantitation ions for the $^{13}C_{12}$ -labeled quantitation standards, A_{is} was the sum of the integrated ion abundances of the quantitation ions for the $^{13}C_{12}$ -labeled internal standards, Q_{qs} was the amount in pg of the $^{13}C_{12}$ -labeled quantitation standard added to the sample immediately prior to extraction, Q_{is} was the amount in pg of the $^{13}C_{12}$ -labeled internal standard added to the sample extract in the last step of preparation, $\overline{RRF(m)}$ was the mean relative response factor for $^{13}C_{12}$ -labeled quantitation standard relative to its $^{13}C_{12}$ -labeled internal standard [$\overline{RRF(m)}$, with $m = 1$ to 15].

A sample specific estimated detection limit (EDL) was calculated for each 2,3,7,8-substituted congener that was not identified, regardless of whether or not other non-2,3,7,8-substituted isomers were present. The EDL was the concentration of a given analyte required to produce a signal with a peak height of at least 3 times the background signal level. The quantitation software (OPUSQuan) supplied by the instrument manufacturer calculated a sample specific-EDL using an algorithm based on the criteria outlined in EPA Method 8290, Section 7.9.5.1.1.

The relative percent difference (RPD) between duplicate sample results was calculated.

The percent recovery of analytes in the MS and MSD samples were reported.

7.3. Confirmation analysis

If the 2,3,7,8-TCDF isomer was identified at a concentration which was above the minimum level, the 2,3,7,8-TCDF concentration was confirmed on a second analytical column (DB225).

Following the steps outlined in 7.1 through 7.6, the sample extract was reanalyzed on a DB225 analytical column. Use the GC conditions described in Section 3.3.2, and the mass spectral conditions described in section 2.1.1, only the ions listed in the first descriptor (Table 4) were monitored.

The concentration of 2,3,7,8-TCDF were calculated using Equation 8.

The lower of the two calculated concentrations was reported for 2,3,7,8-TCDF (DB5 and DB225).

8. INSTRUMENT MAINTENANCE

8.1. Gas chromatograph maintenance

The syringe was cleaned by rinsing with appropriate solvent after each injection. A new injection port liner and septum were installed at the beginning of each new run sequence. A new injection port base plate was installed as needed when chromatographic resolution fell below the QC acceptance criteria. One to two feet of the analytical column were removed as needed when chromatographic resolution fell below the QC acceptance criteria. The tank of carrier gas (He) was replaced when the pressure falls below 500 psi. All maintenance was recorded.

8.2. Mass spectrometer maintenance

The emission filament was replaced as necessary. The inner ion source assembly was cleaned and replaced as necessary. The outer ion source assembly was cleaned and replaced as necessary. The septum on the PFK reservoir was replaced as necessary. The transfer line/re-

entrant assembly was disassembled, cleaned, repaired and reassembled as necessary. The rotary pump oil was changed yearly, or more frequently if indicated. The diffusion pump oil was changed as necessary. All maintenance was recorded.

9. CONCLUSIONS

This method provides procedures for the detection and quantitative measurement of polychlorinated dibenzo-*p*-dioxins (tetra- through octa-chlorinated homologues; PCDDs) and polychlorinated dibenzofurans (tetra- through octa-chlorinated homologues; PCDFs) in sediments and bivalves at pg/g concentrations. The sensitivity of this method is dependent upon the level of interferences within a given matrix. The calibration range of the method for a 10 g sediment or tissue sample is 1.0 to 400 ppt for TCDD/TCDF, 5.0 to 2,000 ppt for PeCDD/PeCDF, HxCDD/HxCDF and HpCDD/HpCDF, and 10 to 4,000 ppt for OCDD/OCDF. The actual limits of detection will differ from the lower method calibration limit, depending on the complexity of the matrix.

10. REFERENCES

EPA (1992) EPA Method 8290: Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High-Resolution Gas Chromatography/High Resolution Mass Spectrometry (HRGC/HRMS), Revision 0, SW-846, U.S. Environmental Protection Agency, Office of Solid Waste, November 1992.

EPA (1994) EPA Method 1613: Tetra- Through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRCG/HRMS, Revision B, EPA 821-B-94-005, U.S. Environmental Protection Agency, Office of Water, October 1994.

Table 7. Concentration of stock and spiking solutions containing PCDDs and PCDFs labeled compounds.

CDD/CDF	Labeled Compound spiking solution (LCSS) (pg/ μ L)	PAR spiking solution (pg/ μ L)
2,3,7,8-TCDD	----	40
2,3,7,8-TCDF	----	40
1,2,3,7,8-PeCDD	----	200
1,2,3,7,8-PeCDF	----	200
2,3,4,7,8-PeCDF	----	200
1,2,3,4,7,8-HxCDD	----	200
1,2,3,6,7,8-HxCDD	----	200
1,2,3,7,8,9-HxCDD	----	200
1,2,3,4,7,8-HxCDF	----	200
1,2,3,6,7,8-HxCDF	----	200
1,2,3,7,8,9-HxCDF	----	200
2,3,4,6,7,8-HxCDF	----	200
1,2,3,4,6,7,8-HpCDD	----	200
1,2,3,4,6,7,8-HpCDF	----	200
1,2,3,4,7,8,9-HpCDF	----	200
OCDD	----	400
OCDF	----	400
$^{13}\text{C}_{12}$ -2,3,7,8-TCDD	100	----
$^{13}\text{C}_{12}$ -2,3,7,8-TCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,7,8-PeCDD	100	----
$^{13}\text{C}_{12}$ -1,2,3,7,8-PeCDF	100	----
$^{13}\text{C}_{12}$ -2,3,4,7,8-PeCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,4,7,8-HxCDD	100	----
$^{13}\text{C}_{12}$ -1,2,3,6,7,8-HxCDD	100	----
$^{13}\text{C}_{12}$ -1,2,3,4,7,8-HxCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,6,7,8-HxCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,7,8,9-HxCDF	100	----
$^{13}\text{C}_{12}$ -2,3,4,6,7,8-HxCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,4,6,7,8-HpCDD	100	----
$^{13}\text{C}_{12}$ -1,2,3,4,6,7,8-HpCDF	100	----
$^{13}\text{C}_{12}$ -1,2,3,4,7,8,9-HpCDF	100	----
$^{13}\text{C}_{12}$ -OCDD	200	----
		Concentration (pg/ μ L)
Cleanup standard	$^{37}\text{Cl}_4$ -2,3,7,8-TCDD	0.8
Internal standards	$^{13}\text{C}_{12}$ -1,2,3,4,-TCDD	200
	$^{13}\text{C}_{12}$ -1,2,3,7,8,9-HxCDD	200

Quantitative Determination of Chlorinated Hydrocarbons

J. L. Sericano, P. Gardinali, and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

Chlorinated hydrocarbons are quantitatively determined by gas chromatography with an electron capture detector (ECD). The ECD is very sensitive and allows for detection of the chlorinated hydrocarbons at trace concentrations (ppb).

1. INTRODUCTION

The quantitative method described in this document determines chlorinated hydrocarbons (e.g. chlorinated pesticides and PCBs) in sample extracts. The method is based on high resolution, capillary gas chromatography using electron capture detection (GC/ECD).

Sample collection, preservation, storage and holding times are discussed under the analytical procedures for sample extraction and purification.

2. APPARATUS AND MATERIALS

A gas chromatograph with a split/splitless injection system, capillary column capability and a electron capture detector (ECD) was utilized.

2.1. GC Column

A 30-m long x 0.25-mm i.d. fused silica capillary column with DB-5 and DB-17 HT (confirmation column) bonded phase (J&W Scientific or equivalent) were used. The column provided good resolution of chlorinated hydrocarbons, surrogates and internal standards.

2.2. Autosampler

The autosampler was capable of making 1 - 4 μ L injections.

3. REAGENTS

3.1. Calibration Solution

The calibration solution was comprised of the chlorinated hydrocarbons indicated in Table 1.

Table 1. Chlorinated hydrocarbons of interest.

Chlorinated pesticides	Polychlorinated biphenyls
1,2,4,5-Tetrachlorobenzene *	Dichlorobiphenyls
1,2,3,4-Tetrachlorobenzene *	PCB 8*
Pentachlorobenzene*	
Pentachloroanisole*	Trichlorobiphenyls
Endosulfan II	PCB 18*, PCB 31, PCB 28*
alpha-HCH*	
Hexachlorobenzene*	Tetrachlorobiphenyls
beta-HCH*	PCB 52*, PCB 49, PCB 44*, PCB 74, PCB
gamma-HCH*	70, PCB 66*, PCB 60
delta-HCH*	
Heptachlor*	Pentachlorobiphenyls
Heptachlor Epoxide*	PCB 84, PCB 85, PCB 101*, PCB 87*, PCB
Oxychlordane*	99, PCB 97, PCB 110, PCB 123, PCB 118*,
gamma-Chlordane*	PCB 114, PCB 105*
alpha-Chlordane*	
<i>trans</i> -Nonachlor*	Hexachlorobiphenyls
<i>cis</i> -Nonachlor*	PCB 153*, PCB 138*, PCB 158, PCB 128*,
Aldrin*	PCB 167, PCB 156, PCB 157
Dieldrin*	
Endrin*	Heptachlorobiphenyls
Mirex*	PCB 187*, PCB 183, PCB 174, PCB 177, PCB
2,4'-DDE*	180*, PCB 170*, PCB 189
4,4'-DDE*	
2,4'-DDE*	Octachlorobiphenyls
4,4'-DDE*	
2,4'-DDT*	PCB 201, PCB 196, PCB 195*, PCB 194,
4,4'-DDT*	PCB 203
	Nonachlorobiphenyl
	PCB 206*
	Decachlorobiphenyl
	PCB 209*

*Analytes in matrix spiking solution.

PCB number from Ballschmiter and Zell (1980).

Calibration standards were prepared in the concentration range of 5 to 200 ng/mL at four concentrations. Internal standard and surrogate compounds were added at 100 ng/mL to all calibration standards.

3.2. Surrogate spiking solution

The surrogate compounds for all sample types were 4,4'-dibromooctafluorobiphenyl (DBOBF), PCB 103, and PCB 198. A surrogate solution was made by weighing an appropriate aliquot of

pure material into a volumetric flask and diluting to volume with hexane. Surrogate standards were added to each sample at a concentration greater than ten times the MDL.

3.3. Internal standard solution

The internal standard for this analysis was tetrachloro-*m*-xylene (TCMX). An internal standard solution was made by weighing an appropriate aliquot of pure material into a volumetric flask and diluting to volume with hexane. Internal standard were added to each sample extract to obtain a final concentration of approximately 100 ng/mL.

3.4. Matrix recovery spiking solution

The matrix spike was added to samples at a concentration of 40 ng/mL.

3.5. Retention index solution

The calibration mixtures were used to determine retention windows. Retention windows were ± 0.07 min for all analytes in Table 1, with the exception of PCB 209, which had a retention window of ± 0.10 min.

4. PROCEDURE

4.1. Sample extraction and purification

Samples were extracted and purified following methods described in other sections of this document.

4.2. High resolution GC-ECD analysis

4.2.1. GC conditions

For the analysis of chlorinated hydrocarbons, the analytical system, or its equivalent, included at a minimum,

Instrument:	Hewlett-Packard 5880A or 5890 or Varian 3500 series
Features:	Split/splitless capillary inlet system, with data acquisition system
Inlet:	Splitless
Detector:	Electron Capture
Column:	0.25-mm i.d. x 30-m DB-5 fused silica capillary column (J&W Scientific) or equivalent
Second Column:	0.25 mm i.d. x 30-m DB-17 HT fused silica capillary column (J&W Scientific) or equivalent
Gases:	
Carrier:	Helium 1 mL/min
Make-Up:	Argon/methane (95/5) or nitrogen, 40 mL/min.

Temperatures:

Injection port:	275 °C
Detector:	325 °C
Oven Program:	100 °C for 1 min., then 5 °C/min. to 140 °C, hold 1 min.; 1.5 °C/min to 250 °C, hold 1 min.; 10 °C/min to 300 °C, hold 5 min. The GC oven temperature program may be modified to improve resolution.
Calibration:	Four-point calibration (5, 20, 80, and 200 ng/mL)
Quantification:	Surrogate standard/calibration

4.2.2. Calibration

Pesticide/PCB calibration was done as part of the analytical run. The four calibration mixtures were interspersed with actual samples during the GC/ECD analyses. The calibration curve was then based on four standards. If the calibration curve had an *r* of 0.9950 or higher for all analytes present in the samples it was accepted, if not the calibration standards as well as all the samples were reanalyzed by GC/ECD. Calibrations with internal standards was superior to the procedure where the instrument was initially calibrated at four points and then mid-level standards were analyzed during the analytical run. This latter calibration only insures that mid-level samples remain in calibration. Since the ECD detector was nonlinear, a single point check on its calibration was not as rigorous as calibration during the GC/ECD run.

4.2.3. Sample Analysis

As discussed in Section 4.2.2 calibration mixture, actual samples, and QA samples (e.g., blanks, matrix spikes, SRM) were analyzed as one analytical sequence.

Sample injections of 1 to 4 µL were made with an autosampling device.

If the response for any peak exceeded the highest calibration solution, the extract was diluted, a known amount of surrogate and TCMX solution added, and the sample reanalyzed for those analytes that exceeded the calibration range. If dilutions were necessary, the final concentration reported was adjusted to account for the surrogate recoveries determined by the initial injections.

4.2.4. Calculations

Concentrations in the samples were based on surrogate standards added. Analyte concentrations were calculated using the PCB 103 surrogate. The internal standard (TCMX) was used to calculate surrogate recoveries. DBOFB or PCB 198 was used to calculate selected analytes concentrations, if it was demonstrated that they produced more reliable data (i.e., if matrix interference occurs with PCB 103) based on percent recoveries in spiked blanks, matrix spikes, or reference materials.

5. QUALITY ASSURANCE/QUALITY CONTROL (QA/QC) REQUIREMENTS

5.1. Calibration checks

A four-point calibration curve was used to establish the response of the detector. The calibration curve was prepared using a non-linear calibration equation of the form:

$$Y = b_0 + b_1x + b_2x^2$$

where Y is the modified area ratio; the ratio of the area of the analyte to the area of the internal standard times the amount of the internal standard in micrograms.

$$Y = \frac{A(a)}{A(s)} C(s)$$

and b_0 , b_1 , and b_2 are coefficients of the quadratic equation.

A solution to the quadratic equation is:

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

which will give you the amount of analyte in $\mu\text{g}/\mu\text{L}$.

Alternatively, the following equation can be used:

$$Y = A x^B$$
$$Y = \frac{C_a}{C_{su}} = A \frac{A_a}{A_{su}}^B$$

where, A is the slope of the line fit, B is the polynomial coefficient for the line fit, C_a is the concentration of the analyte to be measured (ng/mL), C_{su} is the concentration of the surrogate standard (ng/mL) (PCB 103), A_a is the area for the analyte to be measured and A_{su} is the area for the surrogate standard (PCB 103).

The calibration solutions that were analyzed as part of the analytical GC/ECD run, were preceded by no more than six samples and no more than six samples are run between calibration mixtures.

5.2. Method blank analysis

An acceptable method blank contained no more than two target compounds at concentrations three times greater than the MDL. This criteria did not need to be met if the analytes in the blanks were not detected in the associated samples or the analyte concentration was greater than ten times the blank value. If the method blank did not meet these criteria, the analytical system was out of control, and the source of the contamination was investigated, corrective measures taken, and samples reanalyzed.

5.3. Surrogate standard analysis

All samples and quality control samples were spiked with DBOFB, PCB 103 and PCB 198. The surrogate standard solution was spiked into the sample prior to extraction in an attempt to minimize individual sample matrix effects associated with sample preparation and analysis.

The laboratory took corrective action whenever the recovery of the surrogate used for quantitation was outside of 50 to 125% range.

The following corrective action were taken when an out of control event occurs,

- a. Calculations were checked to assure that no errors had been made.
- b. The surrogate standard solutions were checked for degradation, contamination, or concentration, and the instrument performance was checked.
- c. If the surrogate could not be measured because the sample required dilution or only a portion of the sample was analyzed, or matrix interference occurs with only one surrogate, no corrective action was required. The surrogate recovery was properly annotated.
- d. If the steps above failed to reveal a problem, the sample or extract was reanalyzed. If reanalysis of the extract yielded surrogate recoveries within the stated limits, then the reanalyzed data were reported. If upon reinjection QA criteria were still violated, the sample was submitted for re-extraction if sufficient sample was available. If the sample was completely consumed, the data was reported but designated as outside the QA criteria.

5.4. Matrix spike/duplicate analysis

A matrix spike and a duplicate were analyzed with each sample set or every 20 field samples, whichever was more frequent. A sample was randomly chosen and split into subsamples. One subsample was fortified with the matrix spike solution. The acceptable matrix spike recovery criteria were 50 - 125% recovery for at least 80% of the analytes. Criterion for duplicates was 30% RPD.

If the matrix spike criteria were not met, the matrix spike was reinjected on the GC. If the reinjected matrix spike analysis met the criteria, then the reanalysis data was reported. If none of the analytes that were in violation were present in the sample the violation was noted but no action was required. If analytes that were present in the sample were in violation, the entire batch of samples was submitted for re-extraction, if sufficient sample was available. If the sample was completely consumed, the data was reported but designated as outside the QA criteria.

5.5. Method detection limit

The method detection limit was determined following the procedures outlined in Federal Register (1984), Vol. 49, No. 209, 198-199.

5.6. GC resolution

Most target compounds, surrogates and internal standard were resolved from one another and from interfering compounds. When they were not, coelutions were documented.

5.7. Reference material analysis

Standard reference materials (SRM) were analyzed for chlorinated hydrocarbons. One SRM sample was analyzed per batch of samples or every 20 samples whichever was more frequent. Results were within $\pm 30\%$ of the certified or reference value on the average for all analytes, and did not exceed $\pm 35\%$ of the certified or reference value for more than 30% of the individual analytes. If a value was not certified the QC criteria (35% and 30%) apply to either the upper or lower 95% confidence interval reference value.

6. CALCULATIONS

6.1. Chlorinated hydrocarbon calculations

All calculations were based on the surrogate PCB 103 added before extraction and purification. The actual sample concentration (C) for each compound was calculated by the following formula:

$$C = A \frac{A_a}{A_{su}} B \frac{I_{su}}{S_{DW}}$$

where A was the slope of the curve fit, B was the polynomial coefficient for the curve fit, A_a was the area for the analyte to be measured, A_{su} is the area for the surrogate (PCB 103), I_{su} was the amount of surrogate added to the sample, and S_{DW} was the sample dry weight.

6.2. Calculation notes

To each sample, a specific amount of surrogate standard was added. The recovery of these compounds was monitored in each sample using the response of TCMX the internal standard (I_{gc}) added to the final extract just prior to GC/ECD analyses.

$$\text{Percent surrogate recovery} = R_1 R_2 R_3 R_4 100$$

where R_1 was the surrogate peak area/internal standard peak area in sample, R_2 was the surrogate concentration/internal standard concentration in one of the calibration mixtures, R_3 was the internal standard peak area/surrogate peak area in one of the calibration mixtures, and R_4 was the amount of internal standard (I_{gc}) added to sample just prior to GC analysis/amount of surrogate standard added to sample just prior to sample extraction.

7. REPORTING

Data were reported in ng/g dry weight. The minimum method performance standard for tissues was 0.5 ng/g for individual compounds. Results were reported to three significant figures. Surrogate recoveries were reported for each sample analyzed. Matrix spike recoveries and duplicate relative percent difference were reported for each batch of samples analyzed. Results of the analysis of reference materials were reported for each batch of samples analyzed.

8. CONCLUSIONS

The electron capture detector provides a means to detect and quantitate chlorine containing organic contaminants at trace concentrations (ng/g). The detector, however, does not have a linear response over a wide concentration range and must be used by sufficiently trained personnel.

9. REFERENCE

Ballschmiter, K., and M. Zell (1980) Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. Frensenius Z. Anal. Chem., 302:20-31.

Quantitative Determination of Butyltins

J. L. Sericano and T. L. Wade
Geochemical and Environmental Research Group
Texas A&M University
College Station, TX

ABSTRACT

The quantitative determination of tetrabutyltin, tributyltin, dibutyltin, and monobutyltin in sediment and tissue samples by high resolution, capillary gas chromatography using flame photometric detection is described. Final concentrations are reported as ng/Sn/g for sediment and tissue samples.

1. INTRODUCTION

The quantitative method described in this document determined butyltins in extracts of water, sediments and tissues. The method was based on high resolution, capillary gas chromatography using flame photometric detection (GC/FPD). This method quantitatively determined tetrabutyltin (4BT), tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT).

Sample collection, preservation and storage times are discussed under the analytical procedures for sample extraction and purification.

2. APPARATUS AND MATERIALS

A gas chromatograph with a split/splitless injection system, capillary column capability and a flame photometric detector (FPD) equipped with a tin selective 610 nm filter was used. The output from the detector was collected and processed by an automated data acquisition software package.

2.1. GC Column

A 30-m long x 0.32-mm i.d. fused silica capillary column with DB-5 bonded phase (J&W Scientific or equivalent) was used.

2.2. Autosampler

The autosampler was capable of making 1 - 4 μ L injections.

3. REAGENTS

3.1. Calibration solution

The calibration solution was comprised of 4BT, TBT, DBT and MBT.

The calibration standards were prepared in the concentration range of 1.25 to 50 ng Sn/mL (at five concentrations) at a minimum. Internal standard and surrogate compounds were added at 20 ng Sn/mL to all calibration standards.

3.2. Surrogate spiking solution

The surrogate compounds for all sample types were tripropyltin chloride (TPT) and dimethyltin dichloride (DMT). The surrogate solution was prepared by weighing an appropriate amount of pure material into a volumetric flask and diluting to volume with dichloromethane. Surrogates were added to each sample at a concentration of approximately ten times the MDL (i.e., 100 µL of 20 ng Sn/mL to a final volume of 1 mL).

3.3. Internal standard solution

The internal standard for this analysis was tetrapropyltin (4PT). The internal standard solution was prepared by weighing an appropriate amount of pure material into a volumetric flask and diluted to volume with dichloromethane. The internal standard was added to each sample extract to obtain a final concentration of approximately 2 ng Sn/mL.

3.4. Matrix recovery spiking solution

The matrix spiking solution consisted of 4BT, TBT, DBT and MBT (TBT, DBT, and MBT as the chlorides).

Matrix spike solution was added to samples at a concentration approximately 10 times the MDL.

3.5. Retention Index Solution

The calibration mixture was used a retention index solution.

4. PROCEDURE

4.1. Sample extraction and purification

Sediment and tissue samples were extracted and purified following the analytical procedures described in previous sections.

4.2. High resolution GC-FPD analysis

4.2.1. GC conditions

For the analysis of butyltins, the analytical system, or its equivalent, included at a minimum:

Instrument:	Hewlett-Packard 5880A or HP 5890 Gas Chromatograph
Features:	Split/splitless capillary inlet system and data acquisition system
Inlet:	Splitless
Detector:	Flame photometric, 610 nm filter
Column:	0.32-mm i.d. x 30-m DB-5 fused silica capillary column (J&W Scientific) or similar

Gases:

Carrier: Helium 2 mL/min.
Make-Up: Helium 33 mL/min.
Detector: Air 100 mL/min.
Hydrogen 80 mL/min.

Temperatures:

Injection port: 250 °C
Detector: 250 °C
Oven Prog: 80 °C to 300 °C at 13°/min, hold 0 min.
Run Time: 13 min.

The GC oven temperature program may be modified to improve resolution.

Daily Calibration: Mid-level calibration solution;
Retention index solution
(20 mg/mL)

Quantification: Based on surrogate added prior to extraction/calibration.

Note: The GC must be capable of the baseline resolution of all target compounds, surrogates, and internal standards.

4.2.2. Calibration

Prior to analysis, a sample set was used to perform the GC calibration at a minimum of three concentrations. One of the concentration levels was near, but above the MDL. The remaining concentrations corresponded to the expected concentration range of the samples. A concentration range of 1.25 to 50 ng Sn/ μ L was used. The detector was linear within this range. An average calibration factor from the authentic standard of each individual compound was used to calculate sample analyte concentrations. The initial calibration was verified by the measurement of calibration standards after at least every 12 samples.

A mid-level standard was analyzed immediately prior to conducting any analyses, and after each group of samples. The response factor criteria for an in-control calibration check was $\pm 30\%$ for each analyte.

4.2.3. Retention time windows

Retention time windows were established and maintained. Three times the standard deviation of the retention time determined from the calibration solutions was used to calculate the acceptable retention time windows.

4.2.4. Sample analysis

If the instrument was in calibration, the butyltin analyses were initiated with a calibration check, followed by the samples, and ending with a calibration check (Table 1). If the response factor for any analyte in the calibration check failed to meet the criteria established in Section 4.2.2, the instrument was recalibrated. All samples that were injected after the standard exceeding the criteria were reinjected.

Sample injections of 1 to 4 μ L were made with an autosampling device.

Table 1. Sample distribution to meet QA requirements during a typical TBT analysis.

Sample no.	Description	Function
1	Solvent Blank	Establish instrument background
2	Cal Ck*	Confirm "in calibration" condition
3	Sample #1	Unknown Analysis
4	Sample #2	Unknown Analysis
5	Sample #3	Unknown Analysis
6	Sample #4	Unknown Analysis
7	Sample #5	Unknown Analysis
8	Sample #6	Unknown Analysis
9	Sample #7	Unknown Analysis
10	Sample #8	Unknown Analysis
11	Sample #9	Unknown Analysis
12	Sample #10 (duplicate)* *	Unknown Analysis
13	System Blank	Confirm method blank
14	Spiked Blank/Fortified Sample/SRM	Complete Analytical QA* **
15	Cal Ck	Confirm "in calibration" condition

* Criteria ±30% for an individual analyte

** Criteria ±30% for all analytes

Note: The period of time in between Cal Ck did exceed 12 hours therefore the number of samples were adjusted accordingly.

If the response for any peak exceeded the highest calibration solution, the extract was diluted and reanalyzed.

4.2.5. Calculations

Calculations were based on the internal standard methods. Concentrations were reported as µg Sn/g.

5. QUALITY ASSURANCE/QUALITY CONTROL (QA/QC) REQUIREMENTS

5.1. Initial calibration and continuing calibration checks

Prior to the analyses, a three-point response factor linear calibration curve was established.

Each calibration standard was analyzed and the response factor (RF) of each compound was calculated at each concentration level from the area of the peak and the Sn concentration of the standard.

The following formula was used to calculate the response factors of the surrogate standard relative to each of the calibration standards.

$$RF = \frac{A_s C_{su}}{A_{su} C_s}$$

where A_s was the area for the analyte to be measured, A_{su} was the area for the appropriate surrogate standard (tripropyltin or dimethyltin), C_{su} was the concentration of the appropriate surrogate standard (ng/mL), and C_s was the concentration of the analyte in the calibration standard (ng/mL).

The response factor for each compound of interest relative to the internal standard was determined at least once a day.

The daily response factor for each compound was compared to the initial calibration curve. The percent difference was calculated using the following equation:

$$\text{Percent difference} = \frac{\text{RFI} - \text{RFC}}{\text{RFI}} 100$$

where RFI was the average response factor from initial calibration and RFD was the response factor from current verification check standard.

If the average daily response factors for all analytes was within $\pm 15\%$ of the corresponding calibration curve value, the analysis proceeded. If, for any individual analyte, the daily response factor exceeded $\pm 30\%$ of the corresponding calibration curve value, a three-point calibration curve was repeated prior to the analysis of the samples. All samples were calculated based on the initial calibration response factor.

5.2. Method blank analysis

An acceptable method blank analysis did not contain any target compound at concentration 3 times greater than the MDL. If the method blank did not meet these criteria, the analytical system was out of control and the source of the contamination was investigated, corrective measures taken and documented before further sample analysis proceeded.

5.3. Surrogate compound analysis

All samples and quality control samples were spiked with TPT and DMT. The surrogate solutions were spiked into the sample prior to extraction to measure individual sample matrix effects associated with sample preparation and analysis.

The laboratory took corrective action whenever the recovery of the surrogate was below 30 percent.

The following corrective actions were taken when an out of control event occurred:

- a. Calculations were checked to assure that no errors were made.
- b. The internal standard and surrogate solutions were checked for degradation, contamination, or concentration and the instrument performance was checked.
- c. If the surrogate could not be measured because the sample required a dilution, no corrective action was required. The surrogate recovery was properly annotated.
- d. If the steps above failed to reveal a problem, the sample or extract was reanalyzed. If reanalysis of the extract yielded surrogate recoveries within the stated limits, then the reanalyzed data was reported. If upon reinjection QA criteria were still violated, the sample was submitted for re-extraction if sufficient sample was available. If the

sample was completely consumed, the data was reported but designated as outside the QA criteria.

5.4. Matrix spike analysis

The laboratory spiked and analyzed a matrix spike every 12 samples or with every sample set, whichever was more frequent. A sample was randomly chosen, split into two subsamples and the subsample was fortified with the matrix spike. The acceptable matrix spike recovery criteria were $90 \pm 20\%$ for 4BT, TBT, DBT and $60 \pm 25\%$ for MBT. If the matrix spike criteria were not met, the matrix spike was reinjected on the GC and reanalyzed. If the reinjected matrix spike analysis met the criteria, the reanalysis data was reported. If not, the entire batch of samples was submitted for re-extraction if sufficient sample was available. If the sample was completely consumed, the data was reported but designated as outside the QA criteria.

5.5. Method detection limit

The method detection limit was determined following the procedures outlined in Federal Register (1984), Vol. 49, No. 209: 198-199.

5.6. GC resolution

The target compounds, and internal standard were resolved from one another and from interfering compounds.

5.7. Reference sample analysis

When available, one reference material was analyzed per batch of samples, and the results used to construct laboratory QC charts. The result should agree within $\pm 30\%$ of the mean of the previously reported data on the certified concentration.

6. CALCULATIONS

6.1. Butyltin calculations

Concentrations were based on the surrogate added before extractions:

$$C = \frac{A_s I_{su}}{A_{su} RF W}$$

where C was the sample concentration (ng Sn/g), A_s was the area of the analyte measured, A_{su} was the area of the appropriate surrogate standard, RF is the response factor, I_{su} was the amount in ng of appropriate surrogate standard added to each sample, and W is the sample dry weight in grams.

6.2. Calculation notes

A specific amount of surrogate was added to each sample. The recovery of this surrogate was monitored in each sample using the response of the IS. that was added to the final extract.

$$\text{Percent SUR recovery} = \frac{A_{is} C_{is}}{C_{sur} A_{su} RF_{sur}}$$

where A_{su} was the area of tripropyltin or dimethyltin, A_{is} is the area of tetrapropyltin, C_{sur} was the amount (in ng) of Sn in tripropyltin added to the sample, C_{is} was the amount (in ng) of Sn in tetrapropyltin added to the sample extract, and RF_{sur} was the response factor for tripropyltin.

7. REPORTING

Report units were ng Sn/g. Results were reported to three (3) significant figures. Surrogate recoveries were reported for every sample analyzed. Matrix spike recoveries were reported for each batch of samples analyzed. When available, the results of the analysis of reference materials were reported with each batch of samples analyzed.

8. CONCLUSIONS

The flame photometric detector with a 610 nm filter provides a sensitive detector that is specific to tin. Hexylation of the organotin anions provides compounds amenable to the GC/FPD technique and provides reliable quantitation of organotin compounds at low concentrations (ng Sn/g).

Battelle Duxbury Operations Trace Organic Analytical Procedures for the Processing and Analysis of Tissue Samples

C. S. Peven McCarthy and A. D. Uhler
Battelle Duxbury Operations
Duxbury, Massachusetts

ABSTRACT

This document describes the analytical methods for the measurement of trace organic compounds in tissues. The methods presented are modifications of methods initially developed by the NOAA/National Marine Fisheries Service National Analytical Facility. These Battelle methods have been used for multiple monitoring programs, including the analysis of samples collected for the NOAA Status and Trends Mussel Watch Project.

1. INTRODUCTION

This document summarizes the methods used by Battelle for the preparation and analysis of tissues for trace organic contaminants. These methods are based on those developed by Sloan *et al.* (1993). All equipment, apparatus, standards, and reagent necessary to perform the extractions and analyses are described below. These methods were followed by Battelle from 1986 through 1994, as well as in 1996 and 1997 as participants in the Mussel Watch Project. They are also the foundation for multiple monitoring and assessment projects conducted by Battelle worldwide. The methods are continually evolving to reflect the incorporation of new state-of-the-art analytical techniques.

2. EQUIPMENT AND REAGENTS

2.1. Sample processing equipment and apparatus

Tissue preparation equipment

- Stainless steel shucking knives

- Electronic calipers

- 500-mL certified cleaned glass sample jars (ESS, I-Chem or equivalent)

Apparatus for homogenizing tissues

- Tekmar Tissumizer (Cincinnati, OH) with stainless steel probes, or equivalent

Apparatus for shaking tissue extract

- Orbital shaker table; Lab-Line Instruments Inc. Model 3520 or equivalent

Apparatus for determining wet weight and dry weight (tissue and sediment)

- Top-loading balance capable of weighing to 0.01 g; Ohaus, Mettler or equivalent

- Aluminum weighing pan

- Stainless steel spatula

- Drying oven maintained at 105-120 °C, Blue M Model SW-17TA or equivalent

Apparatus for determining lipid weight:

- Class A volumetric pipette/pipette bulb, or 10-mL syringe

- Aluminum weighing pan

General sample processing equipment

250-mL Teflon or glass (certified clean) extraction bottles
Centrifuge capable of holding 250-mL bottles and maintaining 2000 rpm
Glass wool
19-mm chromatography column with ~200-mL reservoir and Teflon stopcock (tissue)
Kuderna-Danish (K-D) apparatus (listed below) or Turbovap concentration units and tubes
 Reservoir, 500 mL
 Snyder column, three-ball macro
 Concentrator tube, 10- or 20-mL
Hot water bath capable of reaching 100 °C, located in fume hood.
Boiling chips, Teflon, solvent rinsed
Nitrogen evaporation apparatus, N-Evap, Turbovap, or equivalent
Glass graduated cylinders, 100- and 500-mL
Erlenmeyer flasks, 250- and 500-mL
Microliter syringes, Hamilton
Vials, 70-, 4-, 1-, 250-mL
Aluminum foil
Pasteur pipettes
Forceps, stainless steel
Electrobalance, Cahn, for standard preparation

High pressure liquid chromatography:

Autosampler, 231/401. Gilson
Phenogel columns, 22.5 x 250 mm, 100 Å size.
UV/VIS detector, Spectra-Physics
Fraction collector, Isco Foxy 200
Integrator, Shimadzu CR-3A

Gas chromatographs (GCs):

GCs - Hewlett Packard 5890 Series II
Mass spectrometer (MS) - Hewlett Packard 5972 MSD
Electron capture detector (ECD) - Hewlett Packard
Fused silica capillary column (30-m x 0.32 mm i.d. DB-5 MS or 0.25 mm i.d. DB-5)

Gases:

Argon/methane (95/5) (99.999% pure)
Helium (99.999% pure)
Hydrogen (99.9999% pure)

2.2. Reagents

Dichloromethane (DCM), pesticide grade or equivalent
Isooctane, pesticide grade or equivalent
Acetone, pesticide grade or equivalent

Sodium sulfate - anhydrous, reagent grade, heated to 400 °C for at least 4 hr, then cooled and stored in a tightly-sealed glass container at room temperature.

Alumina, Sigma F-20 or equivalent, 80-200 mesh. Prepare 2% deactivated alumina by heating to 400 °C for at least 4 hr in a shallow dish, adding 2% water (by volume) and shaking the sealed glass flask for 1 hr. Use within 24 hr.

2.3. Standard solutions

Surrogate internal standards (SIS)

Recovery internal standards (RIS)

Matrix spike solutions

HPLC calibration solution

3. SUMMARY OF PROCESSING PROCEDURES

3.1. Initial extraction

Approximately 30 g of wet tissue homogenate was weighed into a Teflon extraction container (250 mL) and spiked with surrogate internal standards (SIS). An additional 5 g aliquot of the homogenate was placed in a pre-weighed aluminum pan for dry weight determination (see Section 5.0). Fifty g of sodium sulfate and 75 mL DCM were added to the tissue and the mixture homogenized for two min at high speed. The jar was centrifuged, the solvent poured off into a labeled 500-mL Erlenmeyer flask, an additional 75 mL DCM was added to the Teflon jar and the procedure repeated. After the second aliquot of DCM was added to the Erlenmeyer, 50 mL DCM was added to the Teflon jar and the mixture shaken for approximately one hour. The jar was again centrifuged and the solvent added to the other two aliquots. The Erlenmeyer was swirled to mix the solvent extracts, and a 10-mL aliquot removed for lipid weight determination (DCM extractables) using a Class A pipette. The aliquot was placed in a pre-weighed aluminum pan, covered with foil, and allowed to dry at room temperature (see Section 5.0 for lipid weight calculation). Approximately 20 - 50 g sodium sulfate was added to the Erlenmeyer and the mixture swirled. After approximately 1 hr the combined extract was decanted through a prepared alumina column, and the eluent collected into a clean reservoir: Erlenmeyer flask, K-D apparatus, or Turbovap tube. When the extract reached the top of the column packing, 50 mL of DCM is added to the column. The column was completely drained into the reservoir.

3.2. HPLC cleanup

After alumina column cleanup, the extracts are concentrated by KD and nitrogen techniques to 900 μ L (volume measured with a syringe and recorded). 600 μ L of this concentrated extract is fractionated by HPLC following procedures developed by Krahn *et al.* (1988), and modified by Battelle to accommodate local equipment. The system is calibrated prior to fractionation by analyzing two calibration standards containing the retention time markers dibromooctafluorobiphenyl (DBOBF) and perylene. The solution also contains corn oil (lipid material) and sulfur (a common contaminant), to ensure that they will be eliminated from the final cleaned extracts. The fractionation window is set to collect between 1.5 min before the elution of DBOFB until 2 min after the elution of perylene. The pump is set to flow at a rate of 5 mL/min (DCM mobile phase). A calibration check is run every 10 samples to verify the collection window.

After the samples were fractionated, the cleaned extracts were concentrated under nitrogen to approximately 0.5 mL. The extracts were spiked with internal standards and split for GC and GC/MS analysis. The GC/MS split was transferred to a GC vial and submitted for polycyclic aromatic hydrocarbons (PAH) analysis; the GC split was solvent-exchanged with isoctane, transferred to a GC vial, and submitted to GC/ECD for polychlorinated biphenyl (PCB)/pesticide analysis.

4. SUMMARY OF ANALYSIS METHODS

4.1. Calibration

Prior to sample analysis, the gas chromatography system was calibrated using stock standard solutions containing the analytes of interest as well as the appropriate surrogates and internal standards. Five-level calibrations are analyzed by both GC/ECD (for PCB and pesticides) and GC/MS operating in the SIM mode (for PAH). The system was checked prior to each sample set, and the calibration checked every 10 samples (minimum) by analyzing one of the calibration levels and checking target analyte response. The analyte response factor must fall within 25% of the response factor from the initial calibration for the calibrations to be considered acceptable.

4.2. GC/ECD

The GC is fitted with a 30-m x 0.25-mm internal diameter fused silica capillary column with a 0.25- μ m film thickness stationary phase [DB-5 (5% phenyl-, 95% methyl-polysiloxane) (J&W Scientific, Inc.)]. GC conditions are as follows:

Injection port temperature	280 °C
Detector temperature	300 °C
Initial temperature	60 °C
Initial hold	1 min
Ramp 1 rate	15 °C/min to 150 °C
Ramp 2 rate	1 °C/min to 210 °C
Ramp 3 rate	10 °C/min to 290 °C
Final hold	15 min
Carrier gas flow	1.5 - 2.5 mL/min (30 - 40 cm/sec)
Makeup gas flow	30 - 60 mL/min
Purge vent	1.5 - 2.5 mL/min
Split vent	40 - 80 mL/min
	Purge on after 0.50 min

One or 2 μ L injections of the sample extracts are made onto the column. Column degradation checks are made prior to each sample sequence. The degradation solution contains endrin and 4,4'-DDT.

4.3. GC/MS

Polynuclear aromatic hydrocarbons were separated via high resolution capillary gas chromatography, and identified and quantified using electron impact mass spectrometry. A Hewlett Packard system interfaced to the GC/MS is used to control acquisition and to store, retrieve, and manipulate mass spectral data.

Prior to the analysis of analytical standards and/or samples the mass spectrometer must be tuned with perfluorotributylamine (PFTBA).

The gas chromatograph is fitted with a 30-m x 0.25-mm DB-5MS (J&W Scientific, Inc.) or equivalent (0.25- μ m film thickness) fused silica capillary column. The GC oven temperature program is as follows:

Injection Port:	300 °C
Transfer Liner:	280 °C
Initial Temp:	40 °C

Initial Hold:	1 min
Ramp Rate:	6 °C/min
Final Temp:	290 °C
Final Hold:	20 min

A 2 µL injection volume of sample extract was used for analysis. The mass spectrometer is operated in electron impact mode at a manifold pressure of less than 6×10^{-5} torr. The electron multiplier voltage was set a minimum of 100 V above the tune value.

The quantification and confirmation ions used in the analysis of selected PAH were defined in Battelle standard operating procedures. If possible, ion groups should be selected so that no more than 20 ions are monitored in a single group. It should be noted that as the number of ions scanned per group increases and the individual dwell times decrease, sensitivity will also decrease. Each ion in a group should have identical dwell times to ensure that correct ion ratios are preserved. Total group dwell time should not exceed 400 ms, individual dwell times should be a minimum of 20 ms.

Prior to the first analysis of analytical standards and/or samples in SIM, a 10 ng/µL analytical standard and reference sample (e.g. North Slope Crude Oil) is analyzed in full-scan. The full-scan total ion chromatograms from these analyses are used to determine the proper group start and stop times in the SIM acquisition method.

5. CALCULATIONS

Sample dry weight:

$$\% \text{ Dry weight} = \frac{(\text{Aliquot dry wt.} - \text{pan wt.})}{(\text{Aliquot wet wt.} - \text{pan wt.})} \times 100$$

Lipid weight:

$$\text{Sample dry wt.} = \frac{(\% \text{ dry wt.}) (\text{sample wet wt.})}{(100)}$$

$$\text{Total lipid wt. (g)} = \frac{\text{Vol. of combined extracts (mL)}}{\text{Aliquot vol. (mL)}} \times (\text{Aliquot dry wt. (g)} - \text{Tare wt. (g)})$$

$$\text{Sample lipid content (g/g dry wt.)} = \frac{\text{Total lipid wt. (g)}}{\text{Sample dry wt. (g)}}$$

Calibration response factor:

$$\text{RF} = \frac{\text{Area of analyte}}{\text{Area of Internal Standard}} \times \frac{\text{Concentration of Internal Standard}}{\text{Concentration of analyte}}$$

6. CONCLUSION

The method described above has been validated and is continually being reviewed and modified to incorporate improvements in order to keep it current and efficient. The methods are the basis for all tissue monitoring studies where ultra-low detection limits of NS&T and similar organic contaminants are required.

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NIST Methods for the Certification of SRM 1941a and SRM 1974a

M. M. Schantz, B. A. Benner, Jr., M. K. Donais,^{*} M. J. Hays,^{**} W. R. Kelly, R. M. Parris, B. J. Porter, D. L. Poster, L. C. Sander, K. S. Sharpless, R. D. Vocke, Jr., and S. A. Wise

Analytical Chemistry Division
National Institute of Standards and Technology
Gaithersburg, MD

M. Levenson, S. B. Schiller, and M. Vangel
Statistical Engineering Division
National Institute of Standards and Technology
Gaithersburg, MD

ABSTRACT

The replacement Standard Reference Materials (SRMs) for SRM 1941, Organics in Marine Sediment, and SRM 1974, Organics in Mussel Tissue (*Mytilus edulis*), have been prepared and analyzed for the determination of trace organic constituents. SRM 1941a has been issued with certified concentrations for 23 polycyclic aromatic hydrocarbons (PAH), 21 polychlorinated biphenyl (PCB) congeners, 6 chlorinated pesticides, and sulfur. SRM 1974a has been issued with certified concentrations for 15 PAHs, 20 PCB congeners, 7 chlorinated pesticides, and methylmercury. The preparation and certification analyses for these two replacement SRMs are described in this chapter.

1. INTRODUCTION

As part of the Quality Assurance element of the National Status and Trends Program, the National Institute of Standards and Technology (NIST) developed two Standard Reference Materials (SRMs), SRM 1941 (Organics in Marine Sediment) and SRM 1974 [Organics in Mussel Tissue (*Mytilus edulis*)], to assist in the validation of analytical procedures. SRM 1941 was issued in 1989 with certified concentrations for 11 polycyclic aromatic hydrocarbons (PAHs) and sulfur and noncertified concentrations for polychlorinated biphenyl (PCB) congeners and chlorinated pesticides (Schantz *et al.*, 1990). SRM 1974 was issued in 1990 with certified concentrations for nine PAHs and noncertified concentrations for PCB congeners and chlorinated pesticides (Wise *et al.*, 1991). The supplies of these original SRMs were depleted in 1993 (SRM 1941) and 1994 (SRM 1974) so new materials, SRM 1941a and SRM 1974a, have been prepared and analyzed. To increase the usefulness of the new SRMs, three goals were established: (1) prepare a larger quantity of the material to extend the availability, (2) provide certified values for a significantly larger number of PAHs and to reduce the uncertainties associated with the certified PAH mass fractions compared to the original SRMs, and (3) provide certified values for the PCB congeners and chlorinated pesticides rather than noncertified values as in the original materials (Schantz *et al.*, 1995).

At NIST, the certification process for a natural matrix SRM typically requires the use of two or more chemically independent analytical techniques, and the results of these analyses, if in agreement, are used to determine the certified values of the measured analytes. For the measurement of PAHs, Wise *et al.* (1995) described the use of four different analytical techniques: (1) reversed-phase liquid chromatography with fluorescence detection (LC-FI) for analysis of the total PAH fraction, (2) reversed-phase LC-FI analysis of isomeric PAH

^{*} Present address: VG Elemental, 27 Forge Parkway, Franklin, MA 02038.

^{**} Present address: Battelle Columbus, 505 King Ave., Columbus, OH 43201.

fractions isolated by normal-phase LC (i.e., multidimensional LC), (3) gas chromatography/mass spectrometry (GC/MS) analysis of the PAH fraction on a 5% phenyl-substituted methylpolysiloxane stationary phase, and (4) GC/MS of the PAH fraction on a smectic liquid crystalline stationary phase that provides excellent shape selectivity for the separation of PAH isomers. Based on the agreement of results from these four approaches, certified values were determined for 23 PAHs in SRM 1941a and 15 PAHs in SRM 1974a while noncertified values were reported for an additional 14 PAHs in SRM 1941a and 18 PAHs in SRM 1974a.

To provide certified concentrations for the PCB congeners and chlorinated pesticides, the approaches used for SRM 1941a and SRM 1974a were similar to that used for SRM 1945, Organics in Whale Blubber (Schantz *et al.*, 1995). This approach consists of GC analyses with electron capture detection (GC-ECD) on a 5% phenyl-substituted methylpolysiloxane phase and on a dimethylpolysiloxane phase containing 50% methyl C₁₈ and GC/MS analyses on a 5% phenyl-substituted methylpolysiloxane phase. Using this approach, certified values were provided for 21 PCB congeners and six chlorinated pesticides in SRM 1941a and for 20 PCB congeners and 7 chlorinated pesticides in SRM 1974a. Noncertified values were provided for 10 additional PCB congeners/chlorinated pesticides in SRM 1941a and for eight additional PCB congeners/chlorinated pesticides in SRM 1974a.

Additional values included for SRM 1941a are a certified value for sulfur and noncertified values for 27 inorganic constituents and percent total organic carbon (TOC). Additional values included for SRM 1974a are a certified value for methylmercury (Donais *et al.*, 1996) and noncertified values for 28 inorganic constituents.

2. SRM 1941a, ORGANICS IN MARINE SEDIMENT

2.1. Summary

SRM 1941a is intended for use in validating analytical methods for the determination of trace levels of selected PAHs, PCB congeners, chlorinated pesticides, and sulfur in marine sediments. Noncertified values of additional PAHs, PCB congeners, chlorinated pesticides, and inorganic constituents are also provided.

2.2. Collection and preparation

The marine sediment used to prepare this SRM was collected in the Chesapeake Bay at the mouth of the Baltimore (MD) Harbor near the Francis Scott Key Bridge using a modified Van Veen type grab sampler designed to sample to a depth of 10 cm. The sediment was freeze-dried, sieved (150-250 µm particles used for the SRM), homogenized in a cone blender, radiation (⁶⁰Co) sterilized, and then packaged in screw-capped amber glass bottles (50 g/bottle).

2.3. Moisture determination

The results for both the organic and inorganic constituents are reported on a dry weight basis; however, the material as received contains residual moisture. The amount of moisture in SRM 1941a was determined by measuring the weight loss after oven drying at 90 °C for 18 hr for subsamples of 1-2 g. The moisture content in SRM 1941a at the time of the certification analyses was 2.2%.

2.4. Polycyclic aromatic hydrocarbons

SRM 1941a was analyzed for selected PAHs using GC/MS and reversed-phase LC-FI. GC/MS analyses were performed on two columns with different selectivities for the separation of PAHs: a 5% phenyl-substituted methylpolysiloxane stationary phase and a smectic liquid crystalline stationary phase.

For the GC/MS analyses, two sets of six samples (10-25 g each) from twelve randomly selected bottles were Soxhlet extracted for 18 - 20 hr using dichloromethane. A silica or aminopropylsilane solid phase extraction column was eluted with 1:50 (volume ratio) dichloromethane in n-hexane to remove the polar interferences from each sediment extract. Finely divided copper was then added to the extracts to remove elemental sulfur. The PAH fraction was isolated from each sediment extract by normal-phase liquid chromatography using a semi-preparative aminopropylsilane column (Wise *et al.*, 1977). The GC/MS analyses were performed on one set of six sample extracts using a 0.25 mm i.d. x 60 m fused silica capillary column with a 5% phenyl-substituted polysiloxane phase (0.25 μm film thickness) (DB-5MS, J&W Scientific, Folsom, CA). The second set of six sample extracts was prepared as described above and then analyzed by GC/MS on a 0.2 mm i.d. x 25 m fused silica capillary column with a smectic liquid crystalline phase (0.15 μm film thickness) (SB-Smectic, Dionex, Inc., Salt Lake City, UT).

For the LC-FI analyses, subsamples of 10 g of sediment from six randomly selected bottles were Soxhlet extracted for 20 hr using 1:1 (volume ratio) n-hexane:acetone. Each concentrated extract was placed on an aminopropylsilane solid phase extraction column and eluted with 1:50 (volume ratio) dichloromethane in n-hexane to remove the polar constituents. The extract was then analyzed by reversed-phase LC using a polymeric octadecylsilane (C_{18}) column (4.6 mm i.d. x 25 cm, 5 μm particle size, Hypersil PAH, Keystone Scientific, Inc. Bellefonte, PA) with wavelength programmed fluorescence detection (May and Wise, 1984; Kline *et al.*, 1985; Wise *et al.*, 1988). To quantify several PAHs that have low fluorescence sensitivity and /or selectivity, six additional subsamples were extracted and prepared as described above, and then the extract was fractionated on a semi-preparative aminopropylsilane column to isolate isomeric PAH fractions as described previously (May and Wise, 1984; Kline *et al.*, 1985; Wise *et al.*, 1988). These isomeric PAH fractions were analyzed by reversed-phase LC-FI on a similar octadecylsilane column.

For both the GC/MS and LC-FI analyses, selected perdeuterated PAHs were added to the sediment prior to extraction for use as the internal standards for quantification purposes. For GC/MS analyses the following internal standards were used: naphthalene- d_8 , acenaphthene- d_{10} , fluorene- d_{10} , phenanthrene- d_{10} , fluoranthene- d_{10} , benz[a]anthracene- d_{12} , benzo[e]pyrene- d_{12} , dibenz[a,h]anthracene- d_{14} , and benzo[ghi]perylene- d_{12} . For the LC-FI analyses the following internal standards were used: naphthalene- d_8 , phenanthrene- d_{10} , fluoranthene- d_{10} , and perylene- d_{12} for the LC-FI (Total) analyses and triphenylene- d_{12} , benz[a]anthracene- d_{12} , and benzo[ghi]perylene- d_{14} for the LC-FI (Fraction) analyses. Calibration response factors for the analytes relative to the internal standards were determined by analyzing aliquot of SRMs 1491 (Aromatic Hydrocarbons in Hexane/ Toluene) and 2260 (Aromatic Hydrocarbons in Toluene, Nominal Concentration 60 $\mu\text{g}/\text{mL}$) in the case of GC/MS analyses or SRM 1647b (Priority Pollutant PAHs in Acetonitrile) in the case of LC-FI analyses; gravimetrically prepared solutions of additional analytes not contained in SRMs 1491, 2260, or 1647b; and the internal standards.

2.5. Polychlorinated biphenyl congeners and chlorinated pesticides

SRM 1941a was analyzed for selected PCB congeners and chlorinated pesticides using GC-ECD and GC/MS using the general approach described previously (Schantz *et al.*, 1993). For the GC-ECD analyses, a portion of the extract prepared for the PAH analyses by GC/MS (see above) was used. For the GC/MS analyses, additional samples of approximately 100 g each from six pairs of randomly selected bottles were Soxhlet extracted for 18 hr using 1:1 (volume ratio) n-hexane:acetone. In preparation for both the GC-ECD and GC/MS analyses, the extracts were fractionated on the semi-preparative aminopropylsilane column to isolate two fractions containing (1) the PCBs and lower polarity pesticides and (2) the more polar pesticides. GC-ECD analyses of the two fractions were performed on two columns of different selectivities for PCB separations: 0.25 mm x 60 m fused silica capillary column with a 5% phenyl-substituted methylpolysiloxane phase containing 50% methyl C₁₈ (0.1 µm film thickness) (CP Sil 5 C18 CB, Chrompack International, Middelburg, The Netherlands). The GC/MS analyses were performed on a 5% phenyl-substituted methylpolysiloxane phase as described above for the GC-ECD analyses.

Two PCB congeners, which are not present at significant concentrations in the sediment extract (PCB 103 and PCB 198) (Ballschmiter and Zell, 1980; Schulte and Malisch, 1983), and perdeuterated 4,4'-DDT were added to the sediment prior to extraction for use as internal standards for quantification purposes. Calibration response factors for the analytes relative to the internal standards were determined by analyzing aliquot of SRMs 2261 (Chlorinated Pesticides in Hexane, Nominal Concentration 2 µg/mL), SRM 2262 (Chlorinated Biphenyl Congeners in 2,2,4-Trimethylpentane, Nominal Concentration 2 µg/mL), gravimetrically prepared solutions of additional analytes not contained in SRMs 2261 and 2262, and the internal standards.

2.6. Certified and noncertified concentrations

Statistically combining the results from the different methods, SRM 1941a has been issued with certified values for 23 PAHs (Table 1), 21 PCB congeners (Table 2), and 6 chlorinated pesticides (Table 3). Noncertified values are available for an additional 14 PAHs (Table 4), 7 PCB congeners (Table 5), and three chlorinated pesticides (Table 5). In addition to the organic contaminants, the sulfur content was determined to be 0.9589 ± 0.0058% (by weight) using isotope dilution thermal ionization MS and the total organic carbon was determined to be 4.8 ± 1.2% (by weight) from an interlaboratory comparison. Also, SRM 1941a was analyzed for 27 inorganic constituents using instrumental neutron activation analysis (Wise *et al.*, 1995).

3. SRM 1974a, ORGANICS IN MUSSEL TISSUE (*Mytilus edulis*)

3.1. Summary

SRM 1974a is intended for use in validating analytical methods for the determination of trace levels of selected PAHs, PCB congeners, chlorinated pesticides, and methylmercury in mussel tissues. Noncertified values of additional PAHs, PCB congeners, chlorinated pesticides, and inorganic constituents are also provided.

Table 1. Certified concentrations (mass fractions) for selected PAHs in SRM 1941a and SRM 1974a^a ($\mu\text{g}/\text{kg}$ dry mass basis^b).

Compound	SRM 1941a		SRM 1974a	
Naphthalene	1010	± 140	23.5	$\pm 4.4^{\text{C}}$
Fluorene	97.3	± 8.6		
Phenanthrene	489	± 23	22.2	± 2.4
Anthracene	184	± 14	6.1	$\pm 1.7^{\text{C}}$
Fluoranthene	981	± 78	163.7	$\pm 9.1^{\text{C}}$
Pyrene	811	± 24	151.6	$\pm 6.6^{\text{C}}$
Benz[<i>a</i>]anthracene	427	± 25	32.5	$\pm 4.7^{\text{C}}$
Chrysene	380	± 24	44.2	± 2.3
Triphenylene	197	± 11	50.7	± 5.9
Benzo[<i>b</i>]fluoranthene	740	± 110	46.4	$\pm 3.7^{\text{C}}$
Benzo[<i>k</i>]fluoranthene	361	± 18	20.18	± 0.84
Benzo[<i>a</i>]fluoranthene	118	± 11		
Benzo[<i>e</i>]pyrene	553	± 59	84.0	± 1.9
Benzo[<i>a</i>]pyrene	628	± 52	15.63	± 0.65
Perylene	452	± 58	7.68	± 0.27
Benzo[<i>ghi</i>]perylene	501	± 72	22.0	$\pm 2.2^{\text{C}}$
Indeno[1,2,3- <i>cd</i>]pyrene	525	± 67	14.2	$\pm 2.8^{\text{C}}$
Dibenz[<i>a,j</i>]anthracene	74.3	± 6.8		
Dibenz[<i>a,c</i>]anthracene	43.1	± 3.7		
Dibenz[<i>a,h</i>]anthracene	73.9	± 9.7		
Pentaphene	42	± 12		
Benzo[<i>b</i>]chrysene	99	± 20		
Picene	80.0	± 9.0		

^a Results reported on dry mass basis. SRM 1941a as received contains approximately 2.2% moisture, and SRM 1974a as received contains $88.61\% \pm 0.08\%$ water.

^b Each certified value is the mean of the equally weighted means from two or more independent analytical methods. Each uncertainty, computed according to the CIPM approach (Guide, 1993), is an expanded uncertainty at the 95% level of confidence that includes random sources of uncertainty within each analytical method as well as uncertainty due to the drying study (for the dry mass basis values). The expanded uncertainty defines a range of values for the certified value within which the true value is believed to lie, at a level of confidence of approximately 95%.

^c An additional allowance for differences between methods was included in the total uncertainty for this analyte.

Table 2. Certified concentrations (mass fractions) for selected PCB congeners in SRM 1941a and SRM 1974a^a ($\mu\text{g}/\text{kg}$ dry mass basis^c).

Chlorinated biphenyls ^b		SRM 1941a	SRM 1974a
PCB 44	(2,2',3,5'-Tetrachlorobiphenyl)	4.80 \pm 0.62	72.7 \pm 7.4
PCB 49	(2,2',4,5'-Tetrachlorobiphenyl)	9.5 \pm 2.1	88.8 \pm 5.0
PCB 52	(2,2',5,5'-Tetrachlorobiphenyl)	6.89 \pm 0.56	115 \pm 11
PCB 66	(2,3',4,4'-Tetrachlorobiphenyl)	6.8 \pm 1.4	101.4 \pm 4.4
PCB 87	(2,2',3,4,5'-Pentachlorobiphenyl)	6.70 \pm 0.37	
PCB 95	(2,2',3,5',6-Pentachlorobiphenyl)	7.5 \pm 1.1	83 \pm 17
PCB 99	(2,2',4,4',5-Pentachlorobiphenyl)	4.17 \pm 0.51	70.9 \pm 4.0
PCB 101	(2,2',4,5,5'-Pentachlorobiphenyl)	11.0 \pm 1.6	128.3 \pm 9.7
PCB 105	(2,3,3',4,4'-Pentachlorobiphenyl)	3.65 \pm 0.27	53.0 \pm 3.4
PCB 110	(2,3,3',4',6-Pentachlorobiphenyl)	9.47 \pm 0.85	127.3 \pm 8.6
PCB 118	(2,3',4,4',5-Pentachlorobiphenyl)	10.0 \pm 1.1	130.8 \pm 3.6
PCB 128	(2,2',3,3',4,4'-Hexachlorobiphenyl)	1.87 \pm 0.32	22.0 \pm 3.4
PCB 138	(2,2',3,4,4',5'-Hexachlorobiphenyl)	13.38 \pm 0.97	133.5 \pm 9.5
	163 (2,3,3',4',5,6-Hexachlorobiphenyl)		
	164 (2,3,3',4',5',6-Hexachlorobiphenyl)		
PCB 149	(2,2',3,4',5',6-Hexachlorobiphenyl)	9.2 \pm 1.1	87.6 \pm 2.3
PCB 151	(2,2',3,5,5',6-Hexachlorobiphenyl)		25.6 \pm 3.5
PCB 153	(2,2',4,4',5,5'-Hexachlorobiphenyl)	17.6 \pm 1.9	145.2 \pm 7.6
PCB 156	(2,3,3',4,4',5-Hexachlorobiphenyl)	0.93 \pm 0.14	7.43 \pm 0.99
PCB 170	(2,2',3,3',4,4',5-Heptachlorobiphenyl)	3.00 \pm 0.46	5.5 \pm 1.1
PCB 180	(2,2',3,4,4',5,5'-Heptachlorobiphenyl)	5.83 \pm 0.058	17.1 \pm 3.8
PCB 183	(2,2',3,4,4',5',6-Heptachlorobiphenyl)		16.0 \pm 2.4
PCB 187	(2,2',3,4',5,5',6-Heptachlorobiphenyl)		34.0 \pm 2.3
	159 (2,3,3',4,5,5'-Hexachlorobiphenyl)		
	182 (2,2',3',4,4',5,6'-Heptachlorobiphenyl)		
PCB 194	(2,2',3,3',4,4',5,5'-Octachlorobiphenyl)	1.78 \pm 0.23	
PCB 206	(2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl)	3.67 \pm 0.87	
PCB 209	(Decachlorobiphenyl)	8.34 \pm 0.49	

^a Results reported on dry mass basis. SRM 1941a as received contains approximately 2.2% moisture, and SRM 1974a as received contains 88.61% \pm 0.08% water.

^b PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell (1980) and later revised by Schulte and Malisch (1983) to conform with IUPAC rules; for the specific congeners mentioned in this SRM, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

^c Each certified value is the mean of the equally weighted means from four to seven different analytical methods. Each uncertainty, computed according to the CIPM approach (Guide, 1993), is an expanded uncertainty at the 95% level of confidence that incorporates within- and between-method uncertainty as well as uncertainty due to the drying study (for the dry mass basis values). The expanded uncertainty defines a range of values for the certified value within which the true value is believed to lie, at a level of confidence of approximately 95%.

Table 3. Certified concentrations (mass fractions) for selected chlorinated pesticides in SRM 1941a and SRM 1974a^a ($\mu\text{g}/\text{kg}$ dry mass basis^b).

Chlorinated pesticides	SRM 1941a	SRM 1974a
Hexachlorobenzene	70 \pm 25	
<i>cis</i> -Chlordane (-Chlordane)	2.33 \pm 0.56	17.2 \pm 2.8
<i>trans</i> -Chlordane		16.6 \pm 1.7
<i>cis</i> -Nonachlor		6.84 \pm 0.90
<i>trans</i> -Nonachlor	1.26 \pm 0.13	18.0 \pm 3.6
2,4'-DDE	0.73 \pm 0.11	
4,4'-DDE	6.59 \pm 0.56	51.2 \pm 5.5
4,4'-DDD	5.06 \pm 0.58	43.0 \pm 6.3
4,4'-DDT		3.91 \pm 0.59

^a Results reported on dry mass basis. SRM 1941a as received contains approximately 2.2% moisture, and SRM 1974a as received contains 88.61% \pm 0.08% water.

^b Each certified value is the mean of the equally weighted means from four to seven different analytical methods. Each uncertainty, computed according to the CIPM approach (Guide,1993), is an expanded uncertainty at the 95% level of confidence that incorporates within- and between-method uncertainty as well as uncertainty due to the drying study (for the dry mass basis values). The expanded uncertainty defines a range of values for the certified value within which the true value is believed to lie, at a level of confidence of approximately 95%.

3.2. Collection and preparation

The mussels (*Mytilus edulis*) used for the preparation of SRM 1974a were collected on October 7, 1992 from Dorchester Bay within Boston Harbor, MA following the same procedures as described previously for the collection of mussels for SRM 1974 (Wise *et al.*, 1991). Approximately 6000 individual mussels were collected by hand at low tide. The samples were transported to the Battelle Ocean Sciences Laboratory (Duxbury, MA) where the mussels were rinsed with water to remove rocks and other debris. The samples were bagged in Ziploc bags and placed in insulated, Teflon-lined wooden containers, frozen and transported to NIST on dry ice. The samples were transferred to Teflon bags and stored in a liquid nitrogen vapor freezer (-120 °C) until they were shucked.

The mussel tissue was removed from the shell using the following procedure. The mussels were allowed to warm up to about 0 °C; the tissue was removed from the shell using a titanium knife and placed in Teflon bags (approximately 1 kg per bag) and immediately returned to a liquid nitrogen vapor freezer. Approximately 81 kg of mussel tissue were prepared for use as the SRM. Approximately 20 kg of the same frozen mussel homogenate were freeze-dried. The freeze-dried material will be issued as a separate material, SRM 2974.

The mussel tissue was removed from the shell using the following procedure. The mussels were allowed to warm up to about 0 °C; the tissue was removed from the shell using a titanium knife and placed in Teflon bags (approximately 1 kg per bag) and immediately returned to a liquid nitrogen vapor freezer. Approximately 81 kg of mussel tissue were prepared for use as the SRM. Approximately 20 kg of the same frozen mussel homogenate were freeze-dried. The freeze-dried material will be issued as a separate material, SRM 2974.

Table 4. Noncertified concentrations (mass fractions) for selected PAHs in SRM 1941a and SRM 1974a^a ($\mu\text{g}/\text{kg}$ dry mass basis^b).

Compound	SRM 1941a	SRM 1974a
1-Methylnaphthalene		5.3 ± 1.8
2-Methylnaphthalene		10.2 ± 1.5
Biphenyl	175 ± 18	5.11 ± 0.33
Acenaphthylene	37 ± 14	5.25 ± 0.38
Acenaphthene	41 ± 10	3.15 ± 0.26
Fluorene		5.72 ± 0.91
Dibenzothiophene	70.0 ± 9.4	
1-Methylphenanthrene	101 ± 27	10.5 ± 4.8
2-Methylphenanthrene	158 ± 32	20.6 ± 8.0
3-Methylphenanthrene	97 ± 32	13.5 ± 9.7
4-Methylphenanthrene/9-Methylphenanthrene		14.7 ± 9.2
4H-Cyclopenta[def]phenanthrene	92 ± 15	
Acephenanthrylene	48.1 ± 1.2	
Benzo[c]phenanthrene	80 ± 39	19.5 ± 6.7
Benzo[ghi]fluoranthene	97.9 ± 3.1	28.3 ± 5.5
Benzo[a]fluoranthene		4.0 ± 1.9
Benzo[j]fluoranthene	341 ± 22	20.5 ± 1.7
Dibenz[a,j]anthracene		1.247 ± 0.075
Dibenz[a,h]anthracene/Dibenz[a,c]anthracene		3.00 ± 0.20
Benzo[b]chrysene		1.60 ± 0.15
Indeno[1,2,3-cd]fluoranthene	20.0 ± 2.3	
Anthanthrene	129 ± 10	1.15 ± 0.31

^a Results reported on dry weight basis. SRM 1941a as received contains approximately 2.2% moisture, and SRM 1974a as received contains 88.61% ± 0.08% water.

^b Each noncertified value is the mean of the equally weighted means from two different analytical methods or the mean determined by one analytical method. Each uncertainty, computed according to the CIPM approach (Guide, 1993), is an expanded uncertainty at the 95% level of confidence that includes random sources of uncertainty within each analytical method as well as uncertainty due to the drying study (for the dry mass basis values). The expanded uncertainty defines a range of values for the certified value within which the true value is believed to lie, at a level of confidence of approximately 95%.

Table 5. Noncertified concentrations (mass fractions) for selected PCB congeners and chlorinated pesticides in SRM 1941a and SRM 1974a^a ($\mu\text{g}/\text{kg}$ dry mass basis^c).

Chlorinated biphenyls ^b		SRM 1941a	SRM 1974a
PCB 8	(2,4'-Dichlorobiphenyl)	1.39 \pm 0.19	
PCB 18	(2,2',5-Trichlorobiphenyl)	1.15 \pm 0.16	33 \pm 11
PCB 28	(2,4,4'-Trichlorobiphenyl)	9.8 \pm 3.7	79 \pm 15
PCB 31	(2,4',5-Trichlorobiphenyl)	6.2 \pm 2.4	76 \pm 21
PCB 87	(2,2',3,4,5'-Pentachlorobiphenyl)		54 \pm 14
PCB 151	(2,2',3,5,5',6-Hexachlorobiphenyl)	2.62 \pm 0.22	
PCB 183	(2,2',3,4,4',5',6-Heptachlorobiphenyl)	1.63 \pm 0.15	
PCB 187	(2,2',3,4',5,5',6-Heptachlorobiphenyl)	7.0 \pm 2.6	
182	(2,2',3,4,4',5,6'-Heptachlorobiphenyl)		
Chlorinated pesticides			
Oxychlorthane		2.59 \pm 0.19	
Dieldrin		1.26 \pm 0.37	6.2 \pm 1.3
2,4'-DDE			5.26 \pm 0.27
2,4'-DDD			13.7 \pm 2.8
2,4'-DDT			8.5 \pm 1.9
4,4'-DDT		1.25 \pm 0.10	

^a Results reported on both wet and dry mass basis; the sample as received contains 88.61% \pm 0.08% water.

^b PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell (1980) and later revised by Schulte and Malisch (1983) to conform with IUPAC rules; for the specific congeners mentioned in this SRM, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch.

^c Each noncertified value is the mean of the equally weighted means from four to seven different analytical methods. Each uncertainty, computed according to the CIPM approach (Guide, 1993), is an expanded uncertainty at the 95% level of confidence that incorporates within- and between-method uncertainty as well as uncertainty due to the drying study (for the dry mass basis values). The expanded uncertainty defines a range of values for the certified value within which the true value is believed to lie, at a level of confidence of approximately 95%.

The frozen mussel tissue was pulverized in batches of approximately 700 g each using a cryogenic procedure described previously (Zeisler *et al.*, 1983). The pulverized material was then homogenized in an aluminum mixing drum in 30 kg batches. The mixing drum was designed to fit inside the liquid nitrogen vapor freezer and to rotate in the freezer thereby mixing the frozen tissue powder. After mixing for 2 hr, subsamples of approximately 15 g of the tissue homogenate were aliquoted into pre-cleaned, pre-cooled glass bottles fitted with screw caps containing Teflon liners.

3.3. Moisture determination

The moisture content of SRM 1974a was determined by measuring the mass loss from freeze-drying. Twenty bottles of SRM 1974a were selected according to a stratified randomization scheme for the drying study. The entire contents of each bottle were transferred to a Teflon bottle and dried for 5 days at 1 Pa with a -20 °C shelf temperature and a -50 °C condenser temperature. The shelf temperature was gradually increased to 5 °C. The sample was considered dry when a stable mass was obtained. Based on these studies, a 95% confidence

interval for the mean moisture content of SRM 1974a is $88.61\% \pm 0.08\%$. Analytical results for the organic constituents were determined on a wet basis and then converted to a dry basis by dividing by the conversion factor of 0.1139 kg dry mass/kg wet mass.

3.4. Polycyclic aromatic hydrocarbons

The general approach used for the determination of PAHs in SRM 1974a is similar to that discussed above for the certification of SRM 1941a. Three sets of GC/MS results, designated as GC/MS (I), GC/MS (II), and GC/MS (Sm), were obtained using two columns with different selectivities for the separation of PAHs. For GC/MS (I) analyses, duplicate subsamples of 7 g to 9 g (wet basis) of the mussel homogenate from 12 randomly selected bottles were mixed with approximately 100 g of sodium sulfate, an internal standard (see below) was added to the sodium sulfate-tissue mixture, and then the mixture was Soxhlet extracted for 18 hr using 250 mL of dichloromethane. The extract was concentrated, and size exclusion chromatography on a semi-preparative divinylbenzene-polystyrene column (10 μm particle size, 100 Å pore size, 2.5 cm i.d. x 60 cm) was used to remove the majority of the lipid and biogenic materials. The eluent was concentrated and injected onto a semi-preparative aminopropylsilane column to isolate the PAH fraction by normal-phase LC. The PAH fraction was then analyzed by GC/MS using a 0.25 mm x 60 m fused silica capillary column with a 5% phenyl-substituted methylpolysiloxane phase (0.25 μm film thickness, DB-5 MS, J&W Scientific, Folsom, CA).

For the GC/MS (II) analyses, subsamples of 14 g to 16 g (wet basis) from three bottles of SRM 1974a were extracted and one half of each extract was analyzed using the same procedure as described above for GC/MS (I). However, these extractions and analyses were performed as part of three different sample sets at different times using different calibrations, method blanks, and quality control samples for each set.

The GC/MS (Sm) results were obtained by analyzing seven of the sample extracts from the GC/MS (I) set using a 0.2 mm i.d. x 25 m (0.15 μm film thickness) smectic liquid crystalline stationary phase (SB-Smectic, Dionex, Lee Scientific Division, Salt Lake City, UT). The liquid crystalline phase provides significantly different selectivity based on molecular shape for the separation of PAH isomers when compared with the 5% phenyl methylpolysiloxane phase. The column has a limited temperature range.

Two sets of LC-FI results, designated as LC-FI (Total) and LC-FI (Fraction), were used in the certification process. For the LC-FI (Total) analyses, a subsample of approximately 15 g (wet basis) of the mussel homogenate from eight randomly selected bottles was mixed with approximately 100 g of sodium sulfate, an internal standard (see below) was added to the sodium sulfate-tissue mixture, and then the mixture was Soxhlet extracted for 20 hr using 200 mL of 1:1 (volume ratio) hexane:acetone. The extract was concentrated and then passed through an aminopropylsilane solid phase extraction (SPE) column using 1:50 (volume ratio) dichloromethane in hexane to remove the lipid and more polar interferences. The eluent from the SPE column was concentrated and the SPE procedure was repeated five times on different SPE columns. The cleaned up extract was then analyzed by reversed-phase LC using a polymeric octadecylsilane (C_{18}) column (4.6 mm i.d. x 15 cm, 3 μm particle size, ChromSpher PAH, Chrompack, Middelburg, The Netherlands) with wavelength programmed fluorescence detection (May and Wise, 1984; Kline *et al.*, 1985; Wise *et al.*, 1988). To quantify several PAHs that have low fluorescence sensitivity and/or selectivity, six additional samples of SRM 1974a were extracted and prepared as described above. The extract was then fractionated on a semi-preparative aminopropylsilane column to isolate isomeric PAH fractions as described previously (May and Wise, 1984; Kline *et al.*, 1985; Wise *et al.*, 1988). These isomeric PAH fractions were analyzed by reversed-phase LC-FI on a similar C_{18} column.

For both the GC/MS and LC-FI measurements, selected perdeuterated PAHs were added to the mussel tissue/sodium sulfate mixture immediately prior to Soxhlet extraction for use as internal standards for quantification purposes. For GC/MS analyses the following internal standards were used: naphthalene-d₈, acenaphthene-d₁₀, fluorene-d₁₀, phenanthrene-d₁₀, fluoranthene-d₁₀, benz[*a*]anthracene-d₁₂, benzo[*e*]pyrene-d₁₂, dibenz[*a,h*]anthracene-d₁₄, and benzo[*ghi*]perylene-d₁₂. For the LC-FI analyses the following internal standards were used: naphthalene-d₈, phenanthrene-d₁₀, fluoranthene-d₁₀, and perylene-d₁₂ for the LC-FI (Total) analyses and triphenylene-d₁₂, benz[*a*]anthracene-d₁₂, and benzo[*ghi*]perylene-d₁₄ for the LC-FI (Fraction) analyses. Calibration response factors for the analytes relative to the internal standards were determined by analyzing aliquot of gravimetrically prepared mixtures of SRMs 1491 (Aromatic Hydrocarbons in Hexane/Toluene) and 2260 (Aromatic Hydrocarbons in Toluene, Nominal Concentration 60 µg/mL) in the case of GC/MS analyses or SRM 1647b (Priority Pollutant PAHs in Acetonitrile) in the case of LC-FI analyses; gravimetrically prepared solutions of additional analytes not contained in SRMs 1491, 2260, or 1647b; and the internal standards.

3.5. Polychlorinated biphenyl congeners and chlorinated pesticides

SRM 1974a was analyzed for selected PCB congeners and chlorinated pesticides using GC-ECD on two columns with different selectivity and using GC/MS. This same approach has been used previously at NIST for the certification of PCB congeners and chlorinated pesticides in other environmental matrices, including SRM 1941a as described above.

For SRM 1974a, two sets of GC-ECD analyses, designated as GC-ECD (I) and GC-ECD (II), were performed using similar procedures. For the GC-ECD (I) analyses, subsamples of 14 g to 16 g (wet basis) from eight bottles were mixed with 100 g of precleaned sodium sulfate and Soxhlet extracted for 18 hr using 250 mL of dichloromethane. Size exclusion chromatography on a preparative-scale divinylbenzene-polystyrene column with dichloromethane as the mobile phase was used to remove the majority of the lipid and biogenic material. The concentrated eluent was then fractionated on a semi-preparative aminopropylsilane column to isolate two fractions containing (1) the PCB congeners and lower polarity pesticides using hexane as the mobile phase and (2) the more polar pesticides using 5:95 (volume ratio) dichloromethane in hexane. GC-ECD analyses of the two fractions were performed on two columns of different selectivities for PCB separations: 0.25 mm x 60 m fused silica capillary column with a 5% phenyl-substituted methylpolysiloxane phase (0.25 µm film thickness) (DB-5, J&W Scientific, Folsom, CA) and a 0.32 mm x 100 m fused silica capillary column with a dimethylpolysiloxane phase containing 50% methyl C₁₈ (0.1 µm film thickness) (CPSil 5 C₁₈ CB, Chrompack International, Middelburg, The Netherlands). For the GC-ECD (II) analyses, subsamples of 14 g to 16 g (wet basis) from three bottles of SRM 1974a were extracted and one half of each extract was analyzed using the same procedure as described above for GC-ECD (I). However, these extractions and analyses were performed as part of three different sample sets at different times using different calibrations, method blanks, and quality control samples for each set.

For the GC/MS analyses of SRM 1974a, subsamples of 14 g to 16 g (wet basis) each from eight randomly selected bottles were Soxhlet extracted for 18 hr using 1:1 (volume ratio) hexane:acetone. The extracts were concentrated and placed on a precleaned silica SPE column and eluted with 15 mL of 1:10 (volume ratio) dichloromethane in hexane. The SPE cleanup procedure was performed sequentially three times on separate SPE columns. The final fraction was analyzed by GC/MS on a 5% phenyl-substituted methylpolysiloxane phase as described above for PAH measurements.

For both the GC-ECD and GC-MS analyses, two PCB congeners that are not significantly present in the mussel tissue extract (PCB 103 and PCB 198), octachloronaphthalene, 4,4'-DDT-d₈, and endosulfan-d₄ were added to the mussel tissue/sodium sulfate mixture prior to extraction for use as internal standards for quantification purposes. Calibration curves for the analytes relative to the internal standards were prepared by analyzing gravimetrically prepared mixtures of SRMs 2261 (Chlorinated Pesticides in Hexane, Nominal Concentration 2 µg/mL), SRM 2262 (Chlorinated Biphenyl Congeners in 2,2,4-Trimethylpentane, Nominal Concentration 2 µg/mL); gravimetrically prepared solutions of additional analytes not contained in SRMs 2261 and 2262; and the internal standards.

In addition to the analyses performed at NIST described above, SRM 1974a was used in two interlaboratory comparison exercises as part of the NIST Intercomparison Exercise Program for Organic Contaminants in the Marine Environment. The results from these two exercises, which were conducted in 1993 and 1994 and included results from 22 and 19 laboratories, respectively, were also used in the determination of the certified values for selected PCB congeners and chlorinated pesticides. The laboratories participating in these exercises used the analytical procedures routinely used in their laboratories to measure PCB congeners and chlorinated pesticides.

3.6. Methylmercury

The certified values for methylmercury are based on results of analyses of SRM 1974a and SRM 2974 at NIST and two other laboratories: Institute of Applied Physical Chemistry, Research Centre of Jülich, (Jülich, Germany) and the Marine Environment Laboratory, International Atomic Energy Agency (Monaco). The results from the analyses of both SRMs (i.e., the frozen tissue and the freeze-dried tissue) were combined to provide one certified value for both materials.

For the determination of methylmercury SRM 1974a and SRM 2974 were analyzed at NIST using liquid-solid extraction under acidic conditions and size exclusion chromatography followed by GC with atomic emission detection (GC-AED) as described in detail by Donais *et al.* (1996; 1997). The GC-AED analyses were performed using a 0.53 mm x 15 m fused silica capillary column with a 14% (mole fraction expressed as percent) cyanopropyl-substituted methylpolysiloxane phase (3.0 µm film thickness) (OV-1701, Quadrex, New Haven, CT). The method used at IAEA consisted of saponification at 70 °C followed by ethylation-room temperature precollection-GC-pyrolysis-cold vapor atomic fluorescence spectrometric detection (Horvat *et al.*, 1993). At the Research Centre of Jülich the analytical procedure for methylmercury consisted of a water steam distillation under acid conditions, anion exchange chromatographic separation of inorganic and methylmercury followed by cold vapor atomic absorption spectrometric detection before and after ultraviolet radiation (May *et al.*, 1987; Ahmed *et al.*, 1987; Padberg *et al.*, 1993). Subsamples from six bottles each of SRM 1974a and SRM 2974 were analyzed by each of the three laboratories.

3.7. Certified and noncertified concentrations

Statistically combining the results from the different methods, SRM 1974a has been issued with certified values for 15 PAHs (Table 1), 20 PCB congeners (Table 2), and 7 chlorinated pesticides (Table 3). Noncertified values are available for an additional 18 PAHs (Table 4), 4 PCB congeners (Table 5), and 4 chlorinated pesticides (Table 5). In addition to the organic contaminants, the methylmercury content was determined to be 77.2 ± 3.8 µg/kg dry mass basis (Donais *et al.*, 1997). Also, SRM 1974a was analyzed for 28 inorganic constituents using instrumental neutron activation analysis (Schantz *et al.*, 1997).

4. CONCLUSION

The materials replacing SRM 1941 and SRM 1974 (SRM 1941a and SRM 1974a) do have an increased number of compounds certified with the uncertainties generally lower for those compounds that were certified in the original materials. An increased amount of sediment and an increased number of mussels were collected to expand the supply for an additional period of time. SRM 1941a is a dry sediment and SRM 1974a is a frozen mussel tissue, both of which have concentrations of the organic contaminants typical of an urban harbor environment.

5. ACKNOWLEDGMENTS AND DISCLAIMER

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The mussel tissue material used for SRM 1974a was collected with the assistance of Julie Seavey from Battelle Ocean Sciences Laboratory, Duxbury, MA and M. Cronise and C. Fales from the NIST Standard Reference Materials Program. The support aspects for the preparation and certification of this SRM were coordinated by J. C. Colbert of the NIST Standard Reference Materials Program.

Certain commercial equipment, instruments, or materials are identified in this report 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 the best available for the purpose.

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HISTOPATHOLOGY

Histopathology Analysis

M. S. Ellis, R. D. Barber, R. E. Hillman,* Y. Kim and E. N. Powell
Haskin Shellfish Research Laboratory
Rutgers University
6959 Miller Ave.
Port Norris, NJ

ABSTRACT

This document describes the procedures followed for histopathological analysis of oysters, mussels, and zebra mussels collected for NOAA's National Status and Trends Mussel Watch Program. Analyses are conducted on paraffin-embedded tissues sectioned at a 5- μ m thickness and stained using a pentachrome staining procedure. Infection intensity of parasites, the occurrence and extensiveness of tissue pathologies, and the intensity of diseases are recorded using semi-quantitative or quantitative measures.

1. INTRODUCTION

The use of bivalves in the National Status and Trends (NS&T) Program is predicated upon their reliability as environmental integrators of contamination. The influence of population health on body burden and, in turn, the influence of contaminant exposure on population health is, as yet, poorly understood. Clearly, certain diseases (normally caused by viruses or single-celled prokaryotes and eukaryotes) and non-disease causing parasites (mostly ciliates and multi-cellular parasites) produce tissue level changes that might be expected to affect contaminant body burden. Gonadal quantity, for example, can be dramatically altered by disease (Hofmann *et al.*, 1995; Barber, 1996; Ford and Figueras, 1988) and by parasites (Hopkins, 1957; Yoo and Kajihara, 1985). Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Mo and Neilson, 1993; Cunningham and Tripp, 1975). Because gametic material can account for 20 to 50% of body weight in target species of oysters and mussels (Sprung, 1991; Choi *et al.*, 1993), the relative proportion of gonadal to somatic tissue and the timing of spawns (an important depuration route for some contaminants) can significantly impact the body burden of contaminants. Evidence, mostly by correlation, continues to mount for a relationship between certain tissue pathologies and contaminant exposure (Bowmer *et al.*, 1991; Weis *et al.*, 1995; Johnson *et al.*, 1992) and the influence of contaminant exposure on the bivalve immune system has been described (Anderson *et al.*, 1992; Cheng, 1988; Winstead and Couch, 1988). Besides contaminants, other environmental factors may facilitate disease or trigger the development of pathologies (e.g. Lee *et al.*, 1996; Landsberg, 1996) with significant consequences to tissue composition and, in all likelihood, subsequent contaminant retention. Thus, contaminant exposure and health, as they feed back upon each other, may have dramatic effects on monitoring programs that use sentinel organisms to define spatial and temporal trends in contamination loadings and contaminant gradients.

Evaluation of the health of specimens collected as a part of the Mussel Watch Program necessitates evaluating the prevalence and intensity of diseases, parasites and pathologies by histological examination. Certain pathological conditions recognized by shell condition (Warburton, 1958; Lawler and Aldrich, 1987), ligament degradation (Dungan *et al.*, 1989), or

* Battelle Ocean Sciences, Environmental Assessment Division, Duxbury, MA 02332.

periostracal abnormalities (Davis and Barber, 1994) will not be identified using this approach and some ectoparasites are lost during collection (e.g. odostomians, White *et al.*, 1985, 1989), however most common diseases, parasites, and pathologies can be evaluated by this method.

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

A measure of overall health has not been applied. A number of measures of overall health have been suggested, based on tissue appearance (Quick and Mackin, 1971), histological grading (Bowmer *et al.*, 1991), or summation of total parasite load (Kennicutt, 1995). Although Laird (1961) argues on theoretical grounds for an underlying relationship between total parasite body burden and environmental quality, generally, these overall measures of health have not proven efficacious because the various parasites, diseases and pathologies originate in different ways and certain parasites, even if abundant, may not have a large impact on organism health.

2. EQUIPMENT, REAGENTS, SOLUTIONS, AND SAMPLE PREPARATION

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

3. ANALYSIS

Prepared slides are examined individually under the microscope using a 10X ocular and a 10X objective. If any tissue needs to be examined more closely, a 25X or 40X objective may be used for closer examination of suspected pathologies or parasites. Major tissue types to be examined include gill, mantle, gonad and gonoducts, digestive gland tubules, stomach/digestive gland ducts, and connective tissue. Thus, a proper tissue cross-section is essential (see Ellis *et al.*, this volume). Normally, oysters are examined starting at the gills, and scanned laterally, working toward the anterior end. In mussels, gonads and gills are examined first, and then the visceral mass. For zebra mussels, the visceral mass, including the gonads and digestive tract are examined, followed by scanning of the gills and mantle.

Conditions evaluated, be they parasites, diseases, or tissue pathologies, are scored for intensity using either a quantitative or semi-quantitative scale, as described subsequently. Conditions scored quantitatively are evaluated by keeping a running count of incidences of the condition as the slide is scanned, to avoid re-examining each slide multiple times for each separate malady. Evaluation of conditions scored semi-quantitatively may require re-scanning

portions of the tissue for each malady type to gain a complete portrayal of the degree of tissue involvement.

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

3.1. Quantitative categories

Most parasites were counted quantitatively. These include *Nematopsis spp.*, ciliates of various types, worms in the gonoducts, worms ectoparasitic or commensal on the gills, cestodes, nematodes, and copepods. We also evaluated a number of other tissue conditions quantitatively: the number of ceroid bodies, incidences of tissue inflammation, rickettsial bodies, incidences of tissue edema, and suspected neoplasms and tumors.

Nematopsis [Photograph: Cheng (1967), p. 148, Fig. 29; Ford (1988), p. 218, Fig. 6G; Gauthier *et al.* (1990), p. 110, Fig. 2, 3] is a sporozoan parasite normally found in oysters, and only rarely in mussels and zebra mussels. Different *Nematopsis* species often have different tissue distributions, and, in particular, show a tissue preferences for either mantle or gill (Sprague and Orr, 1952). Although, species identifications were not conducted, we routinely scored gill and mantle infection intensity separately, following Landau and Galtsoff (1951). Quantification was obtained by counting each individual in each tissue within one representative tissue section.

Ceroid bodies or brown cells [Photograph: Murchelano and MacLean (1990), p. 10, Fig. 1- 9], distinct brown-yellow aggregates that may occur in large clumps, appear to be involved in metabolite accumulation and detoxification (Zarogian and Yevich, 1993). Typically, they occur in greatest abundance in oysters and in lesser numbers in mussels and zebra mussels. Quantification was obtained by counting each ceroid body. Occasionally, a ceroid body appears fractured or split; in this case only one fragment is counted.

A variety of kinds of ciliates have been observed, normally at low prevalence and more frequently in mytilid mussels than in oysters. Ciliates have been quantitated by tissue type (e.g. gut, digestive gland, gill) and by size [e.g. individual ciliates versus xenomas (cells distended with maturing ciliates) or larger attached ciliates]. A more detailed taxonomic differentiation can be conducted if high infection intensity warrants it, but this is rarely the case. If the large xenoma has not burst, it is counted as an individual large ciliate; if it has burst, the individual small ciliates are counted. [Photograph: Cheng (1967), p. 184, Fig. 88, representative gill ciliate; Cheng (1967), p. 193, Fig. 102, representative digestive gland ciliate in lumen; Figueras *et al.* (1991), p. 91, Fig. 2, representative digestive gland ciliate in tubule wall; additional photographs in Gauthier *et al.* (1990)]

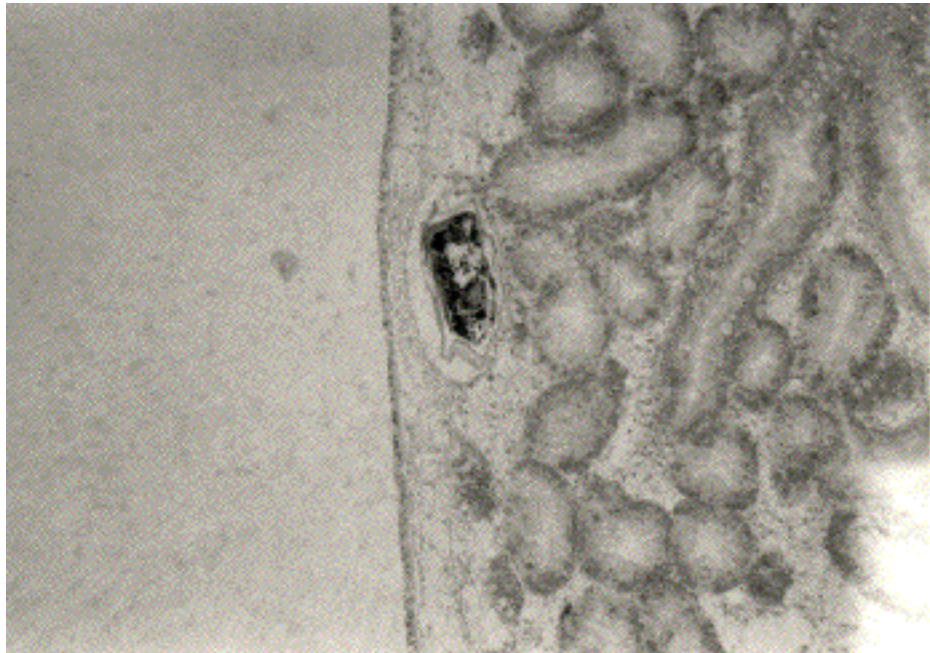


Figure 1. Unidentified worm in the gonoduct of an oyster, *Crassostrea virginica*.

An unidentified, possibly larval arabellid, worm is occasionally seen in the gonoducts of oysters from the Gulf of Mexico (Figure 1). The cross-section is characterized by a distinct cuticle and, occasionally, setae appear to be present. Each occurrence is counted.

Gill nemerteans are occasionally seen between gill filaments. Whether these are commensal or ectoparasitic is unknown. Each individual cross section is counted separately, although, like nematodes, a single individual may be responsible for a number of cross-sections.

A variety of encysted metacestodes and trematodes have been observed, normally at low prevalence (for examples, see Cake, 1977; Cake and Menzel, 1980). Cestodes are more common in oysters, trematodes, except for *Bucephalus*, in mytilid mussels. Cellular reaction in the subepithelial tissues to cestode cysts (e.g., encapsulation of the larval cestodes) has been described by Sindermann (1970) and is normally observed associated with them. Cestode cysts and trematodes have been quantitated by tissue location (e.g. gut, gill, gonad). Each occurrence observed is counted separately. Representative examples are given in Murchelano and MacLean (1990, p. 16, Fig. 1 - 21), Sindermann (1970, p. 128, Fig. 42), and Little *et al.* (1969, p. 455, Fig. 1).

Nematodes [Photograph: Gauthier *et al.* (1990), p. 112, Fig. 9; Murchelano and MacLean (1990), p. 18, Fig. 1 - 25; Sparks (1985), p. 375 - 376, Fig. 10 - 16] are occasionally found in most tissues of oysters. Each cross-section observed is counted; like nemerteans and many other worms, one individual may be responsible for a number of tissue cross-sections, however this method of quantification has proven effective even at high infection intensities (Kennicutt, 1995).

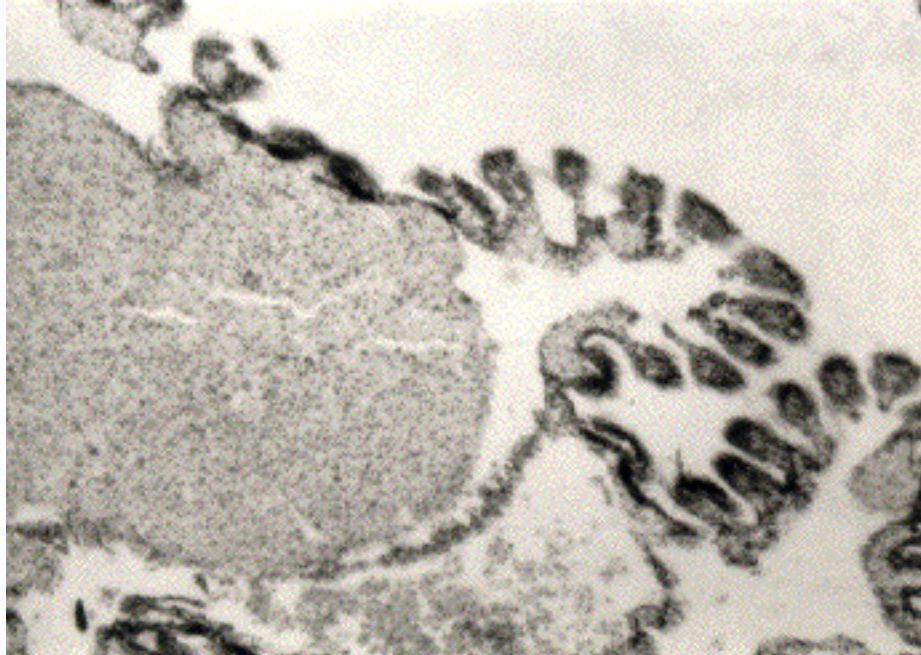


Figure 2. Edema in the gill of an oyster, *Crassostrea virginica*.

Cases of tissue inflammation [Photograph: Murchelano and MacLean (1990), p. 10, Fig. 1 - 10; Sindermann (1970), p. 110, Fig. 34], characterized by intense infiltration of hemocytes, may be focal or diffuse. The type of affected tissue and type of irritation responsible influence the nature of the cellular response (Ford and Tripp, 1996). Each affected area is counted. Diffuse inflammation is differentiated from focal inflammation when the affected area does not appear to have a clear center or focal point of highest hemocyte concentration and hemocytes are abundant and distributed broadly over a large section of tissue.

Amorphous, basophilic, granular inclusions, variously referred to as rickettsial bodies, chlamydial bodies or mycoplasmas, are normally observed in the duct and tubule walls of the digestive gland [Photograph: Murchelano and MacLean (1990), p. 8, Fig. 1 - 6; Harshbarger *et al.* (1977), p. 667, Fig. 1]. Each individual inclusion is tabulated.

Neoplasms and tumors are occasionally observed, particularly in mytilid mussels where disseminated sarcomas, probably of hematopoietic origin, are particularly common in the Puget Sound region (e.g. Elston *et al.*, 1990). Occurrence of neoplasms and tumors in oysters is extremely rare. Examples are described by Farley (1969, 1976), Harshbarger *et al.* (1979) and Ford and Tripp (1996). Representative photographs are provided by Murchelano and MacLean (1990, p. 19 - 20, Fig. 1 - 27, 1 - 29), Peters (1988, p. 81, Fig. C, E, F), and Sparks (1985, p. 107, Fig. 27; p. 109, p. 120, Fig. 51; p. 113, Fig. 42). Each occurrence is tabulated.

Edema (Figures 2, 3) is a localized enlargement of tissue cells without an accompanying response by hemocytes or if hemocytes are present, they too appear to be enlarged. Each affected area is counted separately.

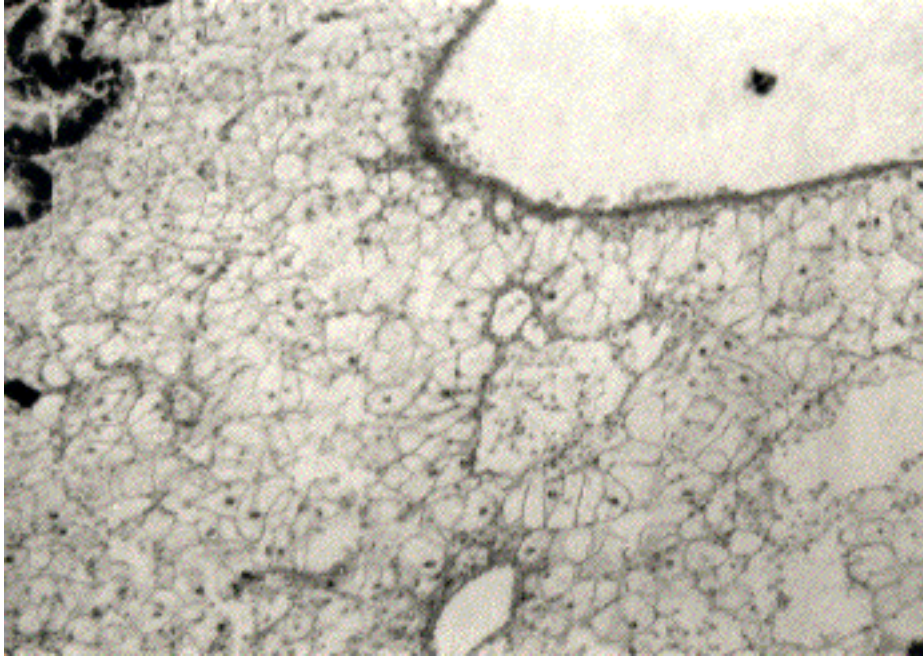


Figure 3. Edema in the connective tissue of an oyster, *Crassostrea virginica*.

Proctoeces sp. [Wolf *et al.* (1987), p. 380, Fig. 1; see also Winstead and Couch (1981)] is occasionally found in the gonoducts. This trematode and occasional nematodes also found in the gonoduct should be distinguished from the more common unidentified, possibly arabellid, worm observed in oysters from the Gulf of Mexico. Each occurrence is tabulated.

Parasitic copepods are occasionally found in the gut lumen (Gee and Davey, 1986). Each occurrence is tabulated.

3.2. Semi-quantitative categories

Some conditions were assigned to a semi-quantitative scale related to the intensity of the effect or the extensiveness of the affected area. With one exception, these were pathologies affecting large tissue areas or diseases characterized by systemic affects and individual counts too large to quantify. One invasive parasite, *Bucephalus*, was also included due to the extensiveness of infections and the difficulty in obtaining quantitative counts of the sporocysts. *Perkinsus marinus* was assayed in oysters by the more precise thioglycollate method, rather than by histology (Powell and Ellis, this volume).

Digestive gland atrophy [Photograph: Gauthier *et al.* (1990), p. 112, Fig. 10; Winstead (1995), p. 107, Fig. 3,4], a condition characterized by the thinning of the lumen of the digestive tubule walls, has been described in a number of bivalve species (Bielefeld, 1991; Marigómez *et al.*, 1990; Axiak *et al.*, 1988). Causes of the condition have been ascribed to a variety of stressors including exposure to contaminants and variations in food supply. Winstead (1995) found that poor nutrition was a key element in producing the condition in oysters and that the digestive gland recovered to its normal state relatively rapidly once food supply improved. Presumably, the thinning of the digestive tubule epithelium affects the normal digestive processes.

The digestive gland is scanned for tubules showing evidence of epithelial thinning. A visual evaluation is made of the average condition of the digestive gland tubules and this average condition is assigned to a semi-quantitative numerical scale (Table 1) according to the degree of thinning of the epithelial wall. The semi-quantitative assessment permits the reading of many samples in a short time. For increased accuracy, the ratio of tubule diameter to wall diameter (e.g. Winstead, 1995) or a direct measure of wall thickness (Marigómez *et al.*, 1990) can be used.

Bucephalus sporocysts [Photograph: Gauthier *et al.* (1990), p. 112, Fig. 8; Murchelano and MacLean (1990), p. 16, Fig. 1-23; Sindermann (1970), p. 125, Fig. 41] occur principally in the gonadal tissue of oysters (Hopkins, 1957). Extensive infections effectively destroy all gametic tissue and the sporocysts begin to invade the remainder of the visceral mass. On rare occasions, the gill tissue may also be involved. The large branching sporocysts are difficult to quantitatively enumerate. Hence, infection intensity is scored on a semi-quantitative scale (Table 2).

Table 1. Semi-quantitative scale for digestive gland atrophy (adapted from scale developed by J. Gauthier).

<u>Score</u>	<u>Description</u>
0	Normal wall thickness in most tubules (0% atrophy), lumen nearly occluded, few tubules even slightly atrophied (Figure 4)
1	Wall thickness averaging less than one-half atrophied, most tubules showing some atrophy, some tubules still normal
2	Wall thickness averaging about one-half as thick as normal (Figure 5)
3	Wall thickness greater than one-half atrophied, most tubules walls significantly atrophied, some walls extremely thin (fully atrophied)
4	Wall extremely thin (100% atrophied), nearly all tubules affected (Figure 6)

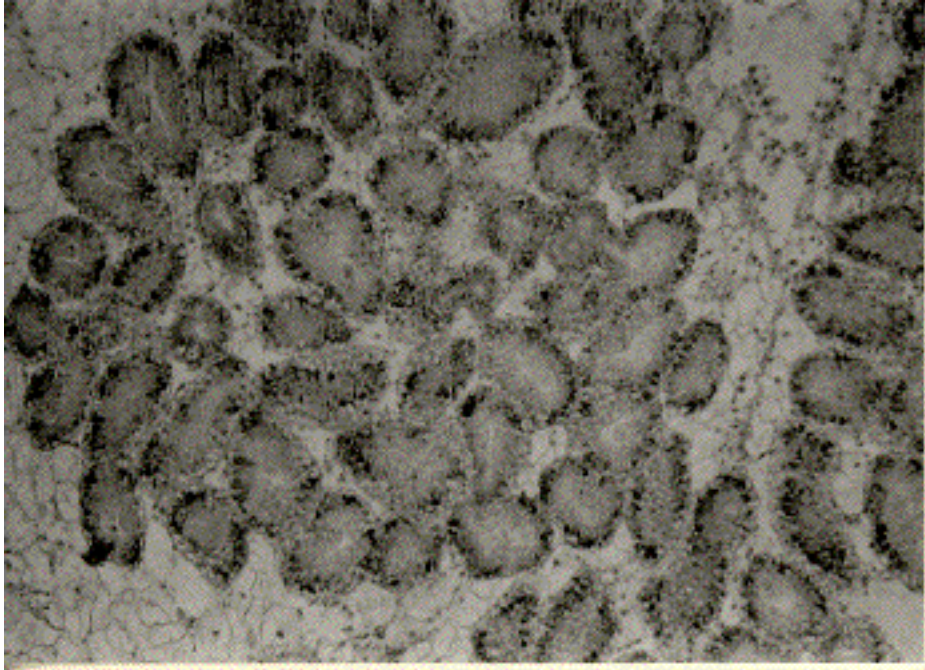


Figure 4. *Crassostrea virginica* normal digestive tubule, scored a 0 according to Table 1.

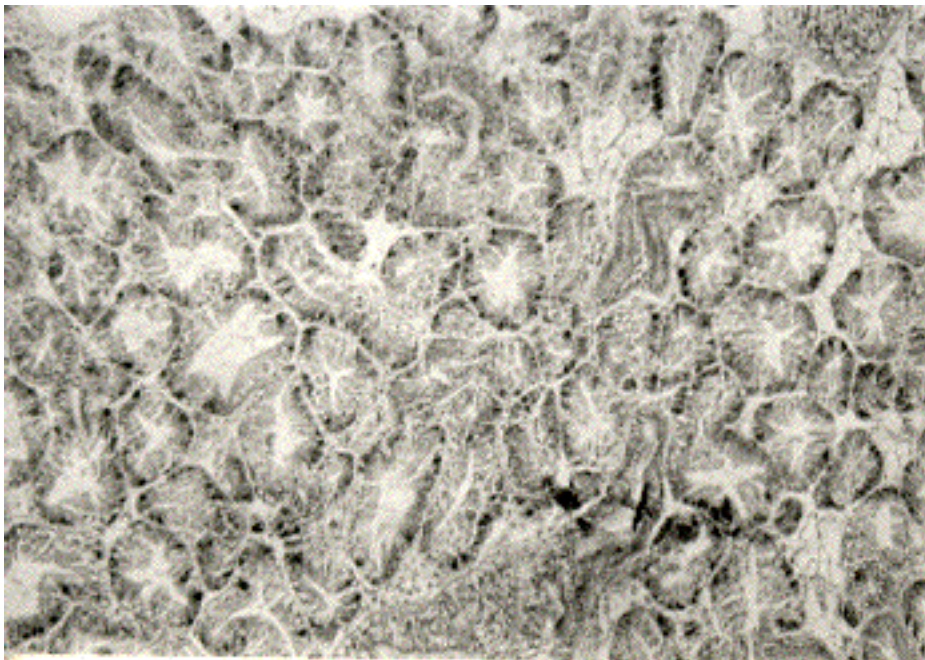


Figure 5. Digestive gland atrophy in *Crassostrea virginica* scored a 2 according to Table 1.

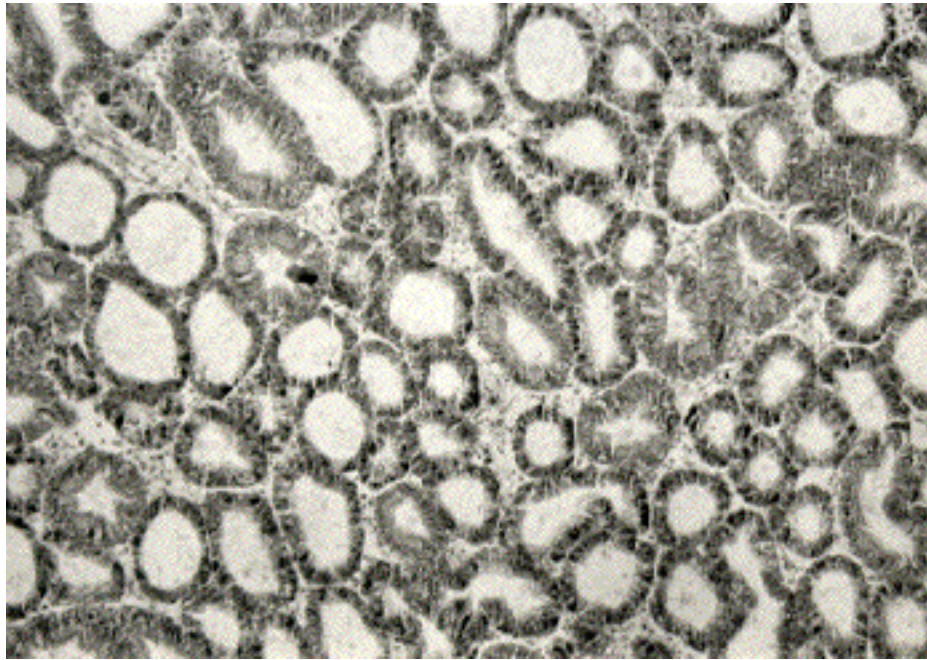


Figure 6. Digestive gland atrophy in *Crassostrea virginica* scored a 4 according to Table 1.

Table 2. Semi-quantitative scale for *Bucephalus* infection.

<u>Score</u>	<u>Description</u>
0	Uninfected
1	Present in the gonads only (some gametic tissue still present) (Figure 7)
2	Completely filling the gonads (no gametic tissue present); may be present in digestive gland or gills in very limited amounts (Figure 8)
3	Completely filling the gonads; extensive invasion of the digestive gland and/or the gills (Figure 9)
4	Completely filling the gonad; substantially filling the digestive gland or gill; individuals appear to be a sac of sporocysts

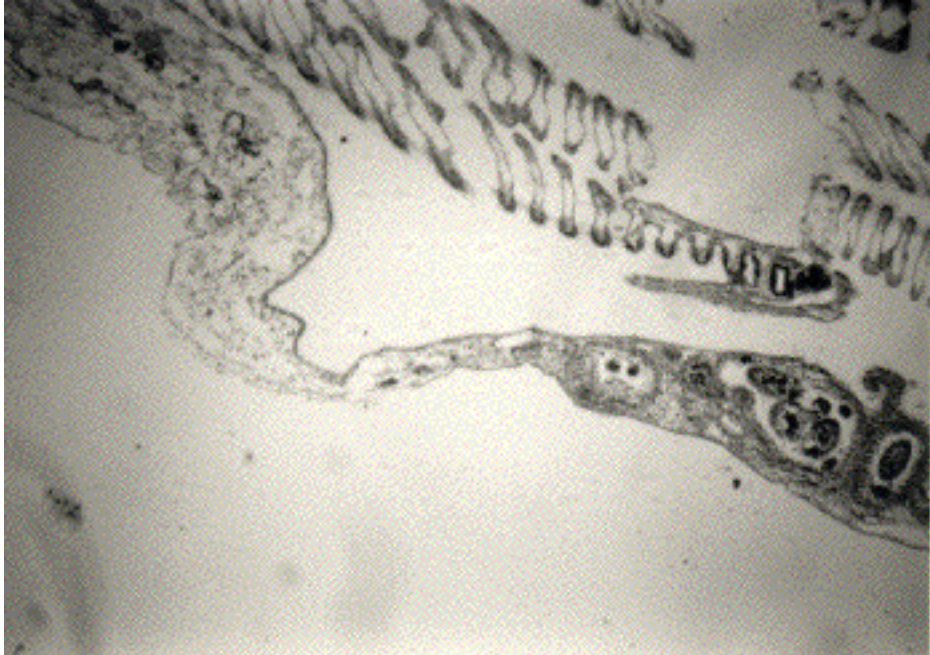


Figure 7. *Bucephalus* infection in *Mytilus edulis* scored a 1 according to Table 2. Some gametic tissue is still present.

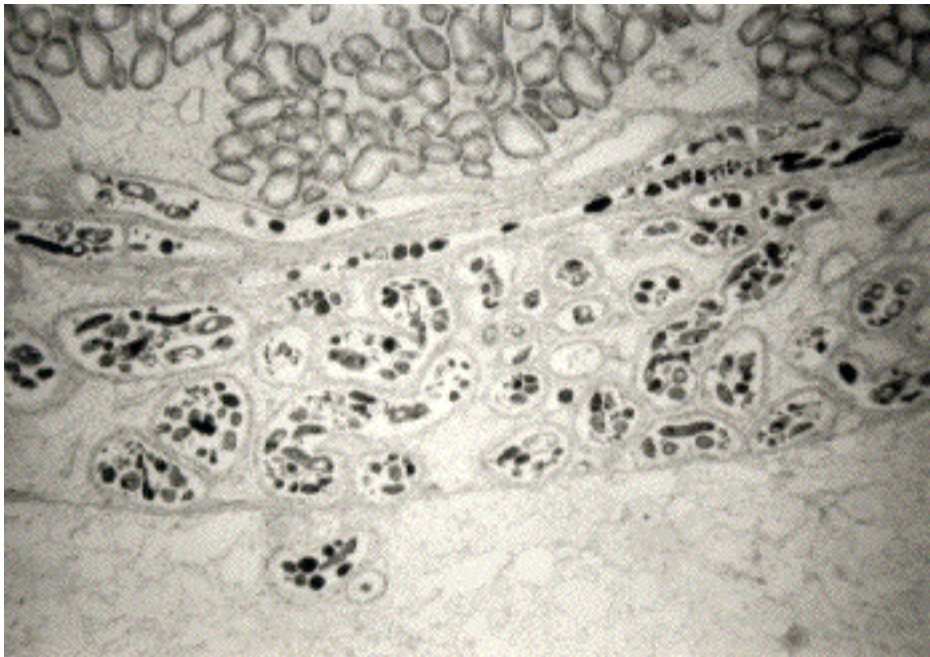


Figure 8. *Bucephalus* infection in *Mytilus edulis* scored a 2 according to Table 2. No gametic tissue is present.

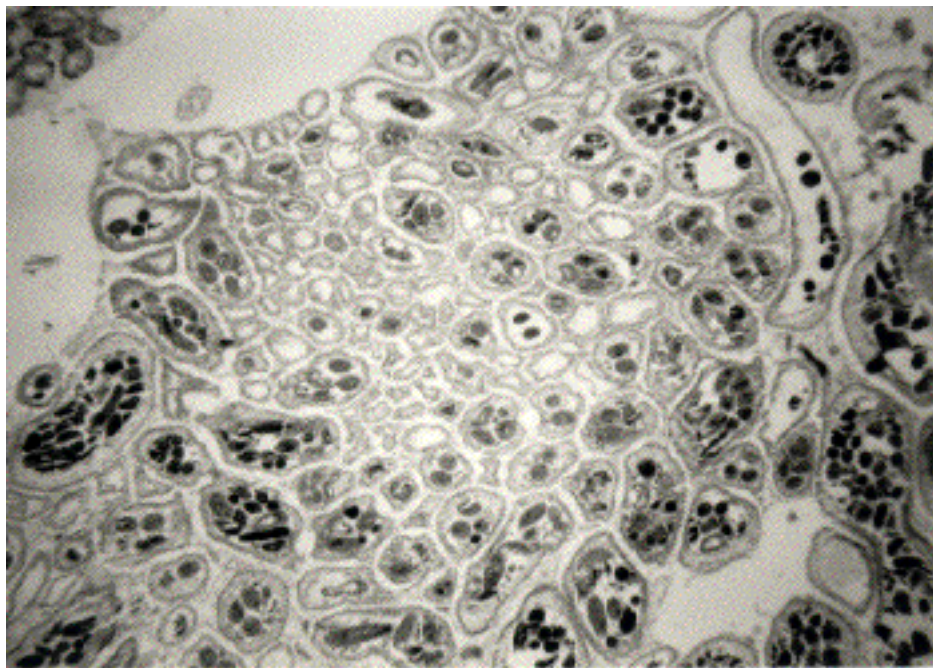


Figure 9. *Bucephalus* infection in *Mytilus edulis* scored a 3 according to Table 2. No gametic tissue is present. *Bucephalus* heavily infiltrating digestive gland.

Haplosporidium nelsoni [photograph: Ford and Tripp (1996), p. 614, Fig. 18] is a haplosporidian protozoan responsible for MSX disease in oysters. The intensity of MSX infection is graded according to a semi-quantitative scale (Table 3) reminiscent of the scale established for *Perkinsus marinus*. Because of the small size of *H. nelsoni*, oyster tissues may need to be examined at a higher power than 10X, in some cases. Because MSX infection normally starts in the gill epithelium, the gill tissue must be carefully examined to score early infections accurately. In cases where the disease has become systemic, examination of the visceral mass is necessary to score infection intensity. More details are provided in Ford (1985, 1986) and Ford and Figueras (1988).

Several kinds of abnormal gonadal development have been observed, sometimes commonly, in mytilid mussels. Often, abnormal gonadal development is characterized by unusual development at the base of the follicles. The cells resemble that of a germinoma and are differentiated from that of normal development by being either enlarged, or appearing to have an enlarged nucleus. This type of abnormal development is more common in males than in females. In other cases, underdeveloped gonadal follicles were observed. Follicles were small and occupied a smaller portion of the mantle tissue. Ova were smaller and male follicles often had very few mature sperm. Sometimes follicles may be filled with anomalous ova or degenerating sperm, and granulocytes, brown cells and cellular debris may clog the follicular lumens (Figure 10). Sometimes cells adhere to each other, forming accumulations and empty spaces among developing cells. Occasionally, fibrosis occurs, with proliferation of fibroblasts inside the follicles and in the interfollicular connective tissue. Abnormal gonadal development is often associated with degeneration of Leydig tissue around the follicles and hemocytic infiltration into the surrounding tissues (Figure 11).

Table 3. Semi-quantitative scale for *Haplosporidium nelsoni* infection [from Ford (1985, 1986) and Ford and Figueras (1988)].

<u>Score</u>	<u>Description</u>
0	Uninfected
1	Epithelial only, rare to lightly infected, 1 - 100 parasites per field of gill tissue
2	Subepithelial, spreading into the visceral mass, lightly to moderately infected with clear subepithelial foci, normally 10 - >100 parasites per field but <5 parasites per 1000X oil field
3	Subepithelial, more evenly distributed in visceral mass, lightly to moderately infected normally 10 - >100 parasites per field but <5 parasites per 1000X oil field
4	Heavy systemic infection, averaging more than 5 parasites per oil field

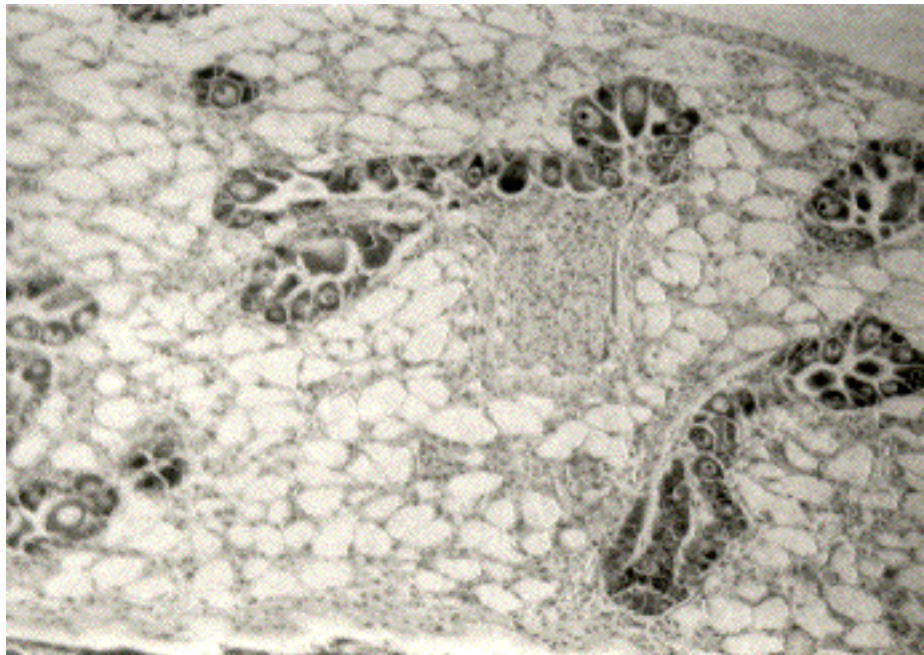


Figure 10. *Mytilus edulis* follicle with abnormal gametic tissue (lower right).

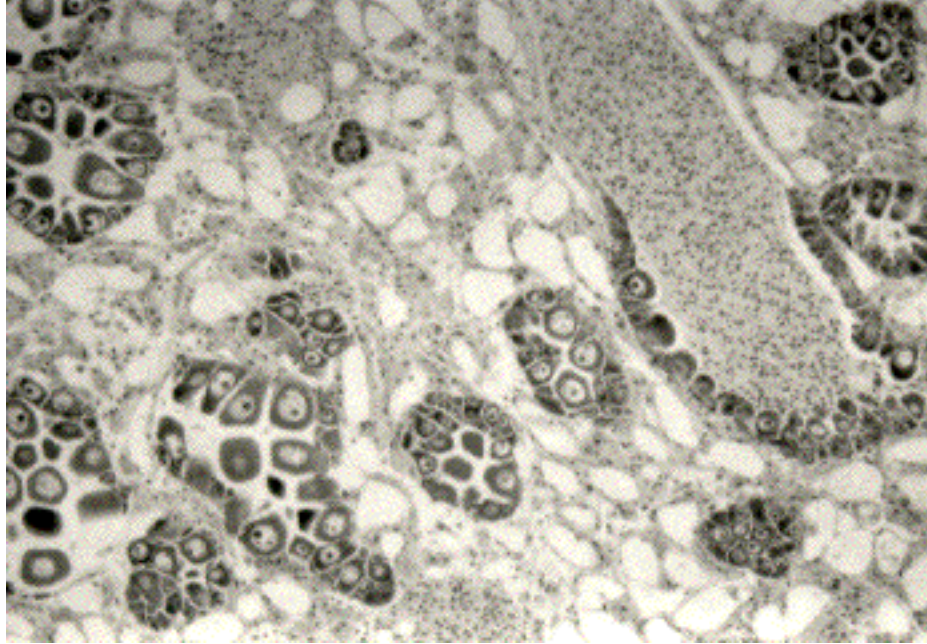


Figure 11. *Mytilus edulis* follicle heavily infiltrated with hemocytes (right).

The approach used to score instances of abnormal gonadal development differs from that used for digestive gland atrophy in that the scale rates the spatial coverage of the condition (e.g. fraction of follicles affected), but not the degree of effect in each follicle (Table 4). Normally, the entire follicle is completely affected or not affected.

4. CONCLUSION

The described technique provides a quantitative or semi-quantitative method to determine the prevalence and intensity of parasites, pathologies, and diseases afflicting oysters, blue mussels and zebra mussels. The described method is a general approach for histopathological analysis targeting a wide range of parasites and pathologies. Certain conditions will be better assessed by other methods; *Perkinsus marinus* infection in oysters being a good example (Powell and Ellis, this volume). The described method emphasizes the quantification of intensity. Prevalence rarely provides an adequate description of the population dynamics of disease and, in practice, often yields ambiguous results. Infection intensity quantitated by counts or the use of a semi-quantitative scale consistently provides a more robust data set for statistical analysis comparing the spatial and temporal distribution of parasites, pathologies, and diseases to contaminant body burden.

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Table 4. Semi-quantitative scale for abnormal gonadal development in mussels.

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

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Gonadal Analysis

M. S. Ellis, R. D. Barber, R. E. Hillman,* and E. N. Powell
Haskin Shellfish Research Laboratory
Rutgers University
6959 Miller Ave.
Port Norris, NJ

ABSTRACT

This document describes the procedures for determining the reproductive stage of oysters, mussels, and zebra mussels collected for NOAA's National Status and Trends Mussel Watch Program. Procedures are documented for preparation of paraffin-embedded tissues sectioned at a 5- μ m thickness and stained using a pentachrome staining procedure.

1. INTRODUCTION

Assessment of the physiological state of bivalve populations requires an analysis of the state of gonadal development. Determination of reproductive stage was included as part of the Mussel Watch Project to give an indication of the amount of gametic material in the bivalves' tissues at the time of chemical analysis. Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Mo and Neilson, 1993; Cunningham and Tripp 1975). Because gametic material can account for 20% to 50% of body weight in target species of oysters and mussels (Sprung, 1992; Choi *et al.*, 1993), the relative proportion of gonadal to somatic tissue and the timing of spawns (an important depuration route for some contaminants) can significantly impact the body burden of contaminants.

The original intent of the determinations of reproductive stage was to assure that sampling was conducted at the same stage of the reproductive cycle so that analysis of neither the lipophilic organic contaminants nor the trace element contaminants were influenced by reproductive state. Unfortunately, the time required for sampling and the wide latitudinal range encompassed by the sites did not permit consistent recovery of individuals in a similar stage of reproductive development at all sites. For example, typically, oysters are undifferentiated in the winter. Gonads begin to develop in early spring and spawning occurs during late spring through early fall. Most Gulf Coast oysters spawn at least twice during this time period. Single spawns tend to occur in the shorter summers of the mid-Atlantic region. The timing of the last spawn varies with latitude and with yearly variations in climate (e.g. Wilson *et al.*, 1990, 1992). Certain sites, particularly southeast Atlantic and southern Gulf sites, routinely yield oysters in reproductive development or ready to spawn in mid-winter during the period when Mussel Watch sampling occurs at these sites. Mid-Atlantic sites typically were characterized by individuals in an undifferentiated state, that thus contained significantly less lipid-rich gametic tissue and that had been accumulating contaminants for a much longer time since their most recent spawn. Mytilids and zebra mussels had the same assortment of problems relating to latitude and interannual changes in climate (Newell, 1989; Seed and Suchanek, 1992; Borcharding, 1991). Thus, analysis of reproductive stage proved important in identifying differences in tissue composition which might affect between site and interannual comparisons

* Battelle Ocean Sciences, Environmental Assessment Division, Duxbury, MA 02332.

of contaminant data. Wilson *et al.* (1990, 1992) discuss the influence of climate on reproductive stage and contaminant body burden in the Mussel Watch Program in more detail.

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

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

2. EQUIPMENT, REAGENTS AND SOLUTIONS

2.1. Equipment

Stainless steel molds, various sizes. Fisher Scientific, Pittsburgh, PA.

Disposable microtome blades, 12-634-11. Fisher Scientific, Pittsburgh, PA..

Cover slips - square no. 1, 22 mm x 22 mm, 12-531-A. Fisher Scientific, Pittsburgh, PA.

Disposable cassettes - HistoPrep tissue capsules, 15-182-218. Fisher Scientific, Pittsburgh, PA.

Paraffin embedding center, 12-653-68. Fisher Scientific, Pittsburgh, PA.

Embedding rings - HistoPrep embedding rings, 12-652-10B. Fisher Scientific, Pittsburgh, PA.

Frosted microslides, 12-552. Fisher Scientific, Pittsburgh, PA.

Microscope, compound, Zeiss, 12-070-20. Fisher Scientific, Pittsburgh, PA.

Microtome, Leica Histocut 820 Rotary microtome, 12-603. Fisher Scientific, Pittsburgh, PA.

Microtome knife sharpener, automatic, Leica 903, 12-643-40. Fisher Scientific, Pittsburgh, PA.

Drying oven, 13-254-1. Fisher Scientific, Pittsburgh, PA.

Paraffin pitcher, electric, Lepshaw 220, M7395. Baxter Scientific, McGaw Park, IL.

Rotary tissue processor, Tissue-Tek, M7286-1. Baxter Scientific, McGaw Park, IL.

Slide warmer, 12-594. Fisher Scientific, Pittsburgh, PA.

Staining dishes, 08-813A. Fisher Scientific, Pittsburgh, PA.

Vacuum oven, 13-264A. Baxter Scientific, McGaw Park, IL.

Water bath - Fisher Tissue PrepModel 134 Flotation Bath, 15-464. Fisher Scientific, Pittsburgh, PA.

2.2. Reagents

Acid fuchsin, certified stain ($C_{20}H_{17}N_3Na_2O_9S_3$) [3244-88-0], A3908. Sigma Chemical Co., St. Louis, MO.

Ammonium hydroxide (NH_4OH) [1336-21-6], A6899. Sigma Chemical Co., St. Louis, MO.

Aniline blue powder, certified stain [28631-66-5], A697-25. Fisher Scientific, Pittsburgh, PA.

Chromotrope powder 2R, ($C_{16}H_{10}N_2Na_2O_8S_2$) [4197-07-3], C3143. Sigma Chemical Co., St. Louis, MO.

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

Fast green FCF, certified stain ($C_{37}H_{34}N_2O_{10}S_3Na_2$) [2353-45-9], F7252. Sigma Chemical Co., St. Louis, MO.

Ferric ammonium sulfate [$NH_4Fe(SO_4)_2 \cdot 12H_2O$] [7783-83-7], F1018. Sigma Chemical Co., St. Louis, MO.

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

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

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

Hematoxylin powder, certified stain ($C_{16}H_{14}O_6$) [517-28-2], H3136. Sigma Chemical Co., St. Louis, MO.

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

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

Paraffin - Paraplast tissue embedding media (melting pt. 56 °C), 12-464-11, Fisher Scientific, Pittsburgh, PA.

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

Phosphomolybdic acid ($24MoO_3 \cdot P_2O_5 \cdot H_2O$) [51429-74-4], P7390. Sigma Chemical Co., St. Louis, MO.

Phosphotungstic acid ($12WO_3 \cdot H_3PO_4 \cdot H_2O$) [12501-23-4], P4006. Sigma Chemical Co., St. Louis, MO.

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

Sulfuric acid (H_2SO_4) [7664-93-9], S1526. Sigma Chemical Co., St. Louis, MO.

Tissue Clear II, SH2-4. Fisher Scientific, Pittsburgh, PA.

Tissue Dry, SH5-20. Fisher Scientific, Pittsburgh, PA.

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

2.3. Solutions

Ferric alum mordant: 25 g ferric ammonium sulfate dissolved in 500 mL distilled water.

Basic ethanol: 26 mL ammonium hydroxide in a solution of 3370 mL 95% ethanol and 630 mL distilled water.

Phosphomolybdic acid solution: 5 g phosphomolybdic acid dissolved in 495 mL distilled water.

1% acetic acid: 20 mL glacial acetic acid in 1980 mL distilled water.

1% acid acetone: 20 mL acetone in 1980 mL distilled water

Groat/Weigert hematoxylin working solution: 245 mL distilled water, 5 mL sulfuric acid, 5 g ferric ammonium sulfate, 245 mL 95% ethanol, and 2.5 g hematoxylin powder.

Acid fuchsin working solution: 2.5 g acid fuchsin powder dissolved in 495 mL distilled water, to which is added 5 mL glacial acetic acid.

Phosphotungstic acid solution: 10 g phosphotungstic acid dissolved in 490 mL distilled water.

Orange G - stock: 4 g orange G powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Chromotrope 2R - stock: 3 g chromotrope powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Orange G/Chromotrope working stock: 400 mL orange G solution and 100 mL chromotrope solution.

Fast green - stock: 8 g fast green FCF dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Aniline blue - stock: 6 g aniline blue powder in 495 mL distilled water.

Fast green/Aniline blue working solution: 150 mL fast green solution and 350 mL aniline blue solution.

Davidson's fixative stock solution: 1 part glycerin, 2 parts 37 - 40% formaldehyde, 3 parts 95% ethanol, and 3 parts isotonic sodium chloride (usually 20 - 30‰). The solution was stored at 4 °C.

Davidson's fixative working solution: Just before use, 1 part of glacial acetic acid was mixed with 9 parts of the stock solution.

3. SAMPLE COLLECTION AND FIXATION

3.1. Sampling

From 1986 to 1994, the same oysters were used for organic contaminant analysis and gonadal analysis at all Gulf coast sites (Powell *et al.*, 1993). The use of the same animals for gonadal analysis and for the analysis of contaminant body burden potentially biases the latter analyses because digestive gland tissue and gonadal tissue, which contribute a disproportionate portion of the tissue taken for histological analysis, may contain a higher than average body burden of certain contaminants. Sericano *et al.* (1993) showed that this source of error resulted in an underestimation of true body burden by no more than 10% under most conditions for the large oysters used in the Mussel Watch Program, if the slice of tissue removed was limited to a 3 to 5 mm section. The potential error would be much larger for the smaller mytilids and zebra mussels. In order to avoid this error, separate samples were obtained for the gonadal analysis portion of the Mussel Watch project for these species (and East coast oysters) in all years, and, beginning in 1995, the same protocol was adopted for the Gulf coast oysters. Thus, the present sampling method requires the assumption in all cases that individuals collected from a common collection area will have experienced similar chemical loading and therefore avoid the error introduced by subsampling the tissue to be analyzed for contaminants.

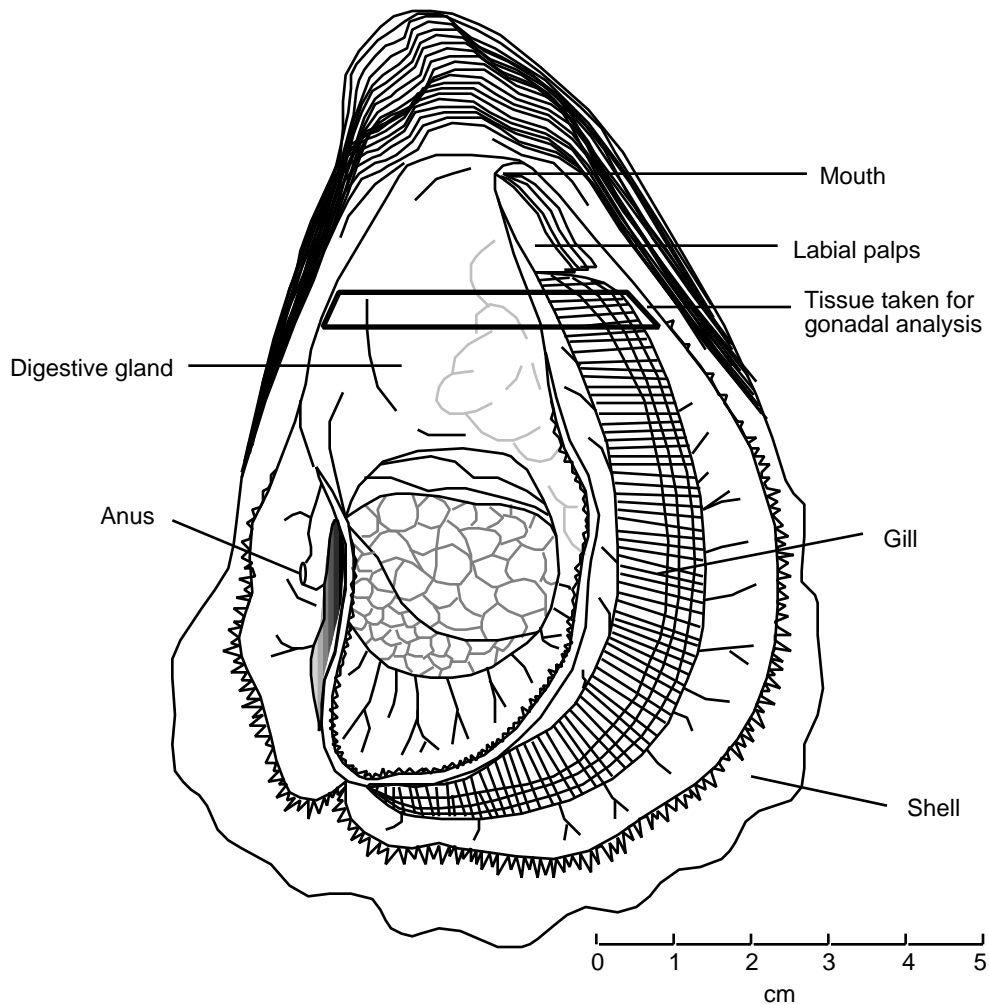


Figure 1. Oyster tissue used for quantifying reproductive stage (adapted from Galtsoff, 1964).

3.2. Tissue preparation

3.2.1. Oyster tissue preparation

Each oyster was opened with an oyster knife by cutting the adductor muscle at its connection with the upper shell. A 3- to 5-mm thick cross-section of tissue was removed from the oyster using a scalpel or scissors (Figure 1). The determination of reproductive stage is based on a histological evaluation of the maturation stage of oyster gonads, which are located around the visceral mass. The tissue section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gill tissue just posterior to the palps. This aspect provides example sections of most oyster tissues for histopathological analysis (Ellis *et al.*, this volume), while also providing a representative cross-section of oyster gonad.

Each section was immediately placed in a tissue cassette and the cassette placed in a jar filled with Davidson's fixative for 48 hr. Bouin's fixative is an adequate alternative in most applications. After 48 hr, the fixative was decanted, 50% ethanol added and the tissues

allowed to stand for 2 hr, during which time the vials are occasionally shaken. After 2 hr, the 50% ethanol solution was replaced with 70% ethanol, and the vials shaken every few hours. After 24 hr, the 70% ethanol was replaced with fresh 70% ethanol for storage until processing.

3.2.2. Mussel tissue preparation

Determination of reproductive stage for mussels was based on a histological evaluation of the maturation stage of mussel gonads, most of which are located in the mantle (Newell, 1989). Ordinary means of removal of mussels from their shells usually resulted in severe damage to the mantle tissue lying next to the shell. Therefore, it was easier to preserve the mussels in their shells. Once preserved, the tissue hardened and become easier to dissect.

The tip of a sharp knife was carefully inserted between the shells at the ventral lip and run dorsally between the shells until the adductor muscle cut so that the shells remain in an open position. Care was taken to cut no further than the adductor muscle to avoid cutting into the digestive gland immediately below the adductor muscle. The mussels were then placed in a wide-mouth jar filled with Davidson's fixative. Because the entire animal was being preserved, the specimens were usually left in fixative for at least a week to ensure preservation of all tissues. After this time, the fixative was decanted, 50% ethanol added, and the tissue allowed to stand for 48 hr, during which time the jar was shaken occasionally. The solution was then poured off, 70% ethanol added and the mixture allowed to stand for 72 hr, after which the 70% ethanol can be replaced with fresh 70% ethanol for storage until processing.

To excise the mussel meat from its shell, a scalpel was carefully run between the mantle and the lip of the shell, detaching the mantle from the shell. This was done around the circumference of one of the shells and the scalpel then used to carefully separate the meat from the shell. This procedure was repeated for the other shell. A 3 - 5 mm thick cross-section, obtained such that the dorsal-ventral aspect passes through the digestive gland at an angle across the body slightly toward the forward edge, was then removed using a scalpel or scissors. Each section was placed in a tissue cassette and processed immediately after dissection.

If the mantle tissue was damaged during the shell-opening procedure, the specimen was discarded because the wound could result in the loss of gametic material, and lead to an erroneous evaluation. Some damage to the gills and viscera was acceptable, but care should be taken to minimize that damage, so the tissues may be used for histopathological evaluation (Ellis et al., this volume) as well as determination of reproductive stage.

3.2.3. Zebra mussel preparation

Most of the gonad of a zebra mussel was concentrated within the visceral mass (Borcherding, 1990). Due to their small size however, dissection of living tissue without destroying the gonads is difficult. Therefore, zebra mussels were preserved whole, in their shells. The animals were placed whole, without bothering to cut the adductor muscle, into a jar with Davidson's fixative and left in fixative for one week to allow adequate time for tissue fixation. After this time, 20 to 30 mL of acetic acid was added to enhance decalcification of the shell. The shell was properly decalcified when examination by hand showed that the shell is no longer hard.

After decalcification was complete, the Davidson's was replaced with ethanol according to the procedure followed for mussels. Once the zebra mussels were placed in the final 70% ethanol solution, they were ready for embedding. Prior to embedding, a small tear was made in a section of the mantle away from the visceral mass to prevent air bubbles from forming within

the body cavity which limits proper infiltration of the paraffin. Byssal threads must also be cut away from the byssal gland to prevent problems when sectioning the tissue.

4. SLIDE PREPARATION

4.1. Tissue embedding

Individual tissue samples were prepared for embedding in paraffin using an established dehydration protocol (Table 1). The solutions used for dehydration, clearing, and infiltration were changed frequently to maintain solution purity.

The tissue embedding sequence used an automated tissue processor which automatically processes tissue through the dehydration-clearing series and into paraffin. Embedding can also be done manually by moving the tissues through the sequence. The paraffin was melted in an oven set at no more than 2 °C above the melting temperature and kept at temperatures no higher than 2 °C above melting during the infiltration process. Newly-melted paraffin should always be used in the final infiltration and embedding steps.

After the tissues were infiltrated with paraffin (Table 1), they were transferred to a paraffin pot and placed under a vacuum for 30 min. The vacuum oven was set to maintain the paraffin at the same carefully-controlled temperature. The tissues were then transferred to stainless steel molds filled with newly-melted paraffin. Oysters and mussels were oriented cross-sectional face down for sectioning, and a plastic mold embedding ring placed on top. Unlike the mytilids and the oysters, zebra mussels were embedded whole. Zebra mussels were placed on their side and, when the paraffin was added, care was taken to ensure that no air bubbles formed within the body cavity. The plastic backing was filled with paraffin and the mold moved to a cold plate. As the tissue/paraffin cooled and hardened, the paraffin shrinks. Care must be taken to use sufficient paraffin to cover the tissue after it cools and hardens. Occasionally, more paraffin must be added. The mold was left on the cold plate until the tissue-paraffin block was removed. The tissue-paraffin block was then placed in a freezer over night before sectioning. When sectioning a number of blocks, the remaining blocks were placed in a tray of ice to keep them cold until sectioned.

Table 1. Tissue embedding sequence*.

Dehydration		Clearing	
Tissue Dry	60 min	Tissue Clear	60 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min		
Tissue Dry	120 min	Infiltration	
Tissue Dry	120 min	paraffin	120 min
Tissue Dry	60 min	paraffin	120 min
		paraffin in vacuum oven	30 min

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

4.2. Tissue sectioning

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

4.3. Tissue staining

The staining procedure was Barber's adaptation of the trichrome stain of Masson (1928) as modified by Gurr (1956). Barber's modifications include the addition of aniline blue to the fast green working solution, substitution of chromotrope 2A/orange G for Ponceau de Xylidene, and the addition of phosphotungstic acid prior to the orange G/chromotrope stain; the procedure is now a pentachrome technique. The addition of these stains yields better differentiation of tissue types and mucins. Times required for each step are flexible in both the staining procedures discussed here and in the previous embedding protocol. Different tissue types may require different times. All tissue baths, especially xylene and ethanol ones, should be changed frequently. Slides should not be allowed to dry during transfers. Solutions to common embedding, sectioning, and staining problems are discussed in Preece (1972) and most other manuals of histological technique.

Sections were deparaffinized and hydrated using a xylene-ethanol series (Table 2). Following hydration, slides were stained in the pentachrome series, dehydrated in a series of acetic acid dips followed by acetone, cleared in xylene and mounted in Permount (Table 2).

5. ANALYSIS

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

For mytilids and zebra mussels, a mean Gonadal Index can be determined from the sum of the individual stage numbers. The index can vary from zero, if the entire population was spent or resting, to five, if the population was fully ripe.

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

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

Table 2. Tissue staining sequence.

Deparaffinization		Phosphotungstic acid	2 min
xylene	5 min	Orange G/Chromotrope stain	1.5 min
xylene	5 min	Running tap water*	5 min
xylene	5 min	Phosphomolybdic acid	2 min
100% ethanol	3 min	Fast Green/Aniline Blue stain	3 min
100% ethanol	2 min		
Hydration		Dehydration	
95% ethanol	2 min	1% acetic acid	20-25 dips
10% ethanol	2 min	1% acetic acid	20-25 dips
distilled water	2 min	1% acetic acid	20-25 dips
Staining series		1% acid acetone	20-25 dips
Ferric Alum Mordant	6 min	1% acid acetone	20-25 dips
Groat/Weigert Hematoxylin	30-45 min	Clearing	
Running tap water	3 min	xylene	5 min
Basic ethanol	15 sec	xylene	5 min
Running tap water	2 min	Mounting	
Acid Fuchsin stain	1.5 min	Mounting in Permount	24 hr to dry
Running tap water*	5 min		

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

Table 3. Oyster development stages.

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

Table 4. Mussel and zebra mussel development stages.

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

6. CONCLUSIONS

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

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

more or less simultaneously with advancement in reproductive stage and because gamete maturation occurs more or less evenly throughout the entire gonad.

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Perkinsus marinus Assay

E. N. Powell and M. S. Ellis
Haskin Shellfish Research Laboratory
Rutgers University
6959 Miller Ave.
Port Norris, NJ
USA

ABSTRACT

This document describes the procedures followed for the assay of *Perkinsus marinus* prevalence and infection intensity in oyster specimens collected on the East and Gulf Coasts of the US and in Puerto Rico by Rutgers University for the National Oceanic and Atmospheric Administration's National Status and Trends Program Mussel Watch Project.

1. INTRODUCTION

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

2. EQUIPMENT AND SUPPLIES

2.1. Reagents

2.1.1. Chemicals

Chloramphenicol (chloromycetin), (C₁₁H₁₂N₂O₅) [56-75-7], 1 g lyophilized powder vials; only available from veterinary supply houses.

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

Iodine, (I₂) [7553-56-2], 13380, purity 99%. Sigma Chemical Company, St. Louis, MO.

Nystatin (mycostatin) (C₄₇H₇₅NO₁₇) [1400-61-9], 500,000 unit lyophilized powder, N3503. Sigma Chemical Company, St. Louis, MO.

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

Potassium phosphate, monobasic, (KH₂PO₄) [7778-77-0], purity 99+%, P5379. Sigma Chemical Company, St. Louis, MO.

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

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

2.1.2. Solutions

2.1.2.1. Thioglycollate medium preparation

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

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

2.1.2.2. Antibiotic solution

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

2.1.2.3. Lugol's iodine solution

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

2.1.2.4. PBS(II)

Enough NaCl and KH_2PO_4 are dissolved in distilled water to produce a 0.15 M NaCl and 0.01 M KH_2PO_4 solution. The pH is adjusted to 7.4 with NaOH or HCl as required.

2.2. Equipment

Autoclave

Automatic pipette or repipettes, 50 μL and 200 mL capacity, 13-707-30. Fisher Scientific, Pittsburgh, PA.

Brinkmann polytron tissue homogenizer, Brinkmann Instrument Co., Westbury, NY.

Heater/stirrer, Corning, 11-495-52C. Fisher Scientific, Pittsburgh, PA.

Microscope, Wild dissecting. Fisher Equivalent Stereomaster Zoom, 12598-14. Fisher Scientific, Pittsburgh, PA.

Repipettes, 10-50 mL adjustable volume, 13-688-70. Fisher Scientific, Pittsburgh, PA.

Slide warmer, 12-594. Fisher Scientific, Pittsburgh, PA.

Vortex mixer, Vortex-Genie 2 mixer, 12-812. Fisher Scientific, Pittsburgh, PA.

3. TISSUE COLLECTION

Twelve large oysters were collected from each field location. Analysis of twelve oysters was sufficient to determine prevalence and infection intensity. The Mussel Watch sampling protocol includes obtaining specimens from three separate locations at each primary site. Comparisons of replicate samples have consistently shown significant differences no more frequently than expected by chance (Craig *et al.*, 1989; Wilson *et al.*, 1990). Thus four oysters were taken from each of the three sampling locations per site to obtain the twelve oysters for analysis. Oysters were immediately put on ice, but not allowed to be submerged in fresh water by allowing meltwater to drain continuously from the containers, and returned to the laboratory where they were assayed within 24 hr of collection.

Each oyster was opened with an oyster knife. Maximum anterior-posterior length, as defined by Morales-Alamo and Mann (1989), was recorded by visual inspection. Using sterile dissecting scissors and forceps, a small piece of approximately 1 cm² of mantle tissue was excised from the lower valve just posterior to the palps. The tissue was placed in a culture tube containing the thioglycollate medium to which 50 µL of chloramphenicol-nystatin antibiotic solution was added.*

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

4. TISSUE ANALYSIS

4.1. Semiquantitative method

After the incubation period, the oyster tissue was removed from the culture tube using a sterile needle and placed on a microscope slide. The tissue sample was teased apart using sterile needles to assure even staining with Lugol's iodine solution. The tissue was flooded with Lugol's solution using a Pasteur pipette, covered with a cover slip and examined microscopically. *P. marinus* hyphospores appear as black spheres of 10 to 200 µm diameter when viewed through a dissecting microscope. Usually, hyphospores exceed 40 µm in size, however, occasionally hyphospores develop without enlargement. An infection intensity was assigned to each tissue sample based on the number or coverage of enlarged *P. marinus* hyphospores observed in the tissue using the scale in Table 1. Note that the scale ranks tissue by the number or coverage of hyphospores after enlargement. Accordingly, those tissues where enlargement occasionally fails to occur must be ranked on Mackin's scale as if the cells were enlarged.

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

Table 1. Semiquantitative scale of infection intensity for *Perkinsus marinus* [adapted from Mackin (1962) by Craig *et al.* (1989)].

Letter designation	Infection intensity	Numerical value	Description
N	Negative	0.00	No hypnospores present
VL	Very light	0.33	1-10 hypnospores
L-	Light	0.67	11-74 hypnospores
L		1.00	75-125 hypnospores
L+		1.33	>125 hypnospores but much less than 25% of tissue is hypnospores
LM-	Light/moderate	1.67	<25% of tissue is hypnospores
LM		2.00	25% of tissue is hypnospores
LM+		2.33	>25% but much less than 50% of tissue is hypnospores
M-	Moderate	2.67	>25% but <50% of tissue is hypnospores
M		3.00	50% of tissue is hypnospores
M+		3.33	>50% but much less than 75% of tissue is hypnospores
MH-	Moderately heavy	3.67	>50% but <75% of tissue is hypnospores
MH		4.00	75% of tissue is hypnospores
MH+		4.33	>75% but much less than 100% of tissue is hypnospores
H-	Heavy	4.67	>75% of tissue is hypnospores but some oyster tissue is still visible
H		5.00	Nearly 100% of tissue is hypnospores

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

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

4.2. Quantitative method

The assessment of infection intensity using a piece of mantle tissue is rapid, inexpensive, and can be done with little tissue loss on animals destined for body burden analysis, permitting a

direct comparison. However, use of a single tissue section introduces three potentially significant inaccuracies.

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

Tissue-to-tissue heterogeneity in infection intensity was typically as large as two levels on Mackin's scale (Table 1) (e.g., from L- to L+).

False negatives were frequently encountered at low infection levels. A lethal infection from a single infective cell required on the order of 22 to 28 doublings of the *P. marinus* population. The first 10 to 12 were likely to be read as negative because cell density was low enough that a piece of mantle tissue examined may easily not contain any cells. This error reduced the value of prevalence as a primary determinant of health because the method used was particularly poor at identifying truly uninfected oysters.

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

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

5. CALCULATIONS

Data were reported as prevalence (percent infected) and mean or median infection intensity calculated using the semiquantitative scale in Table 1. The calculation of median infection was frequently desirable because the semiquantitative scale used was truncated at both extremes and infection intensity in a sampled population was often not normally distributed. In particular, a few relatively heavily infected individuals were frequently encountered in populations characterized by low overall infection intensities, and uninfected individuals could be collected in most populations at certain times of the year because transmission rates are sufficiently slow. If the quantitative scale was desired, data were reported as number of *P. marinus* cells per gram wet weight of oyster tissue and then converted into the semiquantitative equivalent using the formula:

Fisher and Oliver (1996) have recently described a modification of this method.

number of hypospores = 1409.9 ($10^{0.64296x}$)

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

6. CONCLUSIONS

The described technique provides a quantitative or semi-quantitative method to determine the presence and infection intensity of *Perkinsus marinus* in oysters. South of Long Island Sound, *Perkinsus marinus* analysis is a good indicator of population health because *P. marinus* is a principal cause of mortality in most populations. The thioglycollate method is considerable more accurate than examination of tissue sections in routine histopathological analysis, and thus should be a standard component of histopathological analysis for determining population health.

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