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National Coastal Condition Assessment

Laboratory Methods Manual

November 2010

NOTICE

The goal of the National Coastal Condition Assessment (NCCA) is to provide a comprehensive assessment of the Nation's freshwater, marine shoreline and estuarine waters. The complete documentation of overall project management, design, methods, and standards is contained in four companion documents, including:

National Coastal Condition Assessment: Quality Assurance Project Plan (EPA 841-R-09-004)
National Coastal Condition Assessment: Field Operations Manual (EPA 841-R-09-003)
National Coastal Condition Assessment: Laboratory Methods Manual (EPA 841-R-09-002)
National Coastal Condition Assessment: Site Evaluation Guidelines (EPA-841-R-09-00X)

The suggested citation for this document is:

> USEPA. 2009. National Coastal Condition Assessment: Laboratory Methods Manual. EPA 841-R-09-002. U.S. Environmental Protection Agency, Office of Water and Office of Research and Development, Washington, DC.

This document (*Laboratory Methods Manual*) contains information on the methods for analyses of the samples to be collected during the survey, quality assurance objectives, sample handling, and data reporting. These methods are based on established methods and/or guidelines developed and followed in the Agency's Environmental Monitoring and Assessment program. Methods described in this document are to be used specifically in work relating to the NCCA. The method outlined in Section 3.0 of this Manual entitled, *Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay*, is unpublished and provided as DRAFT. Copies of this draft method are available upon request. All published references are available from the National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161. Mention of trade names or commercial products in this document does not constitute endorsement or recommendation for use by EPA. Details on specific methods for sampling and sample processing and handling prior to sending to the laboratory can be found in the companion document *Field Operations Manual* listed above.

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1.0 INTRODUCTION AND GENERAL INSTRUCTIONS

1.1 INTRODUCTION

This manual describes methods for analyses of the samples collected during the 2010 National Coastal Condition Assessment (NCCA), including quality assurance objectives, sample handling, and data reporting. The NCCA is a statistical survey of the condition of our nation's coastal waters, estuaries, and shorelines. Probability-based surveys are used to determine the state of populations or resources of interest using a representative sample of relatively few members or sites. This random selection design allows data from the subset of sampled sites to be applies to the larger target populations (*i.e*., our coastal waters) and assessments with known confidence boundaries to be made.

Along with EPA, states, tribes, and other partners will participate in the survey every five years as part of the National Aquatic Resource Surveys (NARS) Program. The goals of the NARS are threefold:

- Address key questions about the quality of the nation's coasts
    - What percentage of US coastlines is in good condition with respect to ecological integrity, recreational safety, and other key parameters?
    - What is the relative importance of identified stressors such as nutrients, metals, *etc*.?
- Promote collaboration and build strong state/tribal capacity for monitoring programs.
- Provide a nationally consistent data set to examine water quality and develop baseline and trend information to evaluate the effectiveness of water protection/remediation programs effectiveness.

With input from the states and other partners, EPA used an unequal probability design to select 682 marine sites along the coasts of the continental United States and 225 freshwater sites from the nearshore regions of the Great Lakes. Field crews will collect a variety of measurements and samples from these predetermined sampling areas which have been assigned longitude and latitiude coordinates. Additional sites were also identified for Puerto Rico, Hawaii, Alaska and the Pacific Territory islands to provide an equivalent design for these coastal areas if these states and territories choose to sample them.

1.2 LIST OF INDICATORS

Indicators for the 2010 survey are presented in Table 1.1. They will remain the same as those used previously for the National Coastal Condition Report with a few modifications. The most prominent change in the 2010 survey is the inclusion of coasts along the Great Lakes; therefore both sample collection methods and laboratory methods will reflect freshwater and saltwater matrices.

Based on recommendations from a state workshop held in 2008, the NCCA workgroup decided on a few improvements to the original indicators. The changes include: 1) measuring *Enterococcus* levels as a human health indicator; 2) requiring the measurement of photosynthetically active radiation (PAR) using instrumentation to help standardize the water clarity indicator; 3) for sediment toxicity testing, labs will use *Leptochirus* instead of *Ampelisca*

sp. for marine sites, and will use *Hyalella* for freshwater sites; 4) tissue studies will be conducted using whole fish, and 5) fish community structure, Total Suspended Solids (TSS), and PAHs in fish tissue will no longer be included.

Table 1.1. National Coastal Condition Assessment Indicators

Measure/Indicator		Specific data type	Assessment outcome
Water Quality	Dissolved oxygen	Observable on-site	Hypoxia/anoxia
	pH Temperature Depth Conductivity (freshwater) Salinity (marine)	Observable on-site	Water column characterization
	Secchi/light measurements PAR	Observable on-site	Societal value and ecosystem production
	Nutrients	Filtered surface sample for dissolved inorganic NO_2 NO_3 NH_4 ,PO_4; Unfiltered surface sample for Total N and P	Nutrient enrichment
	Chlorophyll	chlorophyll *a*	
Sediment Quality	Grain size	Silt/Clay content	Influencing factor for extent and severity for contamination
	Total organic carbon	Sediment total organic carbon	Influencing factor for extent and severity for contamination
	Sediment chemistry	15 metals 25 PAHs 20 PCBs 14 pesticides 6 DDT metabolites	Potential biological response to sediment contamination
	Sediment toxicity	10-day static bioassay with *Leptochirus* or *Hyalella*	Biological response to sediment exposure
Biological Quality	Tissue Contaminants	13 metals (no SB or MN) 20 PCBs 14 pesticides 6 DDT metabolites	Environmentally available contaminant exposure
	Benthic community structure	One sediment grab target for benthic abundance enumeration and species identification	Biological response to site conditions

1.3 TRAINING AND QUALIFICATIONS

These methods should be used only by trained, qualified laboratory technicians experienced in the theory and application of aquatic resource methodology. A minimum of 2 years experience in the particular laboratory technique and associated analytical equipment is required.

1.4 SAFETY

This manual describes procedures which may involve hazardous materials, operations, and equipment, and it does not purport to address the associated safety issues. While some safety considerations are included, it is beyond the scope of the manual to encompass all safety measures necessary to conduct each test.

Development and maintenance of an effective health and safety program in the laboratory requires an ongoing commitment by laboratory management. It is the laboratory's responsibility to maintain a safe work environment and a current awareness file of OSHA and other applicable regulations regarding the safe handling of the samples, chemicals and machinery. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved with these analyses.

The collection and handling of sediment samples could subject personal to health and safety risks. Contaminants in field-collected sediments may include carcinogens, mutagens, and other potentially toxic compounds. Inasmuch as sediment testing is often begun before chemical analysis can be completed, worker contact should be kept minimal. Personnel collecting sediment samples and conducting tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through the skin, and asphyxiation due to lack of oxygen or the presence of noxious gases.

1.5 PROTOCOLS

Participating laboratories must be prepared to receive all or a portion of 1200 samples. Prior to receiving samples, the laboratory must contact Marlys Cappaert at the Information Management Center by phone (541-754-4467) or e-mail (cappaert.marlys@epa.gov) to arrange access to EPA's sample tracking system. All samples must be logged into EPA's tracking system by the contractor upon receipt. Samples will be tracked according to each unique site_id and sample number. The laboratory must adhere to strict sample tracking procedures throughout the laboratory analysis phase. If expected samples do not arrive, the laboratory must immediately contact Marlys Cappaert.

All cooperating laboratories must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets and/or data reporting spreadsheets are compatible with the electronic deliverables the lab will need to submit. For taxonomic analyses, the laboratory must use a standard, agreed upon key for identification and all organisms are to be identified to the lowest correct taxonomic level, usually genus or species. Any questions regarding the standard operating procedure must be directed to the appropriate Project Officer for the particular contract laboratory.

Weekly tallies of samples received and samples processed are required via e-mail. More detailed progress reports summarizing work performed and financial expenditures are required monthly.

1.6 QUALITY CONTROL AND LABORATORY AUDITS

Laboratories participating in the NCCA must adhere to and document the quality control (QC) elements prescribed for each analytical method. QC requirements routinely associated with analytical chemistry measurements include: calibration standards, reagent blanks, duplicates, check samples (spike/recovery), Standard Reference Materials (SRMs), and continuing calibration curve check samples. Other types of laboratory procedures or tests (*e.g*., grain size determination and toxicity testing) will also be conducted for the NCCA; the QC elements appropriate for each of these methods are specified in this manual.

To ensure that a laboratory is technically competent to perform a particular analysis or procedure, the NCCA will require that laboratory to conduct an initial demonstration of capability prior to the laboratory being authorized to analyze NCCA field samples. For most analytical processes, the demonstration will include documentation of the laboratory's calculated Method Detection Limits (MDLs) for each analyte of interest and a "blind" analysis of an appropriate performance evaluation (PE) sample (*e.g*., an SRM). For other types of laboratory determinations, appropriate PE exercises will be described.

QC results provide the analyst with an immediate indication to the overall validity of the analytical process, affording the opportunity to make necessary adjustments to bring the analysis into control. Post-analysis, documented QC results enable data users to define the level of quality and reliability of that data.

Quality assurance procedures and practices will include an independent laboratory audit. Each laboratory is required to maintain at its facility performance records, raw data, and preserved samples for a minimum of two years from the date the final results are submitted to EPA.

2.0 WATER QUALITY

2.1 PERFORMANCE-BASED METHODOLOGIES

Suggested analytical methods for water quality indicators are described in section 2.2 - 2.7 of this manual. However, some laboratories participating in the survey may choose to employ other analytical methods. Laboratories engaged by EPA or the State may use a different analytical method as long as the lab is able to achieve the same performance requirements as the standard methods. Performance data must be submitted to EPA prior to initiating any analyses. Methods performance requirements for this program identify detection limit, precision and accuracy objectives for each indicator. Method performance requirements for water chemistry and chlorophyll *a* sample analysis are shown in Table 2.1

Table 2.1. Laboratory method performance requirements for water chemistry and chlorophyll a sample analysis

Analyte	Units	Potential Range of Samples[1]	Method Detection Limit Objective[2]	Transition Value[3]	Precision Objective[4]	Accuracy Objective[5]
Ammonia (NH_3)	mg N/L	0 to 17	0.01 marine (0.7 µeq/L) 0.02 freshwater	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Nitrate-Nitrite (NO_3-NO_2)	mg N/L	0 to 360 (as nitrate)	0.01 marine 0.02 freshwater	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Nitrogen (TN)	mg/L	0.1 to 90	0.01	0.10	± 0.01 or ±10%	± 0.01 or ±10%
Total Phosphorous (TP) and ortho-Phosphate	µg P/L	0 to 22,000 (as TP)	2.0	20.0	± 2 or ±10%	± 2 or ±10%
Nitrate (NO_3)	mg N/L	0. to 360	0.01 marine (10.1 µeq/L) 0.03 freshwater	0.1	± 0.01 or ±5%	± 0.01 or ±5%
Chlorophyll-*a*	µg/L in extract	0.7 to 11,000	1.5	15	± 1.5 or ±10%	± 1.5 or ±10%

[1] Estimated from samples analyzed at the WED-Corvallis laboratory between 1999 and 2005
[2] The method detection limit is determined as a one-sided 99% confidence interval from repeated measurements of a low-level standard across several calibration curves.
[3] Value for which absolute (lower concentrations) vs. relative (higher concentrations) objectives for precision and accuracy are used.
[4] For duplicate samples, precision is estimated as the pooled standard deviation (calculated as the root-mean square) of all samples at the lower concentration range, and as the pooled percent relative standard deviation of all samples at the higher concentration range. For standard samples, precision is estimated as the standard deviation of repeated measurements across batches at the lower concentration range, and as percent relative standard deviation of repeated measurements across batches at the higher concentration range.
[5] Accuracy is estimated as the difference between the measured (across batches) and target values of performance evaluation and/or internal reference samples at the lower concentration range, and as the percent difference at the higher concentration range.

2.2 DISSOLVED INORGANIC NITROGEN – AMMONIA

2.2.1 Saltwater

2.2.1.1 Scope and Application

This method may be used for estuarine and coastal waters. The method is based upon the indophenol reaction adapted to automated gas-segmented continuous flow analysis. A statistically determined method detection limit of 0.3 µg N/L has been determined from seawater of four different salinities. The method is linear to 4.0 mg N/L using a Flow Solution System.

2.2.1.2 Method Summary

The automated gas segmented continuous flow colorimetric method is used for the analysis of ammonia concentration. Ammonia in solution reacts with alkaline phenol and NaDTT at 60°C to form indophenol blue in the presence of sodium nitroferricyanide as a catalyst. The absorbance of indophenol blue at 640 nm is linearly proportional to the concentration of ammonia in the sample. A small systematic negative error caused by differences in the refractive index of seawater and reagent water, and a positive error caused by the matrix effect (the change in the colorimetric response of ammonia due to the change in the composition of the solution) on the color formation, may be corrected for during data processing.

2.2.1.3 Interferences

1. Hydrogen sulfide at concentrations greater than 2 mg S/L can negatively interfere with ammonia analysis. Hydrogen sulfide in samples should be removed by acidification with sulfuric acid to a pH of about 3, then stripping with gaseous nitrogen.
2. The addition of sodium citrate and EDTA complexing reagent eliminates the precipitation of calcium and magnesium when calcium and magnesium in seawater samples mix with high pH (about 13) reagent solution.
3. Sample turbidity is eliminated by filtration or centrifugation.
4. As noted, refractive index and salt error interferences occur when sampler wash solution and calibration standards are not matched with samples in salinity. For low concentration samples (<20 µg N/L), low nutrient seawater with salinity matched to samples, sampler wash solutions and calibration standards is recommended to eliminate matrix interferences.

2.2.1.4 Safety

Chloroform should be used it in a properly ventilated area, such as in a fume hood.

2.2.1.5 Equipment and Supplies

1. Gas Segmented Continuous Flow Autoanalyzer
    - Automatic sampler
    - Analytical cartridge with reaction coils and heater
    - Proportioning pump
    - Nitrogen gas (high-purity grade, 99.99%)
    - Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 640 nm interference filter (max. 2 nm bandwidth)
    - Strip chart recorder or computer based data acquisition system

2. Glassware
   - Pipettes
   - 60-ml glass or high density polyethylene sample bottles, glass volumetric flasks and glass sample tubes.

Ammonia is known for its high surface reactivity. When working at low levels (<20 µg N/L), additional cleansing of labware is mandatory. Plastic bottles and glass volumetric flasks should be cleaned in an ultrasonic bath with reagent water for 60 minutes. Bottles and sample tubes made of glass should be cleaned by boiling in reagent water.

3. Supplies
   - Analytical balance with accuracy to 0.1 mg
   - Drying oven
   - Desiccator
   - Membrane filters (0.45 µm nominal pore size)
   - Syringes with syringe filters
   - Centrifuge
   - Ultrasonic water bath cleaner

2.2.1.6 Reagents and Standards

2.2.1.6.1 Stock Reagent Solutions

1. Complexing Reagent. Dissolve 140 g of sodium citrate dehydrate ($Na_3C_6H_5O_7 \bullet 2H_2O$), 5 g of sodium hydroxide (NaOH) and 10 g of disodium EDTA ($Na_2C_{10}H_{14}O_8N_2 \bullet 2H_2O$) in approximately 800 ml reagent water, mix and dilute to 1 L with reagent water. The pH of this solution is 13. This solution is stable for 2 months.
2. Stock Ammonium sulfate Solution (100 mg N/L). Transfer 0.4721 g of pre-dried (105°C for 2 hours) ammonium sulfate $(NH_4)_2SO_4$ to a 1000 mL volumetric flask containing approximately 800 ml of reagent water and dissolve. Add a few drops of chloroform as a preservative. Dilute the solution to 1 L with reagent water. Store the solution in a glass bottle at 4°C. This solution is stable for 2 months.
3. Low Nutrient Sea Water. Obtain natural or commercially available low nutrient seawater from surface water (salinity 36‰, < 7 µg N/L) and filter it through 0.3 micron pore size glass fiber filters. Do not use artificial seawater.

2.2.1.6.2 Working Reagents

1. Brij-35 Start-up solution. Brij-35 is a trade name for polyoxyehtylene(23) lauryl ether ($C_{12}H_{25}(OCH_2CH_2)_{23}OH$) and is commercially available. Add 2 mL of Brij-35 surfactant to 1000 mL reagent water and mix gently.
2. Working Complexing Reagent. Add 1 mL Brij-35 to 200 mL of stock complexing reagent. Prepare daily. This volume of solution is sufficient for an 8-hour run.
3. Sodium Nitroferricyanide Solution. Dissolve 0.25 g of sodium nitroferricyanide ($Na_2Fe(CN)_5NO \bullet 2H_2O$) in 400 mL reagent water, dilute to 500mL. Store in an amber bottle at room temperature.
4. Phenol Solution. Dissolve 1.8 g solid phenol (C_6H_5OH) and 1.5 g of sodium hydroxide in 100 mL of reagent water. Prepare fresh daily.
5. NaDTT Solution. Dissolve 0.5 g of sodium hydroxide and 0.2 g dichloroisocyanuric acid sodium salt (NaDTT, $NaC_3Cl_2N_3O_3$) in 100 mL reagent water. Prepare fresh daily.

6. Colored SYNC Peak Solution. Add 50 µL of blue food coloring to 1000 mL reagent water and mix. Further dilute to obtain a peak of between 25 to 100 percent full scale according to the AUFS setting used for refractive index measurement.

7. Primary Dilution Standard Solution (5 mg N/L). Prepare by diluting 5.0 mL stock standard solution to 100 mL with reagent water. Prepare fresh daily.

   Note. *This solution should be prepared as an intermediate concentration appropriate for further dilution in preparing calibration solutions. Therefore the concentration must be adjusted according to the desired calibration concentration range.*

8. Calibration Standards. Prepare a series of calibration standards by diluting suitable volumes of a primary dilution standard to 100 mL with reagent water or low nutrient seawater. The concentration range should bracket the expected concentrations of samples and not span more than two orders of magnitude. At least five calibration standards with equal concentration increments should be used to construct the calibration curve.

   Note. *When working with samples of a narrow range of salinities (± 2‰) or samples containing low ammonia concentrations (< 20 µg N/L), it is recommended that the calibration solutions be prepared in low nutrient seawater diluted to the salinity of samples, and the sampler wash solution also be low nutrient seawater diluted to the same salinity. If this procedure is employed, it is not necessary to perform the matrix effect and refractive index corrections outlined in sections 2.1.10.1 and 2.1.10.2. When analyzing samples of moderate or high ammonia concentrations (> 20 µg N/L) and of varying salinities, the calibration standard solutions and sampler wash solution can be prepared with reagent water. The corrections for matrix effect and refractive index should be subsequently applied (sections 2.1.10.1 and 2.1.10.2).*

9. Saline Ammonia Standards. If the calibration standards are not prepared to match sample salinity, then saline ammonia standards must be prepared in a series of salinities in order to quantify the matrix effect. The following dilutions in 100 mL reagent water are recommended:

Salinity (‰)	Low nutrient seawater (mL)	Conc. primary dilution standard (mL)	mg N/L
0	0	2	0.10
9	25	2	0.10
18	50	2	0.10
27	75	2	0.10
35	98	2	0.10

2.2.1.7 Quality Control

Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements consist of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples.

2.2.1.7.1 Initial Demonstration of Performance

1. The method detection limit (MDL) must be established for the method analyte using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots of water which have been processed through the entire analytical method. Calculate the MDL as:

MDL = (t)(S)

where,

S = the standard deviation of the replicate analysis

t = t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

2. The linear dynamic range (LDR) must be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (absorbance units full scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to the samples to avoid the necessity to correct for salt error or refractive index. Normalize responses by multiplying the response by the absorbance units full scale output range setting. Perform the linear regression of normalized response vs. concentration, and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response (R) of a standard no longer yields a calculated concentration (C_C) that is within 100 ± 10% of known concentration (C), where

$$C_C = (R-b)/m$$

This concentration defines the upper limit of the LDR. If samples have a concentration that is ≥90% of the upper limit of the LDR, they must be diluted and reanalyzed.

2.2.1.7.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB). The lab should analyze at least one LRB with each set of samples. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be reprepared and analyzed after the source of contamination has been corrected and acceptable LRB values have been obtained.
2. Laboratory Fortified Blank (LFB). The lab should analyze at lease one LFB with each set of samples. The LFB must be at a concentration within the daily calibration range. The LFB data are used to calculate percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the source of the problem should be identified and resolved before continuing the analysis.
3. The laboratory must use LFB data to assess lab performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

   Upper Control Limit = x + 3S

   Lower Control Limit = x – 3S

   The optional control limits must be equal to or better than the required control limits of 90-11-%. After each 5-10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentration included in the LFB. These data must be kept on file and available for review.

2.2.1.7.3 Assessing Analyte Recovery-Laboratory Fortified Sample Matrix (LFM)

1. The laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one LFM per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater than the MDL.
2. Calculate percent recovery of anlayte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

where,

R = percent recovery

C_S = measured fortified sample addition in mg N/L

C = sample background concentration in mg N/L

S = concentration in mg N/L added to the environmental sample

3. If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be the matrix related and the sample data should be flagged.

2.2.1.8 Calibration and Standardization

1. At least five calibration standards should be prepared fresh daily for system calibration.
2. A calibration curve should be constructed for each sample set by analyzing a series of calibration standard solutions. A sample set should contain no more than 60 samples. For a large number of samples make several sample sets with individual calibration curves.
3. Analyze the calibration standards in duplicate before the actual samples.
4. The calibration curve containing five data points or more that bracketed the concentration of samples should have a correlation coefficient (r) of 0.995 or better and the range should not be greater than two orders of magnitude.
5. Use a high calibration solution followed by two black cups to quantify system carryover. The difference in peak heights between two blank cups is due to the carryover from the high calibration solution. The carryover coefficient (k) is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}}$$

where,

P_{high} = the peak height of the high ammonia standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample

The carryover coefficient (k) should be measured in seven replicates to obtain a statistically significant number. The carryover coefficient should be remeasured with any change in manifold plumbing or upon replacement of pump tubes.

The carryover correction (CO) of a given peak (i) is proportional to the peak height of the preceding sample P_{i-1}.

$$CO = k \times P_{i-1}$$

To correct a given peak height reading, (P_i), subtract the carryover correction.

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is the corrected peak height.

The correction for carryover should be applied to all the peak heights throughout a run. The carryover coefficient should be less than 5%.

6. Place a high standard solution at the end of each sample run to check for sensitivity drift. Apply sensitivity drift correction to all the samples. The sensitivity drift during a run should be less than 5%.

***Note**. Sensitivity drift correction is available in most data acquisition software supplied with subanalyzers. It is assumed that sensitivity drift is linear with time. An interpolated drift correction factor is calculated for each sample according to the sample position during the run. Multiply the sample peak height by the corresponding sensitivity drift correction factor to obtain the corrected peak height for each sample.*

2.2.1.9 Procedure

1. If samples are stored in a refrigerator, equilibrate to room temperature prior to analysis.
2. Turn on the continuous flow analyzer and data acquisition components and warm up for at least 30 minutes.
3. Set up cartridge and pump tubes as shown in Figure 2.1.

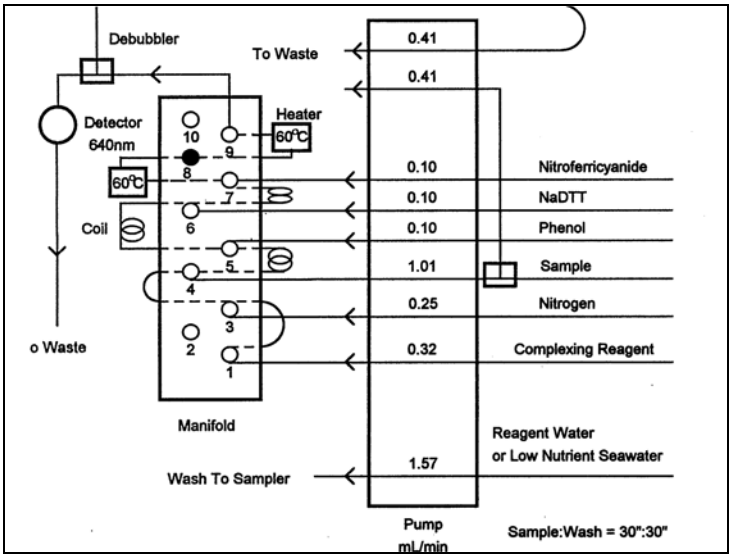

Figure 2.1. Manifold Configuration for Ammonia Analysis

4. Set the spectrophotometer wavelength to 640 nm and turn on lamp.
5. Set the absorbance unit full scale (AUFS) range on the spectrophotometer at the appropriate setting according to the highest concentration of ammonia in the samples. The highest setting appropriate for this method is 0.2 AUFS for 6 mg N/L.
6. Choose an appropriate wash solution for sampler wash. For analysis of samples with a narrow range of salinities (±2‰) or for samples containing low ammonia concentrations (<20 µg N/L), it is recommended that the calibration solutions be prepared in low nutrient seawater diluted to the salinity of samples, and that the sampler wash solution also be low nutrient seawater diluted to the same salinity. For samples with varying salinities and higher ammonia concentrations (>20 µg N/L), it is suggested that the reagent water used for the sampler wash solution and for preparing calibration standards and procedures in sections 2.1.10.1 and 2.1.10.2 be employed.
7. Begin pumping the Brij-35 start-up solution through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has stabilized, reset the baseline.

***Note**. To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the sample line with reagent water, 1 N HCl solution, reagent water, 1 N NaOH solution for a few minutes each at the end of the daily analysis. Make sure to rinse the system well to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulates and filter if necessary.*

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flow cell can often be eliminated by simply attaching a syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohol was found to be effective in removing trapped air.

8. The sampling rate is approximately 60 samples per hour with 30 second of sample time and 30 seconds of wash time. Place a blank after every ten samples.

2.2.1.10 Data Analysis and Calibration

Concentrations of ammonia in samples are calculated from the linear regression, obtained from the standard curve in which the concentration of the calibration standards are entered as the independent variable and their corresponding peak heights are the dependent variable.

2.2.1.10.1 Refractive Index Correction

1. If reagent water is used as the wash solution, the analyst has to quantify the refractive index correction due to the difference in salinity between sample and wash solution. The following procedures are used to measure the relationship between the sample salinity and the refractive index on a particular detector.
2. Analyze a set of ammonia standards in reagent water with color reagent using reagent water as the wash and obtain a linear regression of peak height versus concentration. Then replace reagent water wash solution with low nutrient seawater wash solution.

Note. In ammonia analysis absorbance of the reagent water is higher than that of the low nutrient seawater. When using reagent water as a wash solution, the change in refractive

index causes the absorbance of seawater to become negative. To measure the absorbance due to refractive index change in different salinity samples, low nutrient seawater must be used as the wash solution to bring the baseline down.

3. Replace the phenol solution and NaDTT solution with reagent water. All other reagents remain the same. Replace the synchronization sample with the colored SYNC peak solution.
4. Prepare a series of different salinity samples by diluting the low nutrient seawater. Commence analysis and obtain peak heights for different salinity samples. The peak heights for the refractive index correction must be obtained at the same AUFS range setting and on the same spectrophotometer as the corresponding standards.
5. Using low nutrient seawater as the water wash, a maximum absorbance will be observed for reagent water. No change in refractive index will be observed in the seawater sample. Assuming the absolute absorbance for reagent water (relative to the seawater baseline) is equal to the absorbance for seawater (relative to reagent water baseline), subtract the absorbance of samples of various salinities from that of reagent water. The results are the apparent absorbance due to the change in refractive index between samples of various salinities relative to the reagent water baseline.
6. For each sample of varying salinity, calculate the apparent ammonia concentration due to refractive index from its peak height corrected to the reagent water baseline and the regression equation of ammonia standards obtained with color reagent being pumped through the system. Salinity is entered as the dependent variable. The resulting regression allows the analyst to calculate apparent ammonia concentration due to refractive index when sample salinity is known. Thus, the analyst would not be required to obtain refractive index peak heights for all samples.
7. The magnitude of refractive index correction can be minimized by using a low refractive index flowcell. It is important that the refractive index correction must be calculated for the particular detector. The refractive index must be redetermined whenever a significant change in the design of the flowcell or new matrix is encounter.

   A typical equation is:

   Apparent ammonia (µm N/L) = 0.0134 + 0.0457(S)

   where S is the sample salinity in parts per thousand.

   The apparent ammonia concentration due to refractive index so obtained should then be added to samples of corresponding salinity when reagent water was used as the wash solution for sample analysis.

2.2.1.10.2 Matrix Effect Correction

1. When calculating concentrations of samples of varying salinities from standards and wash solution prepared in reagent water, it is necessary to first correct for refractive index errors, then correct for the change in color development due to the differences in composition between samples and standards (matrix effect). Even where refractive index correction may be small, the correction for matrix effect can be appreciable.
2. Plot the salinity of the saline standards as the independent variable, and the apparent concentration of ammonia (mg N/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for

all saline standards. The resulting regression equation allows the analyst to correct the concentrations of samples of known salinity for the color enhancement due to matrix effect.

3. The matrix effect becomes a single factor independent of sample salinity. A typical equation to correct for matrix effect is:

   Corrected concentration (mg N/L) = Uncorrected concentration / 1.17 (mg N/L)

4. Results of sample analysis should be reported in mg N/L (ppm) or in µg N/L (ppb).

2.2.2 FRESHWATER

2.2.2.1 Scope and Application

1. This method covers the determination of ammonia in freshwater.
2. The applicable range is 0.01-2.0 mg/L NH_3 as N. Higher concentrations can be determined by sample dilution. Approximately 60 samples per hour can be analyzed.
3. This method is described for macro glassware; however, micro distillation equipment may also be used.

2.2.2.2 Summary of Method

1. The sample is buffered at a pH of 9.5 with a borate buffer in order to decrease hydrolysis of cyanates and organic nitrogen compounds, and is distilled into a solution of boric acid. Alkaline phenol and hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonia concentration. The blue color formed is intensified with sodium nitroprusside and measured colorimetrically.
2. Reduced volume versions of this method that use the same reagents and molar ratios are acceptable provided they meet the quality control and performance requirements stated in the method.
3. Limited performance-based method modifications may be acceptable provided they are fully documented and meet or exceed requirements expressed in Section 2.2.2.8 Quality Control.

2.2.2.3 Interferences

1. Cyanate, which may be encountered in certain industrial effluents, will hydrolyze to some extent even at the pH of 9.5 at which distillation is carried out.
2. Residual chorine must be removed by pretreatment of the sample with sodium thiosulfate or other reagents before distillation.
3. Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that bias analyte response.

2.2.2.4 Safety

1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.

2. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data Sheets (MSDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.
3. The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.

   - Sulfuric acid
   - Phenol
   - Sodium nitroprusside

2.2.2.5 Equipment and Supplies

1. Balance - Analytical, capable of accurately weighing to the nearest 0.0001 g.
2. Glassware - Class A volumetric flasks and pipets as required.
3. An all-glass distilling apparatus with an 800-1000 mL flask.
4. Automated continuous flow analysis equipment designed to deliver sample and reagents in the required order and ratios.

   - Sampling device (sampler)
   - Multichannel pump
   - Reaction unit or manifold
   - Colorimetric detector
   - Data recording device

2.2.2.6 Reagents and Standards

1. Reagent water - Ammonia free: Such water is best prepared by passage through an ion exchange column containing a strongly acidic cation exchange resin mixed with a strongly basic anion exchange resin. Regeneration of the column should be carried out according to the manufacturer's instructions.

Note: *All solutions must be made with ammonia-free water.*

2. Boric acid solution (20 g/L): Dissolve 20 g H_3BO_3 in reagent water and dilute to 1 L.
3. Borate buffer: Add 88 mL of 0.1 N NaOH solution to 500 mL of 0.025 M sodium tetraborate solution (5.0 g anhydrous $Na_2B_4O_7$ or 9.5 g $Na_2B_4O_7C_{10}H_2O$ per L) and dilute to 1 L with reagent water.
4. Sodium hydroxide, 1 N: Dissolve 40 g NaOH in reagent water and dilute to 1L.
5. Dechlorinating reagents: A couple of dechlorinating reagents may be used to remove residual chlorine prior to distillation. These include:

   - Sodium thiosulfate: Dissolve 3.5 g $Na_2S_2O_3C_5H_2O$ in reagent water and dilute to 1 L. One mL of this solution will remove 1 mg/L of residual chlorine in 500 mL of sample.
   - Sodium sulfite: Dissolve 0.9 g Na_2SO_3 in reagent water and dilute to 1 L. One mL removes 1 mg/L Cl per 500 mL of sample.

6. Sulfuric acid 5 N: Air scrubber solution. Carefully add 139 mL of conc.sulfuric acid to approximately 500 mL of reagent water. Cool to room temperature and dilute to 1 L with reagent water.
7. Sodium phenolate: Using a 1-L Erlenmeyer flask, dissolve 83 g phenol in 500 mL of distilled water. In small increments, cautiously add with agitation, 32 g of NaOH. Periodically cool flask under water faucet. When cool, dilute to 1 L with reagent water.

8. Sodium hypochlorite solution: Dilute 250 mL of a bleach solution containing 5.25% NaOCl (such as "Clorox") to 500 mL with reagent water. Available chlorine level should approximate 2%-3%. Since "Clorox" is a proprietary product, its formulation is subject to change. The analyst must remain alert to detecting any variation in this product significant to its use in this procedure. Due to the instability of this product, storage over an extended period should be avoided.
9. Disodium ethylenediamine-tetraacetate (EDTA) (5%): Dissolve 50 g of EDTA (disodium salt) and approximately six pellets of NaOH in 1L of reagent water.
10. Sodium nitroprusside (0.05%) Dissolve 0.5 g sodium nitroprusside in 1 L of reagent water.
11. Stock solution: Dissolve 3.819 g of anhydrous ammonium chloride, NH_4Cl, dried at 105°C, in reagent water, and dilute to 1 L. 1.0 mL = 1.0 mg NH_3-N.
12. Standard Solution A: Dilute 10.0 mL of stock solution to 1 L with reagent water. 1.0 mL = 0.01 mg NH_3-N.
13. Standard Solution B: Dilute 10.0 mL of standard solution A to 100.0 mL with reagent water. 1.0 mL = 0.001 mg NH_3-N.

2.2.2.7 Sample Collection, Preservation and Storage

1. Samples should be collected in plastic or glass bottles. All bottles must be thoroughly cleaned and rinsed with reagent water. Volume collected should be sufficient to insure a representative sample, allow for replicate analysis.
2. Samples must be preserved with H_2SO_4 to a pH <2 and cooled to 4°C at time of collection.
3. Samples should be analyzed as soon as possible after collection. If storage is required, preserved samples are maintained at 4°C and may be held for up to 28 days.

2.2.2.8 Quality Control

Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated.

2.2.2.8.1 Initial Demonstration of Performance

1. The initial demonstration of performance is used to characterize instrument performance (determination of LCRs and analysis of QCS) and laboratory performance (determination of MDLs) prior to performing analyses by this method.
2. Linear Calibration Range (LCR) -- The LCR must be determined initially and verified every six months or whenever a significant change in instrument response is observed or expected. The initial demonstration of linearity must use sufficient standards to insure that the resulting curve is linear. The verification of linearity must use a minimum of a blank and three standards. If any verification data exceeds the initial values by ± 10%, linearity must be reestablished. If any portion of the range is shown to be nonlinear, sufficient standards must be used to clearly define the nonlinear portion.

3. Quality Control Sample (QCS) -- When beginning the use of this method (on a quarterly basis or as required to meet data-quality needs) verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS. If the determined concentrations are not within ±10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with on-going analyses.
4. Method Detection Limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit. To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

   MDL = (t) x (S)

   where,

   t = value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates]

   S = standard deviation of the replicate analyses

   MDLs should be determined every six months, when a new operator begins work or whenever there is a significant change in the background or instrument response.

2.2.2.8.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB) - The laboratory must analyze at least one LRB with each batch of samples. Data produced are used to assess contamination from the laboratory environment. Values that exceed the MDL indicate laboratory or reagent contamination should be suspected and corrective actions must be taken before continuing the analysis.
2. Laboratory Fortified Blank (LFB) - The laboratory must analyze at least one LFB with each batch of samples. Calculate accuracy as percent recovery. If the recovery of any analyte falls outside the required control limits of 90-110%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.
3. The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:
   a. Upper Control Limit = x + 3S
   b. Lower Control Limit = x - 3S
4. The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5-10 new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an on-going precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

5. Instrument Performance Check Solution (IPC) -- For all determinations the laboratory must analyze the IPC (a mid-range check standard) and a calibration blank immediately following daily calibration, after every 10th sample (or more frequently, if required) and at the end of the sample run. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subsequent analyses of the IPC solution must verify the calibration is still within ±10%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift, the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

2.2.2.8.3 Assessing Analyte Recovery and Data Quality

1. Laboratory Fortified Sample Matrix (LFM) -- The laboratory must add a known amount of analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis. The analyte concentration must be high enough to be detected above the original sample and should not be less than four times the MDL. The added analyte concentration should be the same as that used in the laboratory fortified blank.
2. Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range 90-110%. Percent recovery may be calculated using the following equation:

$$R = \frac{Cs - C}{s} \times 100$$

where,

R = percent recovery

Cs = fortified sample concentration

C = sample background concentration

s = concentration equivalent of analyte added to sample

3. If the recovery of any analyte falls outside the designated LFM recovery range and the lab performance for that analyte is shown to be in control, the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.
4. Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

2.2.2.9 Calibration and Standardization

1. Prepare a series of at least three standards, covering the desired range, and a blank by diluting suitable volumes of standard solutions to 100 mL with reagent water.
2. Process standards and blanks as described in Section 2.2.2.10, Procedure.
3. Place appropriate standards in the sampler in order of decreasing concentration and perform analysis.

4. Prepare standard curve by plotting instrument response against concentration values. A calibration curve may be fitted to the calibration solutions concentration/response data using computer or calculator based regression curve fitting techniques. Acceptance or control limits should be established using the difference between the measured value of the calibration solution and the "true value" concentration.
5. After the calibration has been established, it must be verified by the analysis of a suitable QCS. If measurements exceed ±10% of the established QCS value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis.
6. Periodic reanalysis of the QCS is recommended as a continuing calibration check.

2.2.2.10 Procedure

1. Preparation of equipment: Add 500 mL of reagent water to an 800 mL Kjeldahl flask. The addition of boiling chips that have been previously treated with dilute NaOH will prevent bumping. Steam out the distillation apparatus until the distillate shows no trace of ammonia.
2. Sample preparation: Remove the residual chorine in the sample by adding the dechlorinating agent equivalent to the chlorine residual. To 400 mL of sample add 1 N NaOH, until the pH is 9.5.
3. Distillation: Transfer the sample, the pH of which has been adjusted to 9.5, to an 800 mL Kjeldahl flask and add 25 mL of the borate buffer. Distill 300 mL at the rate of 6-10 mL/min. into 50 mL of 2% boric acid contained in a 500 mL Erlenmeyer flask.

Note: *The condenser tip or an extension of the condenser tip must extend below the level of the boric acid solution.*

4. Since the intensity of the color used to quantify the concentration is pH dependent, the acid concentration of the wash water and the standard ammonia solutions should approximate that of the samples.
5. Allow analysis system to warm up as required. Feed wash water through sample line.
6. Arrange ammonia standards in sampler in order of decreasing concentration of nitrogen. Complete loading of sampler tray with unknown samples.
7. Switch sample line from reagent water to sampler and begin analysis.

2.2.2.11 Data Analysis and Calculations

1. Prepare a calibration curve by plotting instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve. Multiply answer by appropriate dilution factor.
2. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.
3. Report results in mg NH_3-N/L.

2.3 DISSOLVED INORGANIC NITROGEN – NITRATE-NITRITE

2.3.1 Saltwater

2.3.1.1 Scope and Application

1. This method provides a procedure for determining nitrate and nitrite concentrations in estuarine and saltwater. Nitrate is reduced to nitrite by cadmium and the resulting nitrite determined by formation of an azo dye.
2. A statistically determined method detection limit (MDL) of 0.075 µg N/L has been determined in seawaters of five different salinities. The method is linear to 5.0 mg N/L using a Flow Solution System.
3. Approximately 40 samples per hour can be analyzed.

2.3.1.2 Method Summary

An automated gas segmented continuous flow colorimetric method for the analysis of nitrate concentration is used. Samples are passed through a copper-coated cadmium reduction column. Nitrate in the sample is reduced to nitrite in a buffer solution. The nitrite is then determined by diazotizing with sulfanilamide and coupling with n-1-naphthylethylenediamine dihydrochloride to form a color azo dye. The absorbance measured at 540 nm is linearly proportional to the concentration of nitrite + nitrate in the sample. Nitrate concentrations are obtained by subtracting nitrite values, which have been separately determined without the cadmium reduction procedure, from nitrite + nitrate values.

There is no salt error in this method. The small negative error caused by differences in the refractive index of seawater and reagent water is readily corrected during the data processing.

2.3.1.3 Interferences

1. Hydrogen sulfide at concentrations greater than 0.1 mg S/L can interfere with nitrite analysis by precipitating on the cadmium column. Hydrogen sulfide in samples must be removed by precipitation with cadmium or copper salt.
2. Iron, copper, and other heavy metals at concentrations greater than 1 mg/L alter reduction efficiency of the cadmium column. The addition of EDTA will complex these metal ions.
3. Phosphate at a concentration greater than 0.1 mg/L decreases the reduction efficiency of cadmium. Dilute samples if possible or remove phosphate with ferric hydroxide prior to analysis.
4. Particulates inducing turbidity should be removed by filtration.

2.3.1.4 Equipment and Supplies

1. Gas Segmented Continuous Flow Autoanalyzer
    - Automatic sampler
    - Analytical cartridge with reaction coils and heater
    - Open tubular Cadmium Reactor or laboratory prepared packed copper-coated cadmium reduction column (prepared according to sec. 2.2.5.3 and 2.2.5.4)

- Proportioning pump
- Spectrophotometer equipped with a tungsten lamp (380-800 nm) or photometer with a 540 nm interference filter (max. 2 nm bandwidth)
- Strip chart recorder or computer based data acquisition system
- Nitrogen gas (high-purity grade, 99.99%)

2. Glassware and Supplies
   - Pipettes
   - 60-ml high density polyethylene sample bottles, glass volumetric flasks and glass sample tubes.
   - Analytical balance with accuracy to 0.1 mg
   - Drying oven
   - Desiccator
   - Membrane filters (0.45 µm nominal pore size)
   - Syringes with syringe filters
   - pH meter with a glass electrode and reference electrode.

2.3.1.5 Reagents and Standards

2.3.1.5.1 Stock Reagent Solutions

1. Stock Sulfanilamide Solution. Dissolve 10 g sulfanilamide ($C_6H_8N_2O_2S$) in 1 L of 10% HCl.
2. Stock Nitrate Solution (100 mg N/L). Transfer 0.4928 g of pre-dried (105°C for 1 hour) sodium nitrite ($NaNO_2$) to a 1000 mL volumetric flask containing approximately 800 ml of reagent water and dissolve. Dilute the solution to 1 L with reagent water. Store the solution in a polyethylene bottle at 4°C. This solution is stable for 6 months.
3. Stock Nitrite Solution (100 mg N/L). Transfer 0.7217 g of pre-dried (105°C for 1 hour) potassium nitrate (KNO_3) to a 1000 mL volumetric flask containing approximately 800 ml of reagent water and dissolve. Dilute the solution to 1 L with reagent water. Store the solution in a polyethylene bottle at 4°C. This solution is stable for 3 months.

Note. *High purity nitrite salts may not be available. Assays given be reagent manufacturers are usually in the range of 95-97%. The impurity must be taken into account for the weight taken.*

4. For marine samples-- Low Nutrient Sea Water. Obtain natural or commercially available low nutrient seawater from surface water (salinity 35-36‰, < 7 µg N/L) and filter it through 0.3 micron pore size glass fiber filters. Do not use artificial seawater.

2.3.1.5.2 Working Reagents

1. Brij-35 Start-up solution. Brij-35 is a trade name for polyoxyehtylene(23) lauryl ether ($C_{12}H_{25}(OCH_2CH_2)_{23}OH$) and is commercially available. Add 2 mL of Brij-35 surfactant to 1000 mL reagent water and mix gently.
2. Working Sulfanilamide Solution. Add 1 mL Brij-35 to 200 mL of stock sulfanilamide solution.

Note. *Adding surfactant Brij-35 to sulfanilamide solution instead of to the buffer solution is to prevent the Brij-35 from being adsorbed on the cadmium surface, which may result in decreasing surface reactivity of the cadmium and reduce the lifetime of the column.*

3. NED Solution. Dissolve 1 g of NED (N-1-naphthylehtylenediamine dihydrochloride, $C_{12}H_{14}N_2$•HCl) in 1 L of reagent water.

4. Imidazole Buffer Solution. Dissolve 13.6 g of imidazole ($C_3H_4N_2$) IN 4L reagent water. Add 2ml concentrated HCl. Adjust the pH to 7.8 with diluted HCl. Store in a refrigerator.

5. Copper Sulfate Solution (2%). Dissolve 20g of copper sulfate ($CuSO_4$•$5H_2O$) in 1L reagent water.

6. Colored SYNC Peak Solution. Add 50 µL of red food coloring to 1000 mL reagent water and mix. Further dilute to obtain a peak of between 25 to 100 percent full scale according to the AUFS setting used for refractive index measurement.

7. Primary Dilution Standard Solution (5 mg N/L). Prepare by diluting 5.0 mL of stock standard solution to 100 mL with reagent water. Prepare fresh daily.

Note. *This solution should be prepared as an intermediate concentration appropriate for further dilution in preparing calibration solutions. Therefore the concentration must be adjusted according to the desired calibration concentration range.*

8. Calibration Standards. Prepare a series of calibration standards by diluting suitable volumes of a primary dilution standard to 100 mL with reagent water or low nutrient seawater. The concentration range should bracket the expected concentrations of samples and not span more than two orders of magnitude. At least five calibration standards with equal concentration increments should be used to construct the calibration curve. Prepare daily.

   If nitrate + nitrite and nitrite are analyzed simultaneously by splitting a sample into two analytical systems, a nitrate and nitrite mixed standard should be prepared. The total concentration (nitrate+nitrite) must be assigned to the concentrations of calibration standards in the nitrate+nitrite system.

   When analyzing samples of varying salinities, it is recommended that the calibration standard solutions and sampler wash solution be prepared in reagent water and corrections for refractive index be made to the sample concentrations determined.

9. Saline Nitrate and Nitrite Standards. If the calibration standard solutions are not prepared to match sample salinity, then saline nitrate and nitrite standards must be prepared in a series of salinities in order to quantify the salt error, the change in the colorimetric response of nitrate due to the change in the composition of the solution. The following dilutions in 100 mL reagent water are recommended:

Salinity (‰)	Low nutrient seawater (mL)	Conc. primary dilution standard (mL)	mg N/L
0	0	2	0.10
9	25	2	0.10
18	50	2	0.10
27	75	2	0.10
35	98	2	0.10

2.3.1.5.3 Open Tubular Cadmium Reactor

For this method, the lab may use either a commercial Open Tubular Cadmium Reactor (OTCR) or a laboratory-prepared packed copper-coated cadmium reduction column. If an OTCR is used, it should be activated as follows.

Prepare reagent water, 0.5N HCl solution and 2% $CuSO_4$ solution in three 50 mL beakers. Fit three 10-mL plastic syringes with unions. First flush the OTCR with 10 mL reagent water. Then flush it with 10 mL 0.5N HCl solution for 3 seconds, immediately followed by flushing with a couple of syringe volumes of reagent water. Slowly flush with $CuSO_4$ solution until a large amount of black precipitated copper comes out. Finally flush with reagent water with imidazole buffer for short term storage.

2.3.1.5.4 Packed Cadmium Reduction Column

If a laboratory-prepared packed copper-coated cadmium reduction column is to be used, the following procedures should be used.

1. File a cadmium stick to obtain freshly prepared cadmium filings. Sieve the filings and retain the fraction between 25 and 60 mesh size (0.25-0.71 mm). Wash the filings two times with 10% HCl followed with reagent water.
2. Decant the reagent water and add 50 mL of 2% $CuSO_4$ solution. While swirling, brown flakes of colloidal copper will appear and the blue color of the solution will fade. Decant the faded solution and add fresh $CuSO_4$ solution and swirl. Repeat until the blue color no longer fades.
3. Wash the filings with reagent water until all the blue color is gone and the supernatant is free of fine particles. Keep filings submersed in reagent water to avoid exposure to air.
4. The column can be prepared in a plastic or a glass tube 2mm ID. Plug one end with glass wool. Fill the column with water and transfer Cd filings in suspension using a 10 mL pipette tip connected to one end of the column. While gently tapping the tube and pipette tip let Cd filings pack tightly and uniformly in the column without trapping air bubbles.
5. Insert another glass wool plug at the top of the column. If a U-shape tube is used, the pipette tip is connected to the other end and the procedure repeated. Connect both ends of the column using a plastic tube filled with buffer solution to form a closed loop.
6. If a packed cadmium column has not been used for several days, it should be reactivated prior to sample analysis.

2.3.1.5.5 Stabilization of OTCR or Packed Cadmium Reduction Column

1. Pump the buffer and other reagent solutions through the manifold and obtain a stable baseline.
2. Pump 0.7 mg-N/L nitrite standard solution continuously through the sample line and record the steady state signal.
3. Stop the pump and install the column on the manifold. Ensure no air bubbles have been introduced into the manifold during the installation. Resume the pumping and confirm a stable baseline.

4. Pump 0.7 mg-N/L nitrate solution continuously through the sample line and record the signal. The signal will increase slowly and reach steady state in about 10-15 minutes. This steady state signal should be close to the signal obtained from the same concentration of a nitrite solution without the column on line.

5. The reduction efficiency of the column can be determined by measuring the absorbance of a nitrate standard solution followed by a nitrite standard solution of the same concentration. Reduction efficiency is calculated as follows:

$$\text{Reduction efficiency} = \frac{\text{Absorbance of Nitrate}}{\text{Absorbance of Nitrite}}$$

2.3.1.6 Quality Control

Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements consist of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples.

2.3.1.6.1 Initial Demonstration of Performance

1. The method detection limit (MDL) must be established for the method analyte using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots or water which have been processed through the entire analytical method. Calculate the MDL as follows:

$$MDL = (t)(S)$$

where,

S = the standard deviation of the replicate analysis

t = t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

2. The linear dynamic range (LDR) must be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (absorbance units full scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to the samples to avoid the necessity to correct for salt error or refractive index. Normalize responses by multiplying the response by the absorbance units full scale output range setting. Perform the linear regression of normalized response vs. concentration, and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response (R) of a standard no longer yields a calculated concentration (C_C) that is within 100 ± 10% of known concentration (C), where

$$C_C = (R-b)/m$$

This concentration defines the upper limit of the LDR. If samples are found to have a concentration that is ≥ 90% of the upper limit of the LDR, they must be diluted and reanalyzed.

2.3.1.6.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB). The lab should analyze at least one LRB with each set of samples. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be reprepared and analyzed after the source of contamination has been corrected and acceptable LRB values have been obtained.
2. Laboratory Fortified Blank (LFB). The lab should analyze at lease one LFB with each set of samples. The LFB must be at a concentration within the daily calibration range. The LFB data are used to calculate percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the source of the problem should be identified and resolved before continuing the analysis.
3. The laboratory must use LFB data to assess lab performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

   Upper Control Limit = $x + 3S$

   Lower Control Limit = $x – 3S$

   Optional control limits must be equal to or better than required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentration included in the LFB. These data must be kept on file and available for review.

2.3.1.6.3 Assessing Analyte Recovery-Laboratory Fortified Sample Matrix (LFM)

1. The laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one LFM per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater thean the MDL.
2. Calculate percent recovery of anlayte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_S - C)}{S} \times 100$$

   where,

   R = percent recovery

   C_S = measured fortified sample addition in mg N/L

   C = sample background concentration in mg N/L

   S = concentration in mg N/L added to the environmental sample

3. If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the

recovery problem encountered with the fortified sample is judged to be the matrix related and the sample data should be flagged.

2.3.1.7 Calibration and Standardization

1. At least five calibration standards should be prepared fresh daily for system calibration.
2. A calibration curve should be constructed for each sample set by analyzing a series of calibration standard solutions. A sample set should contain no more than 60 samples. For a large number of samples make several sample sets with individual calibration curves.
3. Analyze the calibration standards in duplicate before the actual samples.
4. The calibration curve containing five data points or more that bracketed the concentration of samples should have a correlation coefficient (r) of 0.995 or better and the range should not be greater than two orders of magnitude.
5. Use a high calibration solution followed by two black cups to quantify system carryover. The difference in peak heights between two blank cups is due to the carryover from the high calibration solution. The carryover coefficient (k) is calculated as follows:

$$k = \frac{P_{b1} - P_{b2}}{P_{high}}$$

where,

P_{high} = the peak height of the high ammonia standard

P_{b1} = the peak height of the first blank sample

P_{b2} = the peak height of the second blank sample

The carryover coefficient (k) should be measured in seven replicates to obtain a statistically significant number. The carryover coefficient should be remeasured with any change in manifold plumbing or upon replacement of pump tubes.

The carryover correction (CO) of a given peak (i) is proportional to the peak height of the preceding sample P_{i-1}.

$$CO = k \times P_{i-1}$$

To correct a given peak height reading, (P_i), subtract the carryover correction.

$$P_{i,c} = P_i - CO$$

where $P_{i,c}$ is the corrected peak height. The correction for carryover should be applied to all the peak heights throughout a run. The carryover coefficient should be less than 5%.

6. Place a high standard nitrate solution followed by a nitrite standard solution of the same concentration at the beginning and end of each sample run to check for change in reduction efficiency of the column. The decline of reduction efficiency during a run should be < 5%.
7. Place a high standard solution end of each sample run to check for sensitivity drift. Apply sensitivity drift correction to all the samples. Sensitivity drift during a run should be < 5%.

***Note**. Sensitivity drift correction is available in most data acquisition software supplied with subanalyzers. It is assumed that sensitivity drift is linear with time. An interpolated drift correction factor is calculated for each sample according to the sample position during the run. Multiply the sample peak height by the corresponding sensitivity drift correction factor to obtain the corrected peak height for each sample.*

2.3.1.8 Procedure

1. If samples are stored in a refrigerator, equilibrate to room temperature prior to analysis.
2. Turn on the continuous flow analyzer and data acquisition components and warm up for at least 30 minutes.
3. Set up cartridge according to the type of cadmium reducer used -- OTCR Figure 2.2 or packed cadmium column Figure 2.3. Configuration for analysis of nitrite alone is shown in Figure 2.4.

***Note**. When a gas segmented flow stream passes through the OTCR, particles derived from the column were found to increase baseline noise and to cause interference at low level analysis. Packed cadmium columns are therefore preferred for nitrite analysis at low concentrations.*

4. Set the spectrophotometer wavelength to 540 nm and turn on lamp.
5. Set the absorbance unit full scale (AUFS) range on the spectrophotometer at the appropriate setting according to the highest concentration of nitrate in the samples. The highest setting appropriate for this method is 0.2 AUFS for 0.7 mg N/L.
6. Begin pumping the Brij-35 start-up solution through the system and obtain a steady baseline. Place the reagents on-line. The reagent baseline will be higher than the start-up solution baseline. After the reagent baseline has stabilized, reset the baseline.

***Note**. To minimize the noise in the reagent baseline, clean the flow system by sequentially pumping the sample line with reagent water, 1 N HCl solution, reagent water, 1 N NaOH solution for a few minutes each at the end of the daily analysis. Make sure to rinse the system well to prevent precipitation of $Mg(OH)_2$ when seawater is introduced into the system. Keep the reagents and samples free of particulates and filter if necessary.*

If the baseline drifts upward, pinch the waste line for a few seconds to increase back pressure. If absorbance drops down rapidly when back pressure increases, this indicates that there are air bubbles trapped in the flow cell. Attach a syringe at the waste outlet of the flowcell. Air bubbles in the flow cell can often be eliminated by simply attaching a syringe for a few minutes or, if not, dislodged by pumping the syringe piston. Alternatively, flushing the flowcell with alcohol was found to be effective in removing trapped air.

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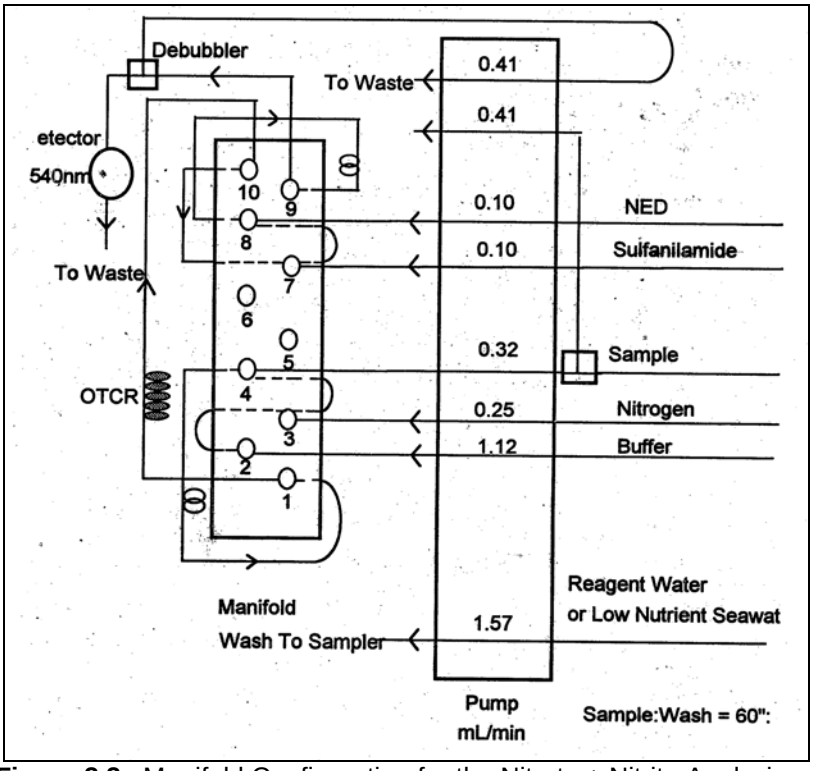

Figure 2.2. Manifold Configuration for the Nitrate + Nitrite Analysis using an Open Tubular Cadmium Reactor

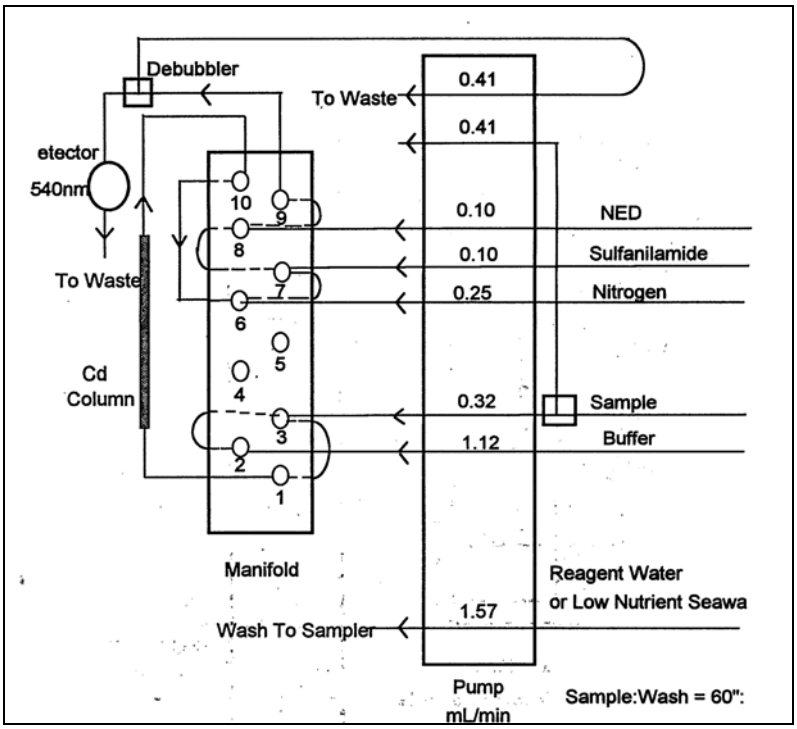

Figure 2.3. Manifold Configuration for Nitrate + Nitrite Analysis using a Laboratory Packed Copper-coated Cadmium Reduction

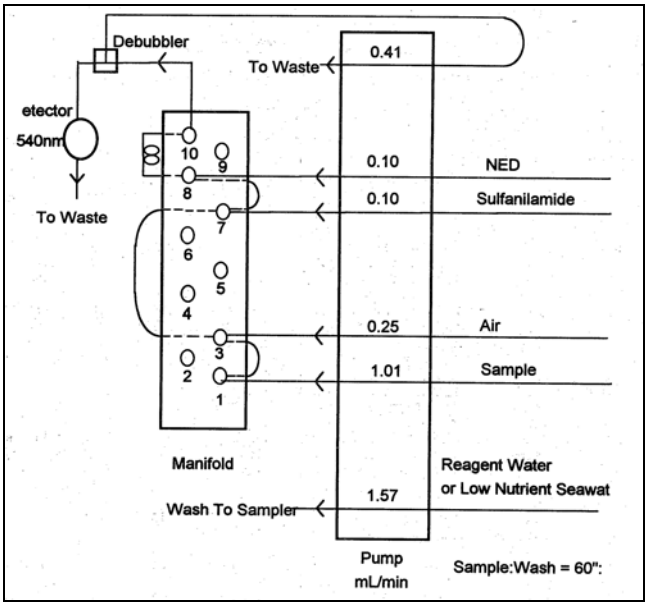

Figure 2.4. Manifold Configuration for Nitrite Analysis

8. Check the reduction efficiency of the OTCR or packed cadmium column. If the reduction efficiency is less than 90%, reactivate and restabilize. Ensure reduction efficiencies reach at least 90% before analyzing samples.
9. The sampling rate is approximately 60 samples per hour with 30 second of sample time and 30 seconds of wash time. Place a blank after every ten samples.

2.3.1.9 Data Analysis and Calibration

Concentrations of nitrate in samples are calculated from the linear regression, obtained from the standard curve in which the concentration of the calibration standards are entered as the independent variable and their corresponding peak heights are the dependent variable.

2.3.1.9.1 Refractive Index Correction

1. If reagent water is used as the wash solution, the analyst has to quantify the refractive index correction due to the difference in salinity between sample and wash solution. The following procedures are used to measure the relationship between the sample salinity and the refractive index on a particular detector.
2. Analyze a set of nitrate or nitrite standards in reagent water with color reagent using reagent water as the wash and obtain a linear regression of peak height vs concentration. Then replace reagent water wash solution with low nutrient seawater wash solution.

***Note**. The change in absorbance due to refractive index is small therefore low concentration standards should be used to bracket the expected absorbances due to refractive index.*

***Note**. In nitrate and nitrite analysis absorbance of the reagent water is higher than that of the low nutrient seawater. When using reagent water as a wash solution, the change in refractive index causes the absorbance of seawater to become negative. To measure the absorbance due to refractive index change in different salinity samples, low nutrient seawater must be used as the wash solution to bring the baseline down.*

3. Replace the NED solution with reagent water. All other reagents remain the same. Replace the synchronization sample with the colored SYNC peak solution.
4. Prepare a series of different salinity samples by diluting the low nutrient seawater. Commence analysis and obtain peak heights for different salinity samples. The peak heights for the refractive index correction must be obtained at the same AUFS range setting and on the same spectrophotometer as the corresponding standards.
5. Using low nutrient seawater as the water wash, a maximum absorbance will be observed for reagent water. No change in refractive index will be observed in the seawater sample. Assuming the absolute absorbance for reagent water (relative to the seawater baseline) is equal to the absorbance for seawater (relative to reagent water baseline), subtract the absorbance of samples of various salinities from that of reagent water. The results are the apparent absorbance due to the change in refractive index between samples of various salinities relative to the reagent water baseline.
6. For each sample of varying salinity, calculate the apparent nitrate or nitrite concentration due to refractive index from its peak height corrected to the reagent water baseline and the regression equation of ammonia standards obtained with color reagent being pumped through the system. Salinity is entered as the dependent variable and the apparent nitrate or nitrite concentration due to refractive index is entered a the dependent variable. The resulting regression allows the analyst to calculate apparent nitrate or nitrite concentration due to refractive index when sample salinity is known. Thus, the analyst would not be required to obtain refractive index peak heights for all samples.
7. The magnitude of refractive index correction can be minimized by using a low refractive index flowcell. It is important that the refractive index correction must be calculated for the particular detector. The refractive index must be redetermined whenever a significant change in the design of the flowcell or new matrix is encounter.

   A typical linear equation is:

   Apparent nitrate (µm N/L) = 0.01047(S)

   Apparent nitrite (µm N/L) = 0.00513 (S)

   where S is the sample salinity in parts per thousand.

   The apparent nitrate and nitrite concentration due to refractive index so obtained should then be added to samples of corresponding salinity when reagent water was used as the wash solution for sample analysis.

   If nitrate and nitrite concentrations are greater than 100 and 50 µm N/L, respectively, the correction for refractive index is negligible and this procedure can be optional.

2.3.1.9.2 Salt Error Correction

1. When calculating concentrations of samples of varying salinities from standards and wash solution prepared in reagent water, it is necessary to first correct for refractive index errors, then correct for the change in color development due to the differences in composition between samples and standards (salt error).
2. Plot the salinity of the saline standards as the independent variable, and the apparent concentration of ammonia (mg N/L) from the peak height (corrected for refractive index) calculated from the regression of standards in reagent water, as the dependent variable for

all saline standards. The resulting regression equation allows the analyst to correct the concentrations of samples of known salinity for color enhancement due to salt error.

3. Results of sample analysis should be reported in mg N/L (ppm) or in µg N/L (ppb).

2.3.2 FRESHWATER

2.3.2.1 Scope and Application

1. This method covers the determination of nitrite singly, or nitrite and nitrate combined in freshwater samples.
2. The applicable range is 0.05-10.0 mg/L nitrate-nitrite nitrogen. The range may be extended with sample dilution.

2.3.2.2 Summary of Method

1. A filtered sample is passed through a column containing granulated copper cadmium to reduce nitrate to nitrite. The nitrite (that was originally present plus reduced nitrate) is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye which is measured colorimetrically. Separate (not combined nitrate-nitrite) values are readily obtained by carrying out the procedure first with, and then without, the Cu-Cd reduction step.
2. Reduced volume versions of this method that use the same reagents and molar ratios are acceptable provided they meet the quality control and performance requirements stated in the method.
3. Limited performance-based method modifications may be acceptable provided they are fully documented and meet or exceed requirements expressed in the NCCA QAPP.

2.3.2.3 Interferences

1. Build up of suspended matter in the reduction column will restrict sample flow. Since nitrate and nitrite are found in a soluble state, samples may be pre-filtered.
2. Low results might be obtained for samples that contain high concentrations of iron, copper or other metals. EDTA is added to the samples to eliminate this interference.
3. Residual chlorine can produce a negative interference by limiting reduction efficiency. Before analysis, samples should be checked and if required, dechlorinated with sodium thiosulfate.
4. Samples that contain large concentrations of oil and grease will coat the surface of the cadmium. This interference is eliminated by pre-extracting the sample with an organic solvent.
5. Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that bias analyte response.

2.3.2.4 Safety

1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure

should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.

2. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of Material Safety Data Sheets (MSDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.
3. The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.
   - Cadmium
   - Phosphoric acid
   - Hydrochloric acid
   - Sulfuric acid
   - Chloroform

2.3.2.5 Equipment and Supplies

1. Balance -- Analytical, capable of accurately weighing to the nearest 0.0001 g.
2. Glassware -- Class A volumetric flasks and pipets as required.
3. Automated continuous flow analysis equipment designed to deliver sample and reagents in the required order and ratios.
   - Sampling device (sampler)
   - Multichannel pump
   - Reaction unit or manifold
   - Colorimetric detector
   - Data recording device

2.3.2.6 Reagents and Standards

1. Granulated cadmium: 40-60 mesh. Other mesh sizes may be used.
2. Copper-cadmium: The cadmium granules (new or used) are cleaned with dilute HCl and copperized with 2% solution of copper sulfate in the following manner:
   a. Wash the cadmium with HCl (Section 7.6) and rinse with distilled water. The color of the cadmium so treated should be silver.
   b. Swirl 10 g cadmium in 100 mL portions of 2% solution of copper sulfate for five minutes or until blue color partially fades, decant and repeat with fresh copper sulfate until a brown colloidal precipitate forms.
   c. Wash the copper-cadmium with reagent water (at least 10 times) to remove all the precipitated copper. The color of the cadmium so treated should be black.
3. Preparation of reduction column. The reduction column is a U-shaped, 35 cm length, 2 mm I.D. glass tube (see Note). Fill the reduction column with distilled water to prevent entrapment of air bubbles during the filling operations. Transfer the copper-cadmium granules to the reduction column and place a glass wool plug in each end. To prevent entrapment of air bubbles in the reduction column, be sure that all pump tubes are filled with reagents before putting the column into the analytical system.

Note: *Other reduction tube configurations, including a 0.081 I.D. pump tube, can be used in place of the 2 mm glass tube, if checked as in.*

4. Reagent water: Because of possible contamination, this should be prepared by passage through an ion exchange column comprised of a mixture of both strongly acidic-cation and strongly basic-anion exchange resins. The regeneration of the ion exchange column should be carried out according to the manufacturer's instructions.
5. Color reagent: To approximately 800 mL of reagent water, add, while stirring, 100 mL conc. phosphoric acid, 40 g sulfanilamide and 2 g N-1-naphthylethylenediamine dihydrochloride. Stir until dissolved and dilute to 1 L. Store in brown bottle and keep in the dark when not in use. This solution is stable for several months.
6. Dilute hydrochloric acid, 6N: Add 50 mL of conc. HCl to reagent water, cool, and dilute to 100 mL.
7. Copper sulfate solution, 2%: Dissolve 20 g of $CuSO_4C_5H_2O$ in 500 mL of reagent water and dilute to 1 L.
8. Wash solution: Use reagent water for unpreserved samples. For samples preserved with H_2SO_4, use 2 mL H_2SO_4 per liter of washwater.
9. Ammonium chloride-EDTA solution: Dissolve 85 g of reagent grade ammonium chloride and 0.1 g of disodium ethylenediamine tetracetate in 900 mL of reagent water. Adjust the pH to 9.1 for preserved or 8.5 for non-preserved samples with conc. ammonium hydroxide and dilute to 1 L. Add 0.5 mL Brij-35 (CASRN 9002-92-0).
10. Stock nitrate solution: Dissolve 7.218 g KNO_3 and dilute to 1 L in a volumetric flask with reagent water. Preserve with 2 mL of chloroform per liter. Solution is stable for six months. 1 mL = 1.0 mg NO_3-N.
11. Stock nitrite solution: Dissolve 6.072 g KNO_2 in 500 mL of reagent water and dilute to 1 L in a volumetric flask. Preserve with 2 mL of chloroform and keep under refrigeration. 1.0 mL = 1.0 mg NO_2-N.
12. Standard nitrate solution: Dilute 1.0 mL of stock nitrate solution to 100 mL. 1.0 mL = 0.01 mg NO_3-N. Preserve with .2 mL of chloroform. Solution is stable for six months.
13. Standard nitrite solution: Dilute 10.0 mL of stock nitrite) solution to 1000 mL. 1.0 mL = 0.01 mg NO_2-N. Solution is unstable; prepare as required.

2.3.2.7 Sample Collection, Preservation and Storage

1. Samples are collected in plastic or glass bottles. All bottles are thoroughly cleaned and rinsed with reagent water. Volume collected must be sufficient to insure a representative sample, allow for replicate analysis.
2. Samples should be analyzed as soon as possible after collection. If storage is required, preserved samples are maintained at 4°C and may be held for up to 28 days.

2.3.2.8 Quality Control

Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated.

2.3.2.8.1 Initial Demonstration of Performance

1. The initial demonstration of performance is used to characterize instrument performance (determination of LCRs and analysis of QCS) and laboratory performance (determination of MDLs) prior to performing analyses by this method.
2. Linear Calibration Range (LCR) -- The LCR must be determined initially and verified every six months or whenever a significant change in instrument response is observed or expected. The initial demonstration of linearity must use sufficient standards to insure that the resulting curve is linear. The verification of linearity must use a minimum of a blank and three standards. If any verification data exceeds the initial values by ± 10%, linearity must be reestablished. If any portion of the range is shown to be nonlinear, sufficient standards must be used to clearly define the nonlinear portion.
3. Quality Control Sample (QCS) -- When beginning the use of this method (on a quarterly basis or as required to meet data-quality needs) verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS. If the determined concentrations are not within ±10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with on-going analyses.
4. Method Detection Limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit. To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

   MDL = (t) x (S)

   where,

   t = value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates]

   S = standard deviation of the replicate analyses

   MDLs should be determined every six months, when a new operator begins work or whenever there is a significant change in the background or instrument response.

2.3.2.8.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one LRB with each batch of samples. Data produced are used to assess contamination from the laboratory environment. Values that exceed the MDL indicate laboratory or reagent contamination should be suspected and corrective actions must be taken before continuing the analysis.
2. Laboratory Fortified Blank (LFB) -- The laboratory must analyze at least one LFB with each batch of samples. Calculate accuracy as percent recovery. If the recovery of any analyte falls outside the required control limits of 90-110%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.
3. The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90-110%. When sufficient internal performance data become

available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

Upper Control Limit = x + 3S

Lower Control Limit = x - 3S

The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5-10 new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an on-going precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

4. Instrument Performance Check Solution (IPC) -- For all determinations the laboratory must analyze the IPC (a mid-range check standard) and a calibration blank immediately following daily calibration, after every 10th sample (or more frequently, if required) and at the end of the sample run. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subsequent analyses of the IPC solution must verify the calibration is still within ±10%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift, the instrument recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

2.3.2.8.3 Assessing Analyte Recovery and Data Quality

1. Laboratory Fortified Sample Matrix (LFM) -- The laboratory must add a known amount of analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis. The analyte concentration must be high enough to be detected above the original sample and should not be less than four times the MDL. The added analyte concentration should be the same as that used in the laboratory fortified blank.
2. Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range 90-110%. Percent recovery may be calculated using the following equation:

$$R = \frac{Cs - C}{s} \times 100$$

where,

R = percent recovery

Cs = fortified sample concentration

C = sample background concentration

s = concentration equivalent of analyte added to sample

3. If the recovery of any analyte falls outside the designated LFM recovery range and the lab performance for that analyte is shown to be in control, the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.

4. Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

2.3.2.9 Calibration and Standardization

1. Prepare a series of at least three standards, covering the desired range, and a blank by diluting suitable volumes of the standard nitrate solution. At least one nitrite standard should be compared to a nitrate standard at the same concentration to verify the efficiency of the reduction column.
2. Set up manifold. Care should be taken not to introduce air into the reduction column.
3. Place appropriate standards in the sampler in order of decreasing concentration and perform analysis.
4. Prepare standard curve by plotting instrument response against concentration values. A calibration curve may be fitted to the calibration solutions concentration/response data using computer or calculator based regression curve fitting techniques. Acceptance or control limits should be established using the difference between the measured value of the calibration solution and the "true value" concentration.
5. After the calibration has been established, it must be verified by the analysis of a suitable quality control sample (QCS). If measurements exceed ±10% of the established QCS value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis. Periodic reanalysis of the QCS is recommended as a continuing calibration check.

Note: Condition column by running 1 mg/L standard for 10 minutes if a new reduction column is being used. Subsequently wash the column with reagents for 20 minutes.

2.3.2.10 Procedure

1. If the pH of the sample is below 5 or above 9, adjust to between 5 and 9 with either conc. HCl or conc. NH_4OH.
2. Allow system to equilibrate as required. Obtain a stable baseline with all reagents, feeding reagent water through the sample line.
3. Place appropriate nitrate and/or nitrite standards in sampler in order of decreasing concentration and complete loading of sampler tray.
4. Switch sample line to sampler and start analysis.

2.3.2.11 Data Analysis and Calculations

1. Prepare a calibration curve by plotting instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve. Multiply answer by appropriate dilution factor.
2. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.
3. Report results in mg/L as nitrogen.

2.4 TOTAL NITROGEN AND PHOSPHORUS

2.4.1 Scope and Application

These methods are intended for determination of total nitrogen (organic nitrogen + ammonium + nitrate + nitrite) and phosphorus (all forms) in filtered and whole-water samples by alkaline persulfate digestion. They were validated for determination of total nitrogen and total phosphorus in drinking water, wastewater, and water-suspended sediment. Their applicability to bottom materials was not investigated. Analytical ranges are 0.03 to 5.00 mg-N/L for dissolved and total nitrogen and 0.01 to 2.00 mg-P/L for dissolved and total phosphorus.

2.4.2 Method Summary and Analytical Considerations

Filtered and whole-water samples are dispensed into glass culture tubes, dosed with alkaline persulfate reagent, capped tightly, and digested in an autoclave at 250ºF (121ºC) and 17 lb/in2 (117.2 kPa) for 1 hour. The alkaline persulfate digestion procedure oxidizes all forms of inorganic and organic nitrogen to nitrate and hydrolyzes all forms of inorganic and organic phosphorus to orthophosphate. Nitrate and orthophosphate in alkaline persulfate digests are determined in parallel with a 2-channel photometric, air-segmented continuous flow analyzer.

Digest preparation protocols and reagent formulations were adapted from previously published procedures (Valderrama, 1981; Hosomi and Sudo, 1986; Ameel and others, 1993; D'Elia and others, 1997; American Public Health Association, 1998b). Two other reports (Nydahl, 1978; Cabrera and Beare, 1993) provided insight into the potential for low nitrogen recovery in samples containing high concentrations of dissolved and particulate organic carbon.

Quantitative recovery of nitrogen and phosphorus by alkaline persulfate digestion depends critically on a progressive decrease in pH (initial pH >12, final pH ≤ 2.2) during the 1-hour course of the digestion (Hosomi and Sudo, 1986). These dynamic reaction conditions are achieved by formulating the digestion reagent with approximately equimolar concentrations of persulfate and hydroxide ions—0.05 ***M***, initial pH >12 after 1 + 2 dilution by samples in this method. Under these initially alkaline conditions, dissolved and suspended nitrogen in samples oxidize to nitrate. As the digestion proceeds, bisulfate ions resulting from thermal decomposition of persulfate first neutralize and then acidify the reaction mixture by the following chemical reaction:

$$S2O8\text{-}2 + H2O \xrightarrow{\Delta} 2\ HSO_4^- + \frac{1}{2}\ O2$$

After all of the persulfate has decomposed, the digest mixture pH approaches 2, and under these acidic conditions, dissolved and suspended phosphorus hydrolyze to orthophosphate. The foregoing discussion indicates that analysis of samples with variable and unknown acidity or alkalinity by alkaline persulfate digestion methods will be problematic. Users of this method are cautioned that amending FCA and WCA samples with concentrations of sulfuric acid other than those specified in USGS field manual protocols (Wilde and others, 1998) likely will result in undetected method failure and possible reporting of erroneous results.

As is the case for Kjeldahl digestion, alkaline persulfate digestion converts all forms of phosphorus to orthophosphate. Thus alkaline persulfate digestion dissolved and total phosphorus (DPAlkP and TPAlkP) concentrations can be compared directly with Kjeldahl digestion dissolved and total phosphorus (KDP and KTP) concentrations by graphical and statistical analysis. This is not the case, however, for Kjeldahl dissolved and total nitrogen (KDN and KTN) concentrations and alkaline persulfate digestion dissolved and total nitrogen (DNAlkP

and TNAlkP) concentrations. In principle, organic nitrogen, but not nitrate or nitrite, is reduced to ammonium during Kjeldahl digestion. Determining ammonium in Kjeldahl digests, therefore, measures organic nitrogen + ammonium. Alkaline persulfate digestion oxidizes all forms of nitrogen to nitrate. Determining nitrate + nitrite in alkaline persulfate digests, therefore, measures total nitrogen (organic nitrogen + ammonium + nitrite + nitrate). To reconcile this difference between the two methods, nitrate + nitrite concentrations were subtracted from DNAlkP and TNAlkP concentrations prior to graphical and statistical comparisons with KDN and KTN concentrations throughout this report. For this purpose and as a quality-control (QC) check, all filtered and whole-water samples selected for alkaline persulfate digestion also were analyzed for dissolved nitrate + nitrite, ammonium, and orthophosphate on the same day that digests were prepared. Particulates were removed from acidified, whole-water samples (WCA bottle type) by 0.45-µm filtration prior to dissolved nutrient determinations.

A 2-channel, air-segmented continuous flow analyzer was configured for simultaneous photometric determination of nitrate + nitrite and orthophosphate in alkaline persulfate digests. Nitrate + nitrite was determined by a cadmium-reduction, Griess-reaction method (Wood and others, 1967) equivalent to U.S. Environmental Protection Agency (USEPA) method 353.2 (U.S. Environmental Protection Agency, 1993) and U.S. Geological Survey (USGS) method I-2545-90 (Fishman, 1993, p. 157) except that sulfanilamide and N-(1-naphthy)ethylenediamine reagents were separate rather than combined. The analytical cartridge diagram is shown in figure 1. Orthophosphate was determined by a phosphoantimonylmolybdenum blue method (Murphy and Riley, 1962; Pai and others, 1990), which is equivalent to the 2-reagent variants (separate molybdate and ascorbic acid reagents) of USEPA method 365.1 (U.S. Environmental Protection Agency, 1993) and USGS method I-2601-90 (Fishman, 1993). The analytical cartridge diagram is shown in Figure 2.

2.4.3 Interferences

2.4.3.1 Alkaline Persulfate Digestion

1. Chloride concentrations up to 1,000 mg/L (the highest tested for this report) do not interfere. Furthermore, because good results are obtained for seawater in 2 + 1 mixture with digestion reagent (D'Elia and others, 1997), chloride concentrations of about 10,000 mg/L apparently are tolerated provided that calibrants are matrix matched. Higher chloride concentrations, however, are likely to interfere because of reaction with persulfate to form oxychlorides or chlorine that might deplete persulfate required to oxidize inorganic and organic nitrogen species to nitrate. Resulting active chlorine species also can interfere in colorimetric reactions used to determine nitrate and orthophosphate in digests.
2. Sulfate concentrations up to 1,000 mg/L (the highest tested for this report) do not interfere.
3. Organic carbon concentrations greater than 150 mg/L interfere because of reaction with persulfate to form carbon dioxide, thus depleting persulfate required to oxidize inorganic and organic nitrogen species to nitrate.
4. Overacidification of FCA and WCA samples at collection sites can result in low recovery of inorganic and organic nitrogen at the NWQL. The possibility of overacidification can be avoided by exclusive use of the sulfuric acid field-amendment solution—one vial containing 1 mL of 4.5 ***N*** H2SO4 (One Stop Shopping number FLD-438) per 120 mL of sample—which is specified in the USGS National Field Manual (Wilde and others, 1998).
5. Nitrate and nitrite do not contribute to KDN and KTN concentrations in principle, but in practice, positive and negative interferences by these ions are well known—see, for example, American Public Health Association, 1998c; Patton and Truitt, 2000. This

interference can confound comparison of KN and NAlkP concentrations when dissolved nitrate concentrations are greater than about 0.1 mg NO3--N/L.

6. Suspended particles remaining in digests must be removed by sedimentation and decantation or filtration prior to colorimetric analyses.

2.4.3.2 Colorimetric Nitrate + Nitrite Determination

1. Typically, concentrations of substances with potential to interfere in cadmium-reduction, Griess-reaction nitrate + nitrite methods are negligible in ambient surface- and ground-water samples. For specific details of inorganic and organic compounds that might interfere in the color reaction, see Norwitz and Keliher (1985, 1986), as well as more general information by the American Public Health Association (1998a).
2. Sulfides, which are often present in anoxic water and well known to deactivate cadmium reduction reactors, are oxidized during the alkaline persulfate digestion and are unlikely to interfere.

2.4.3.3 Colorimetric Orthophosphate Determination

1. Barium, lead, and silver can interfere by forming insoluble phosphates, but their concentrations in natural-water samples usually are less than the interference threshold (Fishman, 1993).
2. Interference from silicate, which also can form reduced heteropoly acids with molybdenum (Zhang and others, 1999), is negligible under reaction conditions used for this report.
3. Arsenate, AsO43, but not arsenite, AsO33, can interfere by forming reduced heteropoly acids analogous to those formed by orthophosphate (Johnson, 1971). Because of the possibility that arsenite might be oxidized to arsenate by persulfate, both species at concentrations up to 20 mg-As/L in deionized water were digested and analyzed. With reference to Table 2, it is apparent that a major fraction of arsenite is oxidized to arsenate during alkaline persulfate digestion and that interference by either species up to 1 mg-As/L is negligible.

Table 2. Data from a study of arsenate and arsenite interference in alkaline persulfate total phosphorus determinations [mg-As/L, milligrams of arsenic per liter; mg-P/L, milligrams of phosphorus per liter; nd, not detected; ≈, nearly equal to; ±, plus or minus]

AsO_4^{3-} added mg-As/L	PO_4^{3-} found mg-P/L	AsO_3^{3-} added mg-As/L	PO_4^{3-} found mg-P/L
0.5	nd	0.5	nd
1.0	nd	1.0	nd
2.0	≈ 0.05	2.0	nd
5.0	0.32 ± 0.01	5.0	0.29 ± 0.04
10.0	1.14 ± 0.13	10.0	0.91 ± 0.06
20.0	Off scale	20.0	Off scale

2.4.4 Instrumentation and Auxiliary Analyses

1. RFA-300™, third-generation, air-segmented continuous flow analyzers (Alpkem) were used to automate photometric determination of nitrate + nitrite and orthophosphate in alkaline persulfate digests and dissolved ammonium, nitrate + nitrite, and orthophosphate in filtered- and whole-water samples prior to digestion. Modules in these systems include 301 samplers, 302 peristaltic pumps, 313 analytical cartridge bases, 314 power modules, 305A photometers, and a personal computer (PC)-based data acquisition and processing system. Alternative instrumentation—flow injection analyzers, sequential injection analyzers, other second- or third-generation continuous flow analyzers, or automated batch analyzers—also could be used to automate photometric finishes.
2. Photometric data were acquired and processed automatically using FASPac™ version 1.34 software (Astoria-Pacific, Clackamas, Ore.). This software operates under Microsoft Windows on a PC platform and includes a model 350 interface box that controls the sampler and digitizes analog photometer outputs with 16-bit resolution. Other data acquisition systems could be used provided that the A/D converter has 16-bit resolution and is capable of acquiring data at frequencies ranging from 0.5 to 2 Hz, that is, from 30 points/min to 120 points/min. As a general rule, data acquisition frequencies for air-segmented continuous flow analyzers should match the roller lift-off frequency of the peristaltic pump (Patton and Wade, 1997), that is, 0.5 Hz for Technicon AutoAnalyzer II™ and 1.5 Hz for Alpkem RFA-300 equipment. Data acquisition frequencies in the range of 2 to 5 Hz are suitable for photometric flow-injection analyzers.
3. Operating characteristics for this equipment are listed in Table 3.
4. Dissolved ammonium, nitrate + nitrite, and orthophosphate in undigested samples were determined photometrically by USGS automated continuous flow methods I-2522-90, I-2545-90 (2-reagent variant), and I-2601-90 (2-reagent variant), respectively. These methods are described in Fishman (1993).
5. The pH of WCA samples was estimated with narrow range (0–2.5) colorimetric pH-indicating test strips to detect improperly acidified samples that had pH values outside the expected range of 1.6 to 1.9.
6. WCA samples were processed through 5-mL capacity UniPrep™ syringeless filters equipped with 0.45-µm nylon membranes (Whatman, Clifton, N.J.) to remove suspended solids prior to determination of dissolved ammonium, nitrate + nitrite, and orthophosphate. These syringeless filters also were used to remove suspended solids from WCA-sample digests prior to photometric analysis when simple sedimentation and decantation into analyzer cups failed to do so.

2.4.5 Apparatus

1. Samples were digested in an autoclave (model number STME, Market Forge Industries, Inc., Everett, Mass.) operated at 250ºF (121ºC) and 17 lb/in2 (117.2 kPa) for 1 hour.
2. Filtered and chilled sample (FCC bottle type) digests were prepared robotically using a large-scale, syringe-pump-based *x-y-z* sample dispenser/diluter module (model number ML-4200, Hamilton Company, Reno, Nev.). This system is equipped with four probes and four 10-mL syringe pumps that operate in tandem under control of DOS-based *Eclipse*™ software (Hamilton Company, Reno, Nev.). Custom modifications to the ML-4200 system, including a pneumatically actuated probe expander, fixtures, and a variety of bottle and test-tube racks, were obtained from another vendor (Robotics Plus, Houston, Tex.).

3. Whole-water (WCA bottle type) sample digests were prepared manually using EDP Plus™ electronic, digital pipets (Rainin Instruments) equipped with a 10-mL liquid end.
4. Digestion vessels were 20 x 150 mm Pyrex®, screw-cap culture tubes (VWR 53283-810; Fisher 14-957-76E or 14-959-37C; or equivalent), and 18-415 linerless polypropylene caps (Comair Glass, Inc., Vineland, N.J.—Part number 14-0441-004).

Table 3. Settings and operational details of Alpkem RFA-300 continuous flow analyzers used for this study [nm, nanometer; mm, millimeter; mg-N/L, milligrams nitrogen per liter; mg-P/L, milligrams phosphorus per liter; ≈, nearly equal to; min, minute; mL, milliliter; –, not applicable; °C, degrees Celsius; s, second; h, hour]

Instrumental conditions	Nitrate + nitrite	Orthophosphate
Analytical wavelength	540 nm	880 nm
Flow cell path length	10 mm	15 mm
Calibration range	0.05 to 5.0 mg-N/L	0.01 to 2.0 mg-P/L
Standard calibration control setting	≈1.1	≈1.5
Segmentation rate (bubbles min-1)	90	90
Heated reaction coil volume	None used	2 mL
Heated reaction coil temperature	-	37°C
Dwell time (seconds)	140	260
Sample time (volume)	25 s (95 µL)	25 s (31 µL)
Wash time (volume)	10 s (38 µL)	10 s (12 µL)
Analysis rate, sample-to-wash ratio	≈103/h, 5:2	≈103/h, 5:2

2.4.6 Reagents

This section provides detailed instructions for preparing digestion and colorimetric reagents. All references to deionized water (DI) refer to NWQL in-house DI water, which is equivalent to ASTM type I DI water (American Society for Testing and Materials, 2001, p. 107–109) for nutrient analysis. All volumetric glassware and reagent and calibrant storage containers should be triple rinsed with dilute (≈5 percent v/v) hydrochloric acid and DI water just prior to use. Additionally storage containers for reagents and calibrants should be triple rinsed with small portions of the solutions before they are filled.

2.4.6.1 Digestion Reagents

NOTE: The alkaline persulfate digestion reagent for FCA and WCA samples contains an additional amount of sodium hydroxide that is calculated to neutralize the sulfuric acid added to these samples at collection sites.

1. *Sodium hydroxide, 1.5 **M** (for FCC samples)*: Dissolve 60 g of sodium hydroxide (NaOH, FW=40.0) in about 800 mL of DI water in a 1-L volumetric flask. [**Caution:** When NaOH dissolves in water, heat is released.] After dissolution is complete, allow the resulting solution to cool and dilute it to the mark with DI water. Transfer this reagent to a plastic bottle in which it is stable at room temperature for 6 months.
2. *Sodium hydroxide, 2.3 **M** (for FCA and WCA samples)*: Dissolve 92 g of sodium hydroxide (NaOH, FW=40.0) in about 800 mL of DI water in a 1-L volumetric flask. After dissolution is

complete, allow the resulting solution to cool and dilute it to the mark with DI water. Transfer this reagent to a plastic bottle in which it is stable at room temperature for 6 months.

3. *Alkaline persulfate digestion reagent (for FCC samples)*: Add 18.0 g of potassium persulfate (K2S2O8, FW=270.33) and 45 mL of 1.5 ***M*** sodium hydroxide solution to about 350 mL of DI water in a graduated 500-mL Pyrex™ media bottle (Corning number 1395-500 or equivalent). Cap the bottle, swirl its contents, and place it in an ultrasonic bath until potassium persulfate dissolution is complete (about 10 minutes). Remove the bottle from the ultrasonic bath, dry its outer surfaces, and then add enough DI water to bring the volume to 450 mL. (Make a line on the side of the bottle that indicates this volume to within ±5 mL.) Swirl the bottle to mix its contents and then divide the resulting solution among four, 125-mL clear plastic bottles used with the robotic digest preparation system. Prepare this reagent daily.
4. *Alkaline persulfate digestion reagent (for FCA and WCA samples)*: Add 18.0 g of potassium persulfate (K2S2O8, FW=270.33) and 45 mL of 2.3 ***M*** sodium hydroxide solution to about 350 mL of DI water in a graduated 500-mL Pyrex™ media bottle (Corning number 1395-500 or equivalent). Then complete preparation of this reagent exactly as described. Prepare this reagent daily.

NOTE: Reagent volumes in (450 mL) are sufficient to prepare 80 digests plus a 15-percent excess for rinsing and providing a liquid level in the 125-mL bottles necessary to prevent air aspiration during robotic dispensing operations. For manual digest preparation, a 400-mL volume of digestion reagent should be sufficient.

2.4.6.2 Colorimetric Reagents

*Sampler wash reservoir solution (0.05 **M** sodium bisulfate)*: Dissolve 6.9 g of sodium bisulfate (NaHSO4•H2O, FW=138.08) in about 800 mL of DI water in a graduated 1-L Pyrex™ media bottle. Dilute this solution to the mark with DI water, mix it well, and store it tightly capped at room temperature.

NOTE: This solution matches the matrix of sample digests. Use it as the matrix for continuing calibration verification (CCV) solutions and any other undigested check samples.

2.4.6.3 Orthophosphate Determination

1. *Stock potassium antimony tartrate reagent*: Dissolve 3.0 g of antimony potassium tartrate [K(SbO)C4H4O7•½ H2O, FW=333.93] in about 800 mL of DI water in a 1-L volumetric flask. Dilute this solution to the mark with DI water and mix it well. Transfer this reagent to a plastic bottle in which it is stable for 6 months at room temperature.
2. *Stock ascorbic acid reagent*: Dissolve 4.5 g of ascorbic acid (C6H8O6, FW=176.1) in about 200 mL of DI water in a 250-mL volumetric flask. Dilute this solution to the mark with DI water, mix it well, and transfer to a 250-mL glass bottle that has been previously rinsed with 5 percent (v/v) hydrochloric acid solution and DI water. This reagent is stable for 2 weeks at 4°C.
3. *Stock sodium lauryl sulfate reagent (15 percent w/w)*: Add 340 mL of DI water to 60 g of sodium lauryl sulfate [SLS, CH3(CH2)11OSO3Na, FW=288.38] in a 500-mL Pyrex™ media bottle. Cap the bottle and place it in an ultrasonic bath until the SLS dissolves completely (about 30 minutes). Manual inversion of the bottle at 5-minute intervals speeds dissolution. Transfer this solution to a plastic bottle in which it is stable indefinitely at room temperature.

4. *Acidic molybdate-antimony reagent*: Using a graduated cylinder, cautiously add 72 mL of concentrated sulfuric acid (H2SO4, sp. gr. 1.84) to about 700 mL of DI water in a 1-L volumetric flask. Work in a hood and manually swirl or magnetically stir the flask during each addition of sulfuric acid. Next add 7.7 g of ammonium molybdate [(NH4)6Mo7O24•4H2O, FW=1235.86] to the hot sulfuric acid solution. Manually swirl or magnetically stir the contents of the flask until the ammonium molybdate dissolves. Then add 50 mL of stock antimony potassium tartrate solution and again mix the contents of the flask thoroughly. After the resulting solution has cooled, dilute it to the mark with DI water, mix it well, and transfer it to a clean 1-L plastic bottle in which it is stable for 1 year at room temperature.
5. *Sodium lauryl sulfate diluent reagent*: Use a 100-mL graduated cylinder to dispense 10 mL of stock SLS and 90 mL of DI water into a small plastic bottle. Manually swirl the bottle to mix its contents. Prepare this reagent daily.
6. *Ascorbic acid reagent*: Use a 50-mL graduated cylinder to dispense 5 mL of the stock ascorbic acid reagent and 25 mL of DI water into an amber glass reagent bottle. Manually swirl the bottle to mix its contents. Prepare this solution daily.
7. *Startup/shutdown solution*: Add 1 mL of stock SLS reagent to 100 mL of DI water in a small plastic bottle. Thoroughly rinse the bottle and prepare a fresh solution every few days or as needed.

2.4.6.4 Nitrate Determination

1. *Copper (II) sulfate reagent (2 percent w/v)*: Dissolve 20 g of copper sulfate pentahydrate (CuSO4•5H2O, FW=249.7) in about 800 mL of DI water in a 1-L volumetric flask. Dilute this solution to the mark with DI water, mix it well, and transfer it to a 1-L plastic bottle. This reagent is stable for several years at room temperature.
2. *Imidazole buffer*, *0.1* ***M****, (pH 7.5)*: In a hood, cautiously add 5.0 mL of concentrated hydrochloric acid (HCl, ~12 ***M***) and 1.0 mL of 2 percent copper sulfate solution to 1,600 mL of DI water in a 2-L volumetric flask. Mix the contents of the flask thoroughly and then add 13.6 g of imidazole (C3H4N2, FW=68.08). Again swirl or shake the flask until the imidazole dissolves. Dilute the resulting solution to the mark with DI water, mix it well, and transfer it into two 1-L plastic bottles. This reagent is stable for 6 months at room temperature.

NOTE: Add 250 µL of Brij-35 surfactant to 250 mL of imidazole buffer each time its container is refilled on the continuous flow analyzer. Do not add Brij-35 to the bulk buffer solution.

3. *Packed bed cadmium reactor*: Cadmium reactors are prepared by slurry packing 40- to 60-mesh, copperized cadmium granules into 6-cm lengths of PTFE Teflon™ tubing (1.6 mm i.d. × 3.2 mm o.d.). Cadmium granules are retained in the column with hydrophilic plastic frits (40-µm nominal pore size). Detailed instructions for preparing copperized cadmium granules and packing them into columns can be found in NWQL standard operating procedure (SOP) IM0384.0 (or subsequent revisions; available on request).
4. *Sulfanilamide reagent (*"*SAN*"*)*: Use a graduated cylinder to dispense 100 mL of concentrated hydrochloric acid (HCl, 36.5–38.0 percent, ≈12 ***M***) into about 700 mL of DI water in a 1-L volumetric flask. Work in a hood and manually swirl or magnetically stir the flask during each addition of HCl. Add 10.0 g of SAN (C6H8N2O2S, FW=172.20) to the warm hydrochloric acid solution. Manually shake, sonicate, or magnetically stir the contents

of the flask until the SAN dissolves. After the resulting solution has cooled, dilute it to the mark with DI water, mix it well, and transfer it to a clean 1-L plastic bottle in which it is stable for 1 year at room temperature.

5. *N-(1-Naphthyl)ethylenediamine dihydrochloride reagent (*"*NED*")*: Dissolve 1.0 g NED (C12H14N2•2HCl, FW=259.2) in about 800 mL of DI water in a 1-L volumetric flask. Dilute the resulting solution to the mark with DI water and mix well by manually shaking the flask. Transfer this reagent to a 1-L amber glass bottle in which it is stable for 6 months at room temperature.
6. *Startup/shutdown solution*: Add 250 µL of Brij-35 surfactant to 250 mL of DI water in a plastic bottle. Thoroughly rinse the bottle and prepare a fresh solution every few days or as needed.

2.4.7 Calibrants and Quality-Control Solutions

This section provides detailed instructions for preparing calibrants, matrix spike solution, quality-control check solutions, and digestion check solution.

1. *Potassium nitrate stock calibrant solution, 1 mL =2.5 mg-N*: Dissolve 1.805 g of potassium nitrate (KNO3, FW=101.1) in about 80 mL of DI water in a 100-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock calibrant to a 100-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.
2. *Potassium di-hydrogen phosphate stock calibrant solution, 1 mL =1.0 mg-P*: Dissolve 0.4394 g potassium di-hydrogen phosphate (KH2PO4, FW=136.09) in about 80 mL of DI water in a 100-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock calibrant to a 100-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.
3. *Sulfuric acid* ≈1.8 ***M***: Use a 25-mL graduated cylinder to dispense 10 mL of concentrated sulfuric acid (H2SO4, sp. gr. 1.84) into about 75 mL of DI water in a 100-mL volumetric flask. After the solution cools, dilute it to the mark with DI water, mix well, and transfer it to a 125-mL plastic bottle. Make a new batch of this acid each time acidified working calibrants and blanks are prepared and use the remainder to prepare acidified blank solution as needed.
4. *Mixed stock calibrant solution, 1 mL = 1.25 mg-N and 0.5 mg-P*: Dispense equal volumes (minimum of 2 mL each) of nitrate and phosphate stock calibrants into a small beaker and mix them thoroughly. Prepare this solution each time working calibrants are prepared.
5. *Working calibrant solutions (for FCC samples)*: Use two adjustable, digital pipets (ranges 10 to 100 µL and 100 to 1,000 µL) to dispense the volumes of mixed stock calibrant (7.4) listed in table 4 into 250-mL volumetric flasks that each contains about 200 mL of DI water. Dilute the working calibrants to the mark with DI water and mix them thoroughly by manual inversion and shaking. Transfer the working calibrants to 250-mL Pyrex™ media bottles in which they are stable for 4 weeks at 4°C.
6. *Acidified working calibrant solutions (for FCA and WCA samples)*: Prepare these calibrants identically to those described in section 7.5, except add 2.5 mL of 1.8 ***M*** H2SO4 to each flask before diluting it to the mark with DI water.
7. *Check standards (for FCC samples)*: Check standards in three concentration ranges, which were designated *Low*, *High*, and *Very high*, were prepared from a concentrated commercial nutrient QC mixture (*Demand*™, Environmental Resource Associates, Arvada, Colo.), as

listed in table 5. Transfer check standards to 1-L Pyrex™ media bottles in which they are stable for 2 months at 4ºC. Each of these check standards was dispensed, digested, and analyzed along with every batch of filtered and whole-water samples analyzed for this study.

8. *Acidified check standards (for FCA and WCA samples)*: Prepare these check standards identically to those described in section 7.7, except add 10.0 mL of 1.8 ***M*** H2SO4 to the flasks before diluting them to the mark with DI water.

9. Spike Solutions

   a. *Nitrogen stock spike solution (1 mL = 0.50 mg-N)*: Dissolve 0.955 g ammonium chloride (NH4Cl, FW=53.49) in about 400 mL of DI water in a 500-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock spike solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

   b. *Phosphorus stock spike solution (1 mL = 0.20 mg-P)*: Dissolve 0.439 g potassium dihydrogen phosphate (KH2PO4, FW=136.1) in about 400 mL of DI water in a 500-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock spike solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

   c. *Mixed spike solution (100 µL = 0.005 mg-N and 0.002 mg-P)*: Dispense 1 mL each of ammonium chloride and orthophosphate stock spike solutions into a 10-mL volumetric flask and dilute to the mark with DI water. Transfer the mixed spike solution to a 15-mL, screw-cap polyethylene centrifuge tube in which it is stable for 2 weeks at 4°C.

NOTE: An equivalent mixed spike solution can be prepared more conveniently from stock calibrants by diluting 500 µL of each to 25 mL in a volumetric flask.

10. Digest-Check Stock Solutions

   a. *Glycine digest-check stock solution (1 mL = 1.0 mg-N)*: Dissolve 3.98 g glycine (C2H5NO2•HCl, FW=111.5) in about 400 mL of DI water in a 500-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

   b. *Glycerophosphate digest-check stock solution (1 mL = 0.4 mg-P)*: Dissolve 1.976 g glycerophosphate (C3H7O6PNa2•5H2O, FW=306.1) in about 400 mL of DI water in a 500-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

   c. *Glucose digest-check stock solution (1 mL = 1.25 mg-C)*: Dissolve 1.564 g glucose (C6H12O6, FW=180.2) in about 400 mL of DI water in a 500-mL volumetric flask. Dilute this solution to the mark with DI water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

   d. *Mixed digest-check solution (for FCC samples—nominal concentration 4 mg-N/L, 1.6 mg-P/L, and 50 mg-C/L)*: Dispense 1 mL each of glycine and glycerophosphate stock digest-check solutions and 10 mL of the glucose digest-check stock solution into a 250-mL volumetric flask that contains about 200 mL of DI water. Dilute the contents of the flask to the mark with DI water and mix it thoroughly by manual inversion and shaking.

Transfer the stock digest-check solution to a 250-mL Pyrex™ media bottle in which it is stable for 1 month at 4°C.

e. *Acidified mixed digest-check solution (for FCA and WCA samples)*: Prepare this digest-check solution identically to the one described ealier, except add 2.5 mL of 1.8 ***M*** H2SO4 to the flask before diluting its contents to the mark with DI water. Transfer the acidified mixed digest-check solution to a 250-mL Pyrex™ media bottle in which it is stable at 4°C for 1 month.

Table 4. Volumes of mixed calibrant and amendment solution required to prepare working calibrants and blanks for determination of total nitrogen and phosphorus by the alkaline persulfate digestion method. Final volumes are 250 mL [µL, microliter; mL, milliliter; mg-N/L, milligrams nitrogen per liter; mg-P/L, milligrams phosphorus per liter; ***M***, molarity (moles per liter); FCA, filtered, chilled, acidified (bottle type); WCA, whole water, chilled, acidified (bottle type)]

Calibrant identity	Mixed calibrant volume (µL)	Volume 1.8 M H_2SO_4 [1] (mL)	Nominal concentration (mg-N/L)	Nominal concentration (mg-P/L)
C1	1,000	2.5	5.00	2.00
C2	750	2.5	3.75	1.50
C3	500	2.5	2.50	1.00
C4	250	2.5	1.25	0.50
C5	100	2.5	0.50	0.20
C6	6[2]	2.5[2]	0.03	0.012
C7	0	2.5	0	0

[1] Add H2SO4 only to acidified calibrants. [2] Prepare 1 L of C6 (24 µL of mixed calibrant and 10 mL of 1.8 ***M*** H2SO4, if appropriate, diluted to 1 L with DI water) to minimize dispensing error.

Table 5. Volumes of Environmental Resource Associates (ERA) *Demand*™ nutrient concentrate used to prepare 1-liter volumes of check standards used in this study

Check standard identity	ERA *Demand*™ volume (µL)	Volume 1.8 *M* H2SO4 1 (mL)[1]	Nominal concentration (mg–N/L)	Nominal concentration (mg–P/L)
Low	100	10.0	0.22	0.11
High	500	10.0	1.09	0.54
Very high	1,000	10.0	2.20	1.08

[1]Add H2SO4 only to acidified check standards as described in section 7.8.

2.4.8 Sample Preparation

Alkaline persulfate digests are prepared by dispensing samples and digestion reagent into 30-mL, screw-cap, Pyrex™ culture tubes in the volume ratio of 2 + 1. For filtered samples (FCC bottle types) that were prepared robotically, 9.5-mL volumes of samples, blanks, calibrants, and reference materials were dosed with 4.75-mL volumes of alkaline persulfate digestion reagent. This is the maximum sample volume that could be delivered by the robotic dispenser/diluter system's 10.000-mL syringes because 0.500 mL of their capacity is expended in the creation of air gaps that minimize interaction between samples and the DI water carrier fluid. Whole-water

samples (WCA bottle types) that require vigorous shaking (and in a few cases, continuous magnetic stirring) just prior to dispensing operations were prepared manually with conventional, high-precision, hand-held electronic pipets (Rainin EDP *Plus*™). Here dispensed volumes of sample and digestion reagent were 10.000 and 5.000 mL, respectively. After robotic or manual sample and reagent-dispensing operations are complete, 100 µL of mixed spike solutio is added manually to the designated tube. Then all tubes are capped tightly and mixed thoroughly either by manual inversion (three times) or with a vortex mixer (3, 5-second cycles). The capped tubes positioned in a purpose-built, 80-position stainless-steel rack then are placed in an autoclave where they are digested at 121ºC and 117.2 kPa for 1 hour. Table 6 lists the rack protocol suggested for a batch of 80 tubes consisting of up to 64 samples plus six calibrants, four blanks, three quality-control (QC) check solutions, one digest-check solution, one duplicate sample, and one spiked sample. A step-by-step procedure for alkaline persulfate digest preparation is provided in NWQL SOP IM0384.0.

NOTE: When samples contain large quantities of suspended solids, continuous stirring during sample aspiration might provide the only means of obtaining representative aliquots.

When the digestion cycle is complete and pressure and temperature gages on the autoclave indicate 0 kPa and less than 80°C, remove the alkaline persulfate digests from the autoclave and allow them to cool sufficiently to be handled comfortably. Then mix the contents of each capped digestion tube by manual inversion (three times) or with a vortex mixer (three, 5-second cycles). FCC and FCA digests can be poured into analyzer cups immediately after mixing. Wait about 1 hour after mixing WCA digests to allow suspended solids to settle. If it is not possible to decant or pipet a clear supernatant solution from digest tubes into analyzer cups, then suspended solids must be removed by 0.45-µm filtration prior to colorimetric analysis. Note that tightly capped digests can be stored at room temperature for several days (4 days was the maximum delay tested) before their nitrogen and phosphorus concentrations are determined by automated colorimetry.

2.4.9 Instrument Performance

An 80-tube batch of samples, calibrants, and reference materials can be prepared robotically and made ready for digestion in about 1 hour. Digestion time—including warm up, cool down, and postdigestion mixing—is about 2 hours. The NWQL Nutrients Unit has two autoclaves, each of which can hold two, 80-tube racks of alkaline persulfate digests. Nitrate and orthophosphate in alkaline persulfate digests can be determined simultaneously with the 2-channel air-segmented continuous flow analyzer at a rate of about 100 samples per hour with less than 1 percent interaction. Thus, using a combination of robotic and manual sample preparation, up to six racks (384 actual samples out of 480 total tubes) of alkaline persulfate digests can be prepared in an 8-hour day. This estimate assumes the use of both NWQL autoclaves and a combination of robotic (FCC samples) and manual (WCA samples) sample preparation. Likewise, up to six racks of previously digested samples can be analyzed for nitrate and orthophosphate in an 8-hour day. This production rate assumes that digest analysis can lag sample digestion by 1 to 3 days.

2.4.10 Calibration

With a second-order polynomial least-squares curve-fitting function ($y = a+bx+cx2$, where y is the baseline and blank-corrected peak height and x is the nominal concentration), calibration plots with correlation coefficients (r2) greater than 0.999 are achieved routinely. Typical calibration plots for nitrate and orthophosphate in alkaline persulfate digests are shown in Figures 3 and 4.

NOTE: In addition to baseline drift correction, a digestion blank correction must be applied to calibrants, check standards, and samples prior to calculation of final results.

2.4.11 Procedure and Data Evaluation

Set up the continuous flow analyzer analytical cartridges as shown in figures 1 and 2. Turn on electrical power to all system modules and put fresh sampler wash reservoir solution and reagents on-line. After about 10 minutes, verify that the sample and reference outputs of both photometers are set at about 5 volts. A suggested sampler tray protocol for automated determination of nitrate and orthophosphate in alkaline persulfate digests is listed in table 7.

NOTE: To minimize errors that result from contaminated analyzer cups, rinse them several times with the solution they are to contain before placing them on the analyzer sampler tray.

NOTE: The full-scale absorbance range control (STD CAL) of photometers should not require daily adjustment. Between-analysis/between-day variations in baseline-absorbance level and calibration curve slope of about ±5 percent are acceptable. Adjustment of the STD CAL control to compensate for larger variations in sensitivity or baseline (reagent blank) levels will only mask underlying problems, such as incipient light source failure, partially clogged flow cells, or contaminated or improperly prepared reagents, any of which could compromise analytical results.

Table 6. Suggested rack protocol for alkaline persulfate digest preparation [ID, identification; QC, quality control; yyyy, year; ddd, Julian day]

Tube number	ID	Tube number	ID	Tube number	ID	Tube number	ID
1	C1	21	yyyyddd007	41	yyyyddd027	61	yyyyddd047
2	C2	22	yyyyddd008	42	yyyyddd028	62	yyyyddd048
3	C3	23	yyyyddd009	43	yyyyddd029	63	yyyyddd049
4	C4	24	yyyyddd010	44	yyyyddd030	64	yyyyddd050
5	C5	25	yyyyddd011	45	yyyyddd031	65	yyyyddd051
6	C6	26	yyyyddd012	46	yyyyddd032	66	yyyyddd052
7	C7 (blank)	27	yyyyddd013	47	yyyyddd033	67	yyyyddd053
8	blank	28	yyyyddd014	48	yyyyddd034	68	yyyyddd054
9	blank	29	yyyyddd015	49	yyyyddd035	69	yyyyddd055
10	blank	30	yyyyddd016	50	yyyyddd036	70	yyyyddd056
11	QC low	31	yyyyddd017	51	yyyyddd037	71	yyyyddd057
12	Digest check	32	yyyyddd018	52	yyyyddd038	72	yyyyddd058
13	QC high	33	yyyyddd019	53	yyyyddd039	73	yyyyddd059
14	QC very high	34	yyyyddd020	54	yyyyddd040	74	yyyyddd060
15	yyyyddd0001	35	yyyyddd021	55	yyyyddd041	75	yyyyddd061
16	yyyyddd0002	36	yyyyddd022	56	yyyyddd042	76	yyyyddd062
17	yyyyddd0003	37	yyyyddd023	57	yyyyddd043	77	yyyyddd063
18	yyyyddd0004	38	yyyyddd024	58	yyyyddd044	78	yyyyddd064
19	yyyyddd0005	39	yyyyddd025	59	yyyyddd045	79	Duplicate
20	yyyyddd0006	40	yyyyddd026	60	yyyyddd046	80	Spike

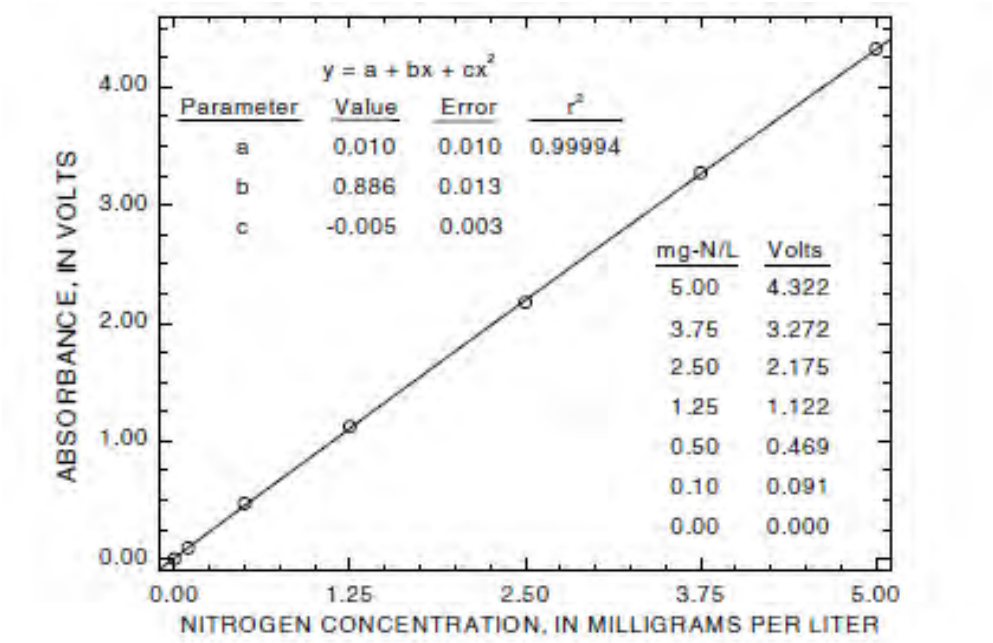

Figure 3. Typical calibration graph for total nitrogen determined as nitrate in alkaline persulfate digests.

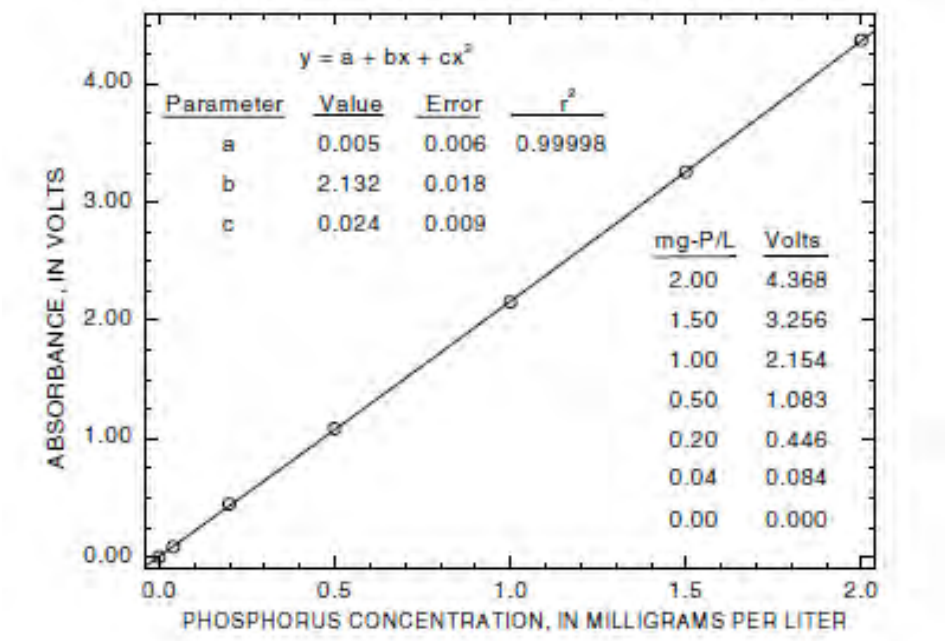

Figure 4. Typical calibration graph for total phosphorus determined as orthophosphate in alkaline persulfate digests.

Table 7. Suggested analyzer sample tray protocol for automated determination of nitrate and orthophosphate in alkaline persulfate digests [#, number; ID, identification; SYNC, synchronization peak; CO, carry-over peak; W, wash; UB, undigested blank; DB, digested blank; CCV, continuing calibration verification; QC, quality control; yyyy, year; ddd, Julian day]

Cup #	ID	Cup #	ID	Cup #	ID	Cup #	ID
1	SYNC	24	yyyyddd006	47	yyyyddd029	70	yyyyddd050
2	CO (C6)	25	yyyyddd007	48	yyyyddd030	71	yyyyddd051
3	(C6)	26	yyyyddd008	49	yyyyddd031	72	yyyyddd052
4	W	27	yyyyddd009	50	yyyyddd032	73	yyyyddd053
5	C1	28	yyyyddd010	51	UB	74	yyyyddd054
6	C2	29	yyyyddd011	52	W (DB)	75	yyyyddd055
7	C3	30	yyyyddd012	53	yyyyddd033	76	yyyyddd056
8	C4	31	yyyyddd013	54	yyyyddd034	77	yyyyddd057
9	C5	32	yyyyddd014	55	yyyyddd035	78	yyyyddd058
10	C6	33	yyyyddd015	56	yyyyddd036	79	yyyyddd059
11	C7	34	yyyyddd016	57	yyyyddd037	80	yyyyddd060
12	W	35	yyyyddd017	58	yyyyddd038	81	yyyyddd061
13	CCV	36	yyyyddd018	59	yyyyddd039	82	yyyyddd062
14	UB[1]	37	yyyyddd019	60	yyyyddd040	83	yyyyddd063
15	QC low[2]	38	yyyyddd020	61	yyyyddd041	84	yyyyddd064
16	Digest check[3]	39	yyyyddd021	62	yyyyddd042	85	Duplicate
17	QC high[2]	40	yyyyddd022	63	yyyyddd043	86	Spike
18	QC very high[2]	41	yyyyddd023	64	yyyyddd044	87	UB
19	yyyyddd0001	42	yyyyddd024	65	yyyyddd045	88	CCV
20	yyyyddd0002	43	yyyyddd025	66	yyyyddd046	89	UB
21	yyyyddd0003	44	yyyyddd026	67	yyyyddd047	90	W (DB)
22	yyyyddd0004	45	yyyyddd027	68	yyyyddd048		
23	yyyyddd0005	46	yyyyddd028	69	yyyyddd049		

1Undigested blank (sampler wash reservoir solution, see section 6.2.1). 2NWQL Check Standard, see sections 7.7 and 7.8. 3Digest-check sample; see sections 7.10.4 and 7.10.5.

2.4.12 Calculations

Instrument calibration requires preparing a set of solutions (calibrants) in which the analyte concentration is known. These calibrants are digested along with samples and used to establish a calibration function that is estimated from a least-squares fit of nominal calibrant concentrations (x) in relation to peak absorbance (y). A second-order polynomial function ($y = a+bx+cx2$) usually provides improved concentration estimates at the upper end of the calibration range than a more conventional linear function ($y = a+bx$). Accuracy is not lost when a second-order fit is used, even if the calibration function is strictly linear, because, in this case, the value estimated for the quadratic parameter c will approach zero.

Before the calibration function can be estimated, the baseline absorbance component of measured peak heights, including drift (continuous increase or decrease in the baseline absorbance during the course of an analysis), if present, needs to be removed. Baseline absorbance in continuous flow analysis is analogous to the reagent blank absorbance in batch analysis. Correction for baseline absorbance is an automatic function of most data acquisition and processing software sold by vendors of continuous flow analyzers.

NOTE: These correction algorithms are based on linear interpolation between initial and intermediate or final baseline measurements, and so they do not accurately correct for abrupt,

step-changes in baseline absorbance that usually indicate partial flow-cell blockage. It is prudent, therefore, to reestablish baseline absorbance at intervals of 20 samples or so.

After peaks are baseline corrected, they need to be digestion-blank corrected. This correction can be applied in several ways:

1. Subtract the baseline-corrected absorbance of the digestion blank—compute an average concentration if multiple digested blanks are included in each block—from the baseline-corrected absorbance of all calibrants, check standard, and samples in the block. Then estimate regression parameters (a, b, and c terms) for the calibration function by using a second-order polynomial least-squares algorithm. For second and higher order calibration functions, use the Newton-Raphson successive approximations algorithm (Draper and Smith, 1966; Swartz, 1976, 1977, 1979) to convert corrected peak heights into concentrations.
2. Designate digestion blanks as a calibrant with a nominal concentration of zero. In this case the resulting calibration function will have a positive *y*-intercept that approximates the baseline-corrected absorbance of the digestion blank. If this method is used, be sure that the curve-fitting algorithm does not force a zero *y*-intercept by including one or more "dummy" (0,0) points in the data set used for calibration.
3. Designate digested blanks as baseline correction samples—that is, "W" in the FasPac™ software used to acquire and process data at the NWQL. In this case initial, intermediate (if included), and final baselines are interpolated between digested blank peak maxima. Thus, baseline and digestion blanks are corrected in a single operation.

NOTE: Digestion blanks were corrected for data in this report by using method 3. However, analytical results calculated by the other two methods should be equivalent. Regardless of the blank correction algorithm chosen, make sure that it is documented in the SOP and that analysts understand it. The SOP for these methods must be updated whenever any changes in data acquisition and processing software or in calculation algorithms are implemented.

Most software packages provide a data base for entering appropriate dilution factors. Usually these factors can be entered before or after samples are analyzed. If dilution factors are entered, reported concentrations will be compensated automatically for the extent of dilution. The dilution factor is the number by which a measured concentration must be multiplied to obtain the analyte concentration in the sample prior to dilution. For example, dilution factors of 2, 5, and 10 indicate that sample and diluent were combined in proportions of 1+1, 1+4, and 1+9, respectively.

2.4.13 Reporting Results

Total nitrogen (lab codes 2754, 2755, 2756)

- 2 decimal places for concentrations up to 5.00 mg-N/L
- 2 significant figures for concentrations greater than 5.00 mg-N/L

Total phosphorus (lab codes 2757, 2758, 2759)

- 2 decimal places for concentrations up to 2.00 mg-P/L
- 2 significant figures for concentrations greater than 2.00 mg-P/L

2.4.14 Detection Levels, Bias, and Precision

1. Method detection limits (MDL) for composited, low-concentration FCC and WCA samples (five of each) were estimated using the U.S Environmental Protection Agency (1997)

protocol—see Table 8. Target concentrations for nitrogen and phosphorus in FCC and WCA composite samples were 0.05 mg-N/L and 0.02 mg-P/L, respectively. The MDL for nitrogen was 0.015 mg-N/L and for phosphorus was 0.007 mg-P/L. Laboratory reporting levels (LRL) will be about twice the MDL concentrations.

2. Table 9 lists the average and standard deviation of 9987L, 9987H, and 9987VH QC check solutions that were included in every rack of alkaline persulfate digests. Most probable values (MPVs) and standard deviations in table 9 were published by the USGS Branch of Quality Systems for the 2002 water year (12-month period ending September 30 each year is called the "water year"). In all cases, total nitrogen and total phosphorus concentrations determined for these reference materials by the alkaline persulfate digestion method were tightly centered around published MPVs and well within published control limits.

3. Spike Recoveries: Median, 90th and 10th percentiles of percent spike recoveries measured in samples collected during high-flow and low-flow conditions are listed in table 10. Median spike recoveries for nitrogen (0.5 mg-N/L as glycine) ranged from about 92 to100 percent and for phosphorus (0.2 mg-P/L as glycerophosphate) from about 86 to 108 percent.

4. Duplication of Results: Median, 10% percentiles, and 90% percentiles for concentration differences for duplicate samples collected during the nominally high- and low-flow conditions are listed in table 11. Median concentration differences between duplicate analyses are about the same as the MDLs. Larger tenth-percentile differences for whole-water samples that were collected during nominally high-flow conditions in relation to those of filtered water samples likely reflect the difficulty of obtaining reproducible aliquots from samples that contain large amounts of suspended solids. Such samples were purposely chosen as duplicates to assess "worst-case" digest-preparation sampling precision.

Table 8. Data and calculations used to estimate method detection limits (MDL) for nitrogen and phosphorus in unacidified (FCC) and acidified (WCA) samples following alkaline persulfate digestion. Low-concentration FCC and WCA samples (five of each) were composited for these determinations [mg-N (-P)/L, milligrams nitrogen (or phosphorus) per liter; %, percent; MDL, method detection limit]

Target concentration [mg-N (-P)/L]	Concentration found (mg-N/L or mg-P/L)			
	Dissolved nitrogen (unacidified)	**Total nitrogen (acidified)**	**Dissolved phosphorus (unacidified)**	**Total phosphorus (acidified)**
0.05 (0.02)	0.064	0.041	0.026	0.033
0.05 (0.02)	.078	.042	.024	.029
0.05 (0.02)	.072	.035	.026	.029
0.05 (0.02)	.066	.035	.029	.027
0.05 (0.02)	.067	.032	.026	.029
0.05 (0.02)	.066	.039	.023	.027
0.05 (0.02)	.071	.026	.022	.026
0.05 (0.02)	.063	.035	.026	.026
Average	**.068**	**.035**	**.025**	**.028**
Standard deviation	.005	.005	.002	.002
Number of values	8	8	8	8
Degrees of freedom	7	7	7	7
t-value (1-sided, 99%)	2.998	2.998	2.998	2.998
MDL	0.15	0.15	.007	.007

Table 9. Most probable values and standard deviations for reference samples 9987L, 9987H, and 9987VH along with averages and standard deviations of these reference materials that were included in every rack of alkaline persulfate digests [ID, identification of reference sample; MPV, most probable value; FCC, filtered, chilled (bottle type); WCA, whole water, chilled, acidified (bottle type); mg-N/L, milligrams nitrogen per liter; mg-P/L, milligrams phosphorus per liter; ±, plus or minus]

ID	MPV	High-flow samples		Low-flow samples	
		FCC[1]	WCA[2]	FCC[3]	WCA[4]
	Alkaline persulfate dissolved and total nitrogen concentration (mg-N/L)				
9987L	0.22 ± 0.08	0.21 ± 0.03	0.21 ± 0.03	0.19 ± 0.03	0.20 ± 0.02
9987H	1.09 ± 0.15	1.09 ± 0.03	1.09 ± 0.03	1.06 ± 0.08	1.04 ± 0.04
9987VH	2.20 ± 0.24	2.27 ± 0.05	2.18 ± 0.06	2.16 ± 0.07	2.13 ± 0.06
	Alkaline persulfate dissolved and total phosphorus concentration (mg-P/L)				
9987L	0.108 ± 0.008	0.105 ± 0.004	0.104 ± 0.004	0.107 ± 0.006	0.105 ± 0.004
9987H	0.54 ± 0.02	0.54 ± 0.01	0.55 8 0.02	0.57 ± 0.02	0.54 ± 0.01
9987VH	1.08 ± 0.05	1.13 ± 0.02	1.10 ± 0.03	1.13 ± 0.03	1.09 ± 0.02

1Number of points: n = 19; 2n = 21; 3n = 21; 4n = 18.

2.5 TOTAL PHOSPHORUS AND FRESHWATER ORTHOPHOSPHATE

2.5.1 Scope and Application

1. This method covers the determination of specified forms of phosphorus in marine or freshwater, and the determination of orthophosphate in freshwater. To determine orthophosphate is saltwater, use the method outlined in section 2.6.
2. The methods are based on reactions specific for the orthophosphate ion. The most commonly measured forms are total and dissolved phosphorus, total and dissolved orthophosphate. Hydrolyzable phosphorus is normally found only in sewage-type samples. Insoluble forms of phosphorus are determined by calculation.
3. The applicable range is 0.01-1.0 mg P/L. 20 - 30 samples per hour can be analyzed.

2.5.2 Summary of Method

1. Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phosphomolybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.
2. Only orthophosphate forms a blue color in this test. Polyphosphates (and some organic phosphorus compounds) may be converted to the orthophosphate form by manual sulfuric acid hydrolysis. Organic phosphorus compounds may be converted to the orthophosphate form by manual persulfate digestion. The developed color is measured automatically.
3. Reduced volume versions of this method that use the same reagents and molar ratios are acceptable provided they meet the quality control and performance requirements stated in the method.

2.5.3 Interferences

1. No interference is caused by copper, iron, or silicate at concentrations many times greater than their reported concentration in seawater. However, high iron concentrations can cause precipitation of, and subsequent loss, of phosphorus.
2. The salt error for marine samples ranging from 5-20% salt content was found to be <1%.
3. Arsenate is determined similarly to phosphorus and should be considered when present in concentrations higher than phosphorus. However, at concentrations found in sea water, it does not interfere.
4. Sample turbidity must be removed by filtration prior to analysis for orthophosphate. Samples for total or total hydrolyzable phosphorus should be filtered only after digestion. Sample color that absorbs in the photometric range used for analysis will also interfere.

2.5.4 Safety

1. Sulfuric acid (Sections 2.5.6.2 and 2.5.6.7) has the potential to be highly toxic or hazardous, consult Material Safety Data Sheet.

2.5.5 Equipment and Supplies

1. Balance -- Analytical, capable of accurately weighing to the nearest 0.000l g.

2. Glassware -- Class A volumetric flasks and pipets as required. All glassware used in the determination must be washed with hot 1:1 HCl and rinsed with distilled water. The acid-washed glassware should be filled with distilled water and treated with all the reagents to remove the last traces of phosphorus that might be adsorbed on the glassware.
3. Hot plate or autoclave.
4. Automated continuous flow analysis equipment designed to deliver and react sample and reagents in the required order and ratios.
5. Sampling device (sampler)
6. Multichannel pump
7. Reaction unit or manifold
8. Colorimetric detector
9. Data recording device

2.5.6 Reagents and Standards

1. Reagent water: Distilled or deionized water, free of the analyte of interest. ASTM type II or equivalent.
2. Sulfuric acid solution, 5N: Slowly add 70 mL of conc. H_2SO_4 (CASRN 7664-93-9) to approximately 400 mL of reagent water. Cool to room temperature and dilute to 500 mL with reagent water.
3. Antimony potassium tartrate solution: Weigh 0.3 g $K(SbO)C_4H_4O_6 \bullet ½H_2O$ (CASRN 28300-74-5) and dissolve in 50 mL reagent water in 100 mL volumetric flask, dilute to volume. Store at 4°C in a dark, glass-stoppered bottle.
4. Ammonium molybdate solution: Dissolve 4 g $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ (CASRN 12027-67-7) in 100 mL reagent water. Store in a plastic bottle at 4°C.
5. Ascorbic acid, 0.1M: Dissolve 1.8 g of ascorbic acid (CASRN 50-81-7) in 100 mL of reagent water. The solution is stable for about a week if prepared with water containing no more than trace amounts of heavy metals and stored at 4°C.
6. Combined reagent: Mix the above reagents in the following proportions for 100 mL of the mixed reagent: 50 mL of 5N H_2SO_4, 5 mL of antimony potassium tartrate solution, 15 mL of ammonium molybdate solution, and 30 mL of ascorbic acid solution. Mix after addition of each reagent. All reagents must reach room temperature before they are mixed and must be mixed in the order given. If turbidity forms in the combined reagent, shake and let stand for a few minutes until the turbidity disappears before processing. This volume is sufficient for a four hour operation. Since the stability of this solution is limited, it must be freshly prepared for each run.

Note: *A stable solution can be prepared by not including the ascorbic acid in the combined reagent. If this is done, the mixed reagent (molybdate, tartrate, and acid) is pumped through the distilled water line and the ascorbic acid solution (30 mL of 7.5 diluted to 100 mL with reagent water) through the original mixed reagent line.*

7. Sulfuric acid solution, 11 N: Slowly add 155 mL conc. H_2SO_4 to 600 mL reagent water. When cool, dilute to 500 mL.
8. Ammonium persulfate (CASRN 7727-54-0).

9. Acid wash water: Add 40 mL of sulfuric acid solution to 1 L of reagent water and dilute to 2 L. (Not to be used when only orthophosphate is being determined).
10. Phenolphthalein indicator solution (5 g/L): Dissolve 0.5 g of phenolphthalein (CASRN 77-09-8) in a solution of 50 mL of isopropyl alcohol (CASRN 67-63-0) and 50 mL of reagent water.
11. Stock phosphorus solution: Dissolve 0.4393 g of predried (105°C for one hour)
12. Potassium phosphate monobasic KH_2PO_4 (CASRN 7778-77-0) in reagent water and dilute to 1000 mL. 1.0 mL = 0.1 mg P.
13. Standard phosphorus solution: Dilute 10.0 mL of stock solution to 100 mL with reagent water. 1.0 mL = 0.01 mg P.
14. Standard phosphorus solution: Dilute 10.0 mL of standard solution to 100 mL with reagent water. 1.0 mL = 0.001 mg P.

2.5.7 Sample Collection, Preservation and Storage

1. Samples must be preserved with H_2SO_4 to a pH <2 and cooled to 4°C at time of collection.
2. Samples should be analyzed as soon as possible after collection. If storage is required, preserved samples are maintained at 4°C and may be held for up to 28 days.

2.5.8 Quality Control

Note. Each laboratory using this method is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability, and the periodic analysis of laboratory reagent blanks, fortified blanks and other laboratory solutions as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data that are generated.

2.5.8.1 Initial Demonstration of Performance

1. The initial demonstration of performance is used to characterize instrument performance (determination of LCRs and analysis of QCS) and laboratory performance (determination of MDLs) prior to performing analyses by this method.
2. Linear Calibration Range (LCR) -- The LCR must be determined initially and verified every six months or whenever a significant change in instrument response is observed or expected. The initial demonstration of linearity must use sufficient standards to insure that the resulting curve is linear. The verification of linearity must use a minimum of a blank and three standards. If any verification data exceeds the initial values by ±10%, linearity must be reestablished. If any portion of the range is shown to be nonlinear, sufficient standards must be used to clearly define the nonlinear portion.
3. Quality Control Sample (QCS) -- When beginning the use of this method, on a quarterly basis or as required to meet data-quality needs, verify the calibration standards and acceptable instrument performance with the preparation and analyses of a QCS. If the determined concentrations are not within ±10% of the stated values, performance of the determinative step of the method is unacceptable. The source of the problem must be identified and corrected before either proceeding with the initial determination of MDLs or continuing with on-going analyses.

4. Method Detection Limit (MDL) -- MDLs must be established for all analytes, using reagent water (blank) fortified at a concentration of two to three times the estimated instrument detection limit. To determine MDL values, take seven replicate aliquots of the fortified reagent water and process through the entire analytical method. Perform all calculations defined in the method and report the concentration values in the appropriate units. Calculate the MDL as follows:

   MDL = (t) x (S)

   where,

   t = value for a 99% confidence level and a standard deviation estimate with n-1 degrees of freedom [t = 3.14 for seven replicates]

   S = standard deviation of the replicate analyses

   MDLs should be determined every six months, when a new operator begins work, or whenever there is a significant change in the background or instrument response.

2.5.8.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB) -- The laboratory must analyze at least one LRB with each batch of samples. Data produced are used to assess contamination from the laboratory environment. Values that exceed the MDL indicate laboratory or reagent contamination should be suspected and corrective actions must be taken before continuing the analysis.
2. Laboratory Fortified Blank (LFB) -- The laboratory must analyze at least one LFB with each batch of samples. Calculate accuracy as percent recovery. If the recovery of any analyte falls outside the required control limits of 90-110%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.
3. The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20-30 analyses), optional control limits can be developed from the percent mean recovery (x) and the standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

   Upper Control Limit = x + 3S

   Lower Control Limit = x - 3S

   The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20-30 data points. Also, the standard deviation (S) data should be used to establish an on-going precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.
4. Instrument Performance Check Solution (IPC) -- For all determinations the laboratory must analyze the IPC (a mid-range check standard) and a calibration blank immediately following daily calibration, after every tenth sample (or more frequently, if required) and at the end of the sample run. Analysis of the IPC solution and calibration blank immediately following calibration must verify that the instrument is within ±10% of calibration. Subsequent analyses of the IPC solution must verify the calibration is still within ±10%. If the calibration cannot be verified within the specified limits, reanalyze the IPC solution. If the second analysis of the IPC solution confirms calibration to be outside the limits, sample analysis must be discontinued, the cause determined and/or in the case of drift the instrument

recalibrated. All samples following the last acceptable IPC solution must be reanalyzed. The analysis data of the calibration blank and IPC solution must be kept on file with the sample analyses data.

2.5.8.3 Assessing Analyte Recovery and Data Quality

1. Laboratory Fortified Sample Matrix (LFM) -- The laboratory must add a known amount of analyte to a minimum of 10% of the routine samples. In each case the LFM aliquot must be a duplicate of the aliquot used for sample analysis. The analyte concentration must be high enough to be detected above the original sample and should not be less than four times the MDL. The added analyte concentration should be the same as that used in the laboratory fortified blank.
2. Calculate the percent recovery for each analyte, corrected for concentrations measured in the unfortified sample, and compare these values to the designated LFM recovery range 90-110%. Percent recovery may be calculated using the following equation:

$$R = \frac{Cs - C}{s} \times 100$$

where,

R = percent recovery

Cs = fortified sample concentration

C = sample background concentration

s = concentration equivalent of analyte added to sample

3. If the recovery of any analyte falls outside the designated LFM recovery range and the laboratory performance for that analyte is shown to be in control, the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.
4. Where reference materials are available, they should be analyzed to provide additional performance data. The analysis of reference samples is a valuable tool for demonstrating the ability to perform the method acceptably.

2.5.9 Calibration and Standardization

1. Prepare a series of at least three standards, covering the desired range, and a blank by pipetting and diluting suitable volumes of working standard solutions into 100 mL volumetric flasks. Suggested ranges include 0.00-0.10 mg/L and 0.20-1.00 mg/L.
2. Process standards and blanks as described in section 2.5.10.
3. Set up manifold as shown in Figure 2.6.
4. Prepare flow system as described in section 2.5.10.
5. Place appropriate standards in the sampler in order of decreasing concentration and perform analysis.
6. Prepare standard curve by plotting instrument response against concentration values. A calibration curve may be fitted to the calibration solutions concentration/response data using computer or calculator based regression curve fitting techniques. Acceptance or control

limits should be established using the difference between the measured value of the calibration solution and the "true value" concentration.

7. After the calibration has been established, it must be verified by the analysis of a suitable quality control sample (QCS). If measurements exceed ±10% of the established QCS value, the analysis should be terminated and the instrument recalibrated. The new calibration must be verified before continuing analysis. Periodic reanalysis of the QCS is recommended as a continuing calibration check.

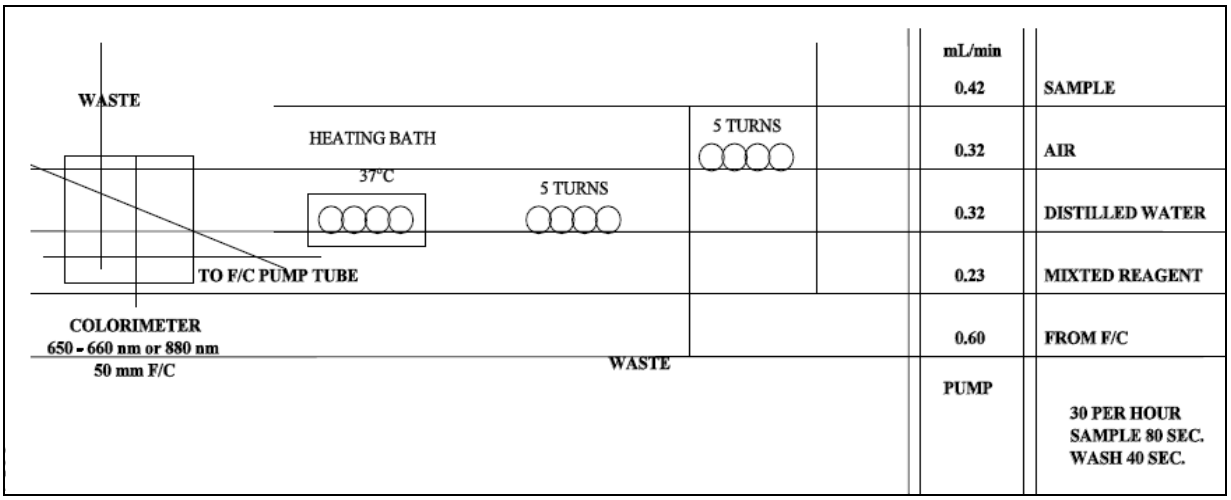

Figure 2.6. Phosporous Manifold

2.5.10 Procedure

2.5.10.1 Phosphorus

1. Add 1 mL of sulfuric acid solution to a 50 mL sample and/or standard in a 125 mL Erlenmeyer flask.
2. Add 0.4 g of ammonium persulfate.
3. Boil gently on a pre-heated hot plate for approximately 30-40 minutes or until a final volume of about 10 mL is reached. Do not allow sample to go to dryness. Alternately, heat for 30 minutes in an autoclave at 21°C (15-20 psi).
4. Cool and dilute the sample to 50 mL. If sample is not clear at this point, filter.
5. Determine phosphorus as outlined in Figure 2.7 with acid wash water in wash tubes.

2.5.10.2 Hydrolyzable Phosphorus

1. Add 1 mL of sulfuric acid solution to a 50 mL sample and/or standard in a 125 mL Erlenmeyer flask.
2. Boil gently on a pre-heated hot plate for 30-40 minutes until a final volume of about 10 mL is reached. Do not allow sample to go to dryness. Alternatively, heat for 30 minutes in an autoclave at 121°C (15-20 psi).
3. Determine phosphorus as outlined in Figure 2.7 with acid wash water in wash tubes.

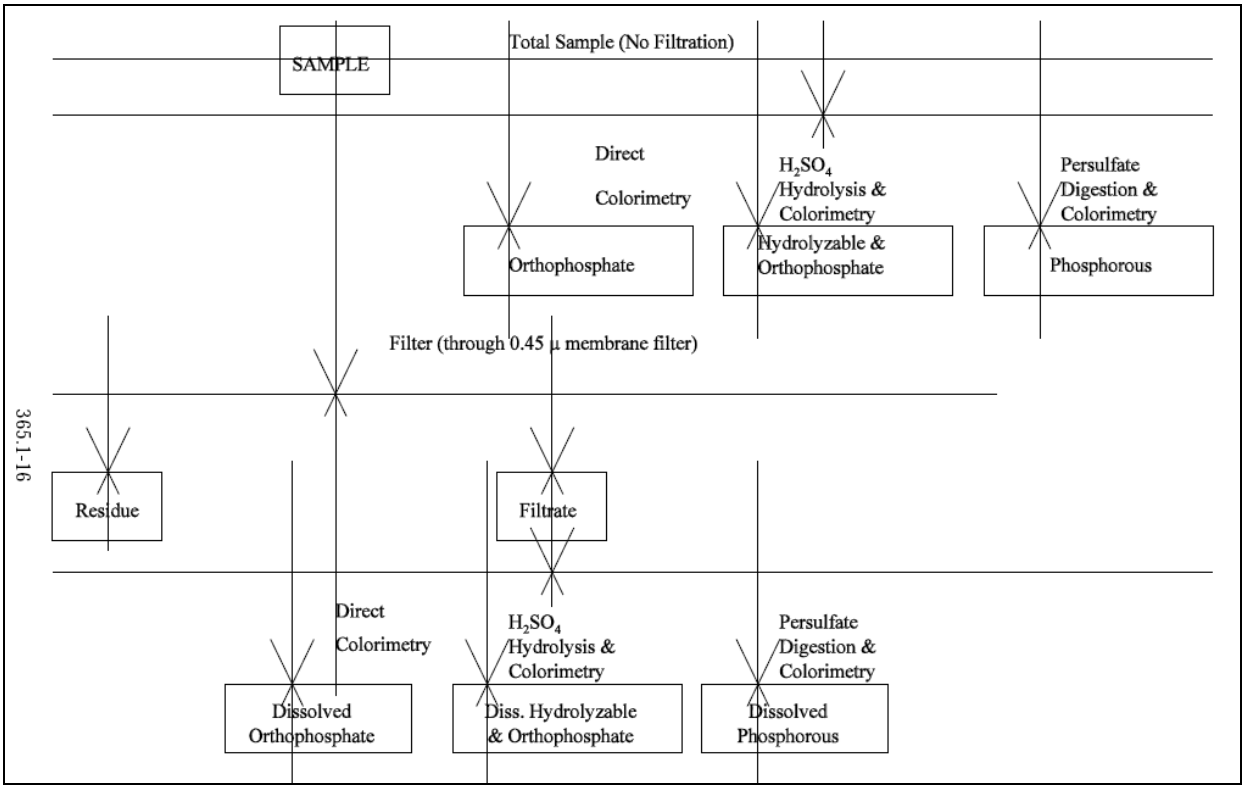

Figure 2.7. Analytical Scheme

2.5.11 Data Analysis and Calculations

1. Prepare a calibration curve by plotting instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve. Multiply answer by appropriate dilution factor.
2. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed. Any sample with a computed value is less than 5% of its immediate predecessor must be rerun.
3. Report results in mg P/L.

2.6 ORTHOPHOSPHATE (*Saltwater Only*)

2.6.1 Scope and Application

This method is for saltwater only. For determination of orthophosphate in freshwater, see method in section 2.5 above.

This method provides a procedure for the determination of low-level orthophosphate concentrations normally found in marine waters by way of automated colorimetric analysis. In this method, the two reagents are added separately for greater reagent stability and facility of sample separation.

2.6.2 Method Summary

Ammonium molybdate and antimony potassium tartrate react in an acidic medium with dilute solutions of phosphate to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color produced is proportional to the phosphate concentration present in the sample. Positive bias caused by differences in the refractive index of seawater and reagent water is corrected for prior to data reporting.

2.6.3 Interferences

1. Interferences caused by copper, arsenate and silicate are minimal relative to the orhtophosphate determination because of the extremely low concentrations normally found in estuarine and coastal waters. High iron concentrations can cause precipitation of and subsequent loss of phosphate from the dissolved phase. Hydrogen sulfide effects, such as occur in samples collected from deep anoxic basins, can be treated by simple dilution of the sample since high sulfide concentrations are most often associated with high phosphate values.
2. Sample turbidity is removed by filtration prior to analysis.
3. Refractive index interferences are corrected for in Section 2.1.9.

2.6.4 Equipment and Supplies

1. Continuous Flow Automated Analytical System:
    - Sampler
    - Manifold of analytical Cartridge equipped with 37°C heating bath
    - Proportioning pump
    - Colorimeter equipped with 1.5 x 50 mm tubular flow cell and 880 nm filter
    - Phototube that can be used for 600-900 nm range
    - Strip chart recorder or computer based data system
2. Phosphate-free glassware and polyethylene bottles
3. Membrane or glass fiber filters, 0.45 µm nominal pore size

2.6.5 Reagent and Standards

2.6.5.1 Stock Reagent Solutions

1. Ammonium Molybdate solution (40 g/L). dissolve 20.0 g of ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}•4H_2O$) in approximately 400 mL of reagent water and dilute to 500 mL. Store in a plastic bottle out of direct sunlight. This reagent is stable for approximately three months.
2. Antimony Potassium Tartrate solution (3.0 g/L). Dissolve 0.3 g of antimony potassium tartrate ($K(SbO)C_4H_4O_6•½H2O$) in approximately 90 mL of reagent water and dilute to 100mL. This reagent is stable for approximately three months.
3. Ascorbic Acid solution (18.0 g/L). Dissolve 18.0 g ascorbic acid ($C_6H_6O_6$) in approximately 800mL of reagent water and dilute to 1L. Dispense approximately 75mL into clean polyethylene bottles and freeze. The stability of frozen ascorbic acid is three month. Thaw overnight in the refrigerator before use. The stability of thawed reagent is less than 10 days.
4. Sodium Lauryl Sulfate solution (30.0 g/L). Sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$). Dissolve 3.0 g of sodium lauryl sulfate in approximately 80 mL of reagent water and dilute to 100 mL. This solution is the wetting agent and its stability is approximately three weeks.
5. Sulfuric Acid solution (4.9 N). Slowly add 136 mL of concentrated sulfuric acid (H_2SO_4) to approximately 800 mL reagent water. After the solution is cooled, dilute to 1 L with reagent water.
6. Stock Phosphorus solution. Dissolve 0.439 g of pre-dried (105°C for 1 hour) monobasic potassium phosphate (KH_2PO_4) in reagent water and dilute to 1000mL (1.0 mL = 0.100 mg P). The stability of this stock standard is approximately 3 months when kept refrigerated.
7. Low Nutrient Seawater. Obtain low nutrient seawater (36‰; <0.0003 mg P/L) or dissolve 31g analytical reagent grade sodium chloride, 10g analytical grade magnesium sulfate, and 0.05g analytical grade sodium bicarbonate in 1L of reagent water.

2.6.5.2 Working Reagents

1. Reagent A. Mix 100 mL of 4.9N H_2SO_4, 30 mL of ammonium molybdate solution, 10 mL of antimony potassium tartrate solution and 2.0 ml of sodium lauryl sulfate for a total volume of 142 mL. Prepare fresh daily.
2. Reagent B. Mix 0.5 mL of the sodium lauryl sulfate solution to 75 mL of ascorbic acid solution. Stability is about 10 days when refrigerated.
3. Refractive Reagent A. Add 50 mL of 4.9N H_2SO_4 to 20 mL of reagent water. Add 1 mL of sodium lauryl sulfate. Prepare fresh daily.
4. Secondary Phosphorous solution. Take 1.0 mL of Stock Phosphorous solution and dilute to 100 mL with reagent water (1.0 mL = 0.0010 ng P). Refrigerate and prepare fresh every 10 days.
5. Prepare a series of standards by diluting suitable volumes of standard solutions to 100 mL with reagent water. Prepare daily. When working with samples of known salinity, it

is recommended that the standard curve concentrations be prepared in low-nutrient seawater diluted to match the salinity of the samples. Doing so obviates the need to perform the refractive index correction outlined in section 2.4.10.1. When analyzing samples of varying salinities, it is recommended that the standard curve be prepared in reagent water and refractive index corrections be made to the sample concentrations. The following dilutions are suggested.

mL Secondary Phosphorous sol'n	Conc. mg P/L
0.1	0.0010
0.2	0.0020
0.5	0.0050
1.0	0.0100
2.0	0.0200
4.0	0.0400
5.0	0.0500

2.6.6 Sample Storage

Sample should be refrigerated and stored at 4°C for up to 24 hours. If samples cannot be analyzed within 24 hours, then freezing at -20°C for a maximum period of two months is acceptable.

2.6.7 Quality Control

Each laboratory using this method is required to implement a formal quality control (QC) program. The minimum requirements consist of an initial demonstration of performance, continued analysis of Laboratory Reagent Blanks (LRB), laboratory duplicates and Laboratory Fortified Blanks (LFB) with each set of samples.

2.6.7.1 Initial Demonstration of Performance

1. The method detection limit (MDL) must be established for the method analyte using a low level seawater sample containing, or fortified at, approximately 5 times the estimated detection limit. To determine MDL values, analyze at least seven replicate aliquots or water which have been processed through the entire analytical method. Calculate the MDL as follows:

   MDL = (t)(S)

   where,

   S = the standard deviation of the replicate analysis

   t = t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

2. The linear dynamic range (LDR) must be determined by analyzing a minimum of eight calibration standards ranging from 0.002 to 2.00 mg N/L across all sensitivity settings (absorbance units full scale output range setting) of the detector. Standards and sampler wash solutions should be prepared in low nutrient seawater with salinities similar to the samples to avoid the necessity to correct for salt error or refractive index. Normalize responses by multiplying the response by the absorbance units full scale output range setting. Perform the linear regression of normalized response vs.

concentration, and obtain the constants m and b, where m is the slope and b is the y-intercept. Incrementally analyze standards of higher concentration until the measured absorbance response (R) of a standard no longer yields a calculated concentration (C_C) that is within 100 ± 10% of known concentration (C), where

$$C_C = (R-b)/m$$

This concentration defines the upper limit of the LDR. If samples are found to have a concentration that is ≥ 90% of the upper limit of the LDR, they must be diluted and reanalyzed.

2.6.7.2 Assessing Laboratory Performance

1. Laboratory Reagent Blank (LRB). The lab should analyze at least one LRB with each set of samples. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When the LRB value constitutes 10% or more of the analyte concentration determined for a sample, duplicates of the sample must be reprepared and analyzed after the source of contamination has been corrected and acceptable LRB values have been obtained.
2. Laboratory Fortified Blank (LFB). The lab should analyze at lease one LFB with each set of samples. The LFB must be at a concentration within the daily calibration range. The LFB data are used to calculate percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the source of the problem should be identified and resolved before continuing the analysis.
3. The laboratory must use LFB data to assess lab performance against the required control limits of 90-110%. When sufficient internal performance data become available (usually a minimum of 20 to 30 analyses), optional control limits can be developed from the percent mean recovery (x) and standard deviation (S) of the mean recovery. These data can be used to establish the upper and lower control limits as follows:

   Upper Control Limit = x + 3S

   Lower Control Limit = x – 3S

   The optional control limits must be equal to or better than the required control limits of 90-110%. After each 5 to 10 new recovery measurements, new control limits can be calculated using only the most recent 20 to 30 data points. Also the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentration included in the LFB. These data must be kept on file and available for review.

2.6.7.3 Assessing Analyte Recovery-Laboratory Fortified Sample Matrix (LFM)

1. The laboratory should add a known amount of analyte to a minimum of 5% of the total number of samples or one LFM per sample set, whichever is greater. The analyte added should be 2-4 times the ambient concentration and should be at least four times greater thean the MDL.
2. Calculate percent recovery of analyte, corrected for background concentration measured in a separate unfortified sample. These values should be compared with the values obtained from the LFBs. Percent recoveries may be calculated using the equation:

$$R = \frac{(C_S - C)}{} \times 100$$

S

where,

R = percent recovery

C_S = measured fortified sample addition in mg N/L

C = sample background concentration in mg N/L

S = concentration in mg N/L added to the environmental sample

3. If the recovery of the analyte falls outside the required control limits of 90-110%, but the laboratory performance for that analyte is within the control limits, the fortified sample should be prepared again and analyzed. If the result is the same after reanalysis, the recovery problem encountered with the fortified sample is judged to be the matrix related and the sample data should be flagged.

2.6.8 Procedure

1. Bring samples to room temperature.
2. Assemble manifold as shown in Figure 2.8 below. The tubing, flow rates, sample wash ratio, sample rate, etc. are based on a Technicon AutoAnalyzer II system. Specifications for similar segmented flow analyzers vary, so slight adjustments may be necessary.

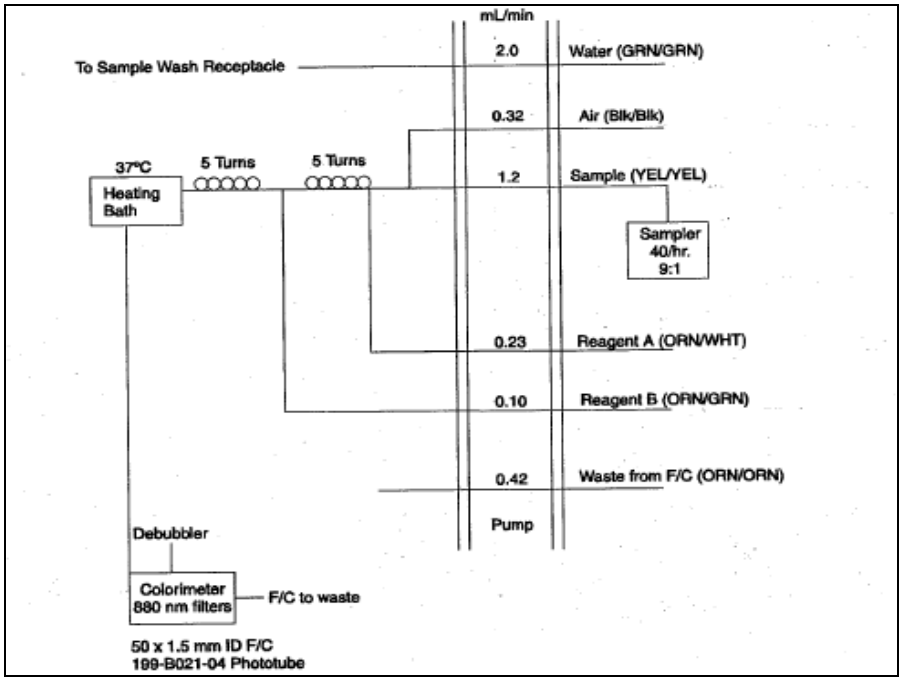

Figure 2.8. Manifold Configuration for Orthophosphate

3. Allow both colorimeter and recorder to warm up for 30 minutes. Obtain a steady baseline with reagent water pumping through the system, add reagents to the sample stream and after the reagent water baseline has equilibrated, note the rise (reagent water baseline) and adjust baseline.

   For analysis of samples with a narrow salinity range, it is advisable to use low nutrient seawater matched to sample salinity as wash water in the sampler in place of reagent

water. For samples with a large salinity range, it is suggested that reagent wash water and procedure be employed.

4. The sampling rate should be about 40 samples per hour with a 9:1 sample:wash ratio.
5. Place standards in sampler in order of decreasing concentration. Complete filling the sampler tray with samples, LRBs, LFBs, and LFMs.

2.6.9 Data Analysis and Calculations

Concentrations of orthophosphate are calculated from the linear regression obtained from the standard curve in which the concentrations of the calibration standards are entered as the independent variable and the corresponding peak height is the dependent variable.

2.6.9.1 Refractive Index Correction

1. Obtain a second set of peak heights for all samples and standards with Refractive Reagent A being pumped through the system in place of Reagent A. This "apparent" concentration due to coloration of the water should be subtracted from the concentrations obtained with Reagent A pumping through. Reagent B remains the same and is also pumped through the system. Peak heights for the refractive index correction must be obtained at the same standard calibration setting and on the same colorimeter as the corresponding samples and standards.
2. Subtract the refractive index peak heights from the heights obtained for the orthophosphate determination. Calculate the regression equation using the corrected sample peak heights.
3. When a large data set has been amassed in which each sample's salinity in which each sample's salinity is known, a regression for the refractive index correction on a particular colorimeter can be calculated. For each sample, the apparent orthophosphate concentration due to refractive index is calculated from its peak height obtained with Refractive Reagent A and Reagent B, and the regression of orthophosphate standards obtained with orthophosphate Reagent A and Reagent B. Its salinity is entered as the independent variable and its apparent orthophosphate concentration due to its refractive index in that colorimeter is entered as the dependent variable. The resulting regression equation allows the analyst to subtract an apparent orthophosphate concentration when the salinity is known, as long as other matrix effects are not present. Thus, the analyst would not be required to obtain the refractive index peak heights for all samples after a large data set has been found to yield consistent apparent orthophosphate concentrations due to salinity.
4. A typical equation is:

   mg P/L apparent PO_4^3 = 0.000087 x Salinity (‰)

   where

   0.000087 is the slope of the line.
5. Results should be reported in mg PO_4^3 – P/L (ppm) or µm PO_4^3 – P/L (ppb).

2.7 CHLOROPHYLL *a*

2.7.1 Scope and Application

This method is for the low level determination of chlorophyll *a* (chl *a*) in marine and freshwater phytoplankton using fluorescence detection. This method may be modified to determine levels of chlorophyll *a* only by using a set of very narrow bandpass excitation and emission filters that nearly eliminate the spectral interference caused by the presence of pheophytin *a* and chlorophyll *b*. Separate equations are used for this modified method.

2.7.2 Method Summary

Chlorophyll-containing phytoplankton are concentrated by filtering at low vacuum through a glass fiber filter. The pigments are extracted from the phytoplankton in 90% acetone with the aid of a mechanical tissue grinder and allowed to steep. The filter slurry in centrifuged to clarify the solution. Fluorescence is measured before and after acidification. Sensitivity calibration factors are used to calculate the concentration in the sample extract. The concentration in the water sample is reported in µg/L.

2.7.3 Interferences

1. Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere with the accurate measurement of both chlorophylls *a* and *b*.
2. The relative amounts of chlorophylls *a*, *b* and *c* vary with the taxonomic composition. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. Due to the spectral overlap of chlorophyll *a* with pheophytin *a* and chlorophyll *a*, underestimation of chlorophyll a occurs. The degree of interference depends on the ration of *a:b.*
3. Quenching effects are observed in highly concentrated solutions or in the presence of high concentrations of other chlorophylls or carotenoids. Minimum sensitivity settings on the fluorometer should be avoided; samples should be diluted instead.
4. Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, blanks and quality control standards must be at the same temperature to prevent errors and/or low precision. Samples should be analyzed at ambient temperature. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.
5. Samples must be clarified by centrifugation prior to analysis.
6. All photosynthetic pigments are light and temperature sensitive. Work must be performed in subdued light and all standards, QC materials and filter samples must be stored in the dark to prevent degradation.

2.7.4 Safety

The grinding of filters during the extraction process should be conducted in a fume hood due to the volatilization of acetone by the tissue grinder.

2.7.5 Equipment and Supplies

1. Fluorometer. Equipped with a high intensity F4T.5 blue lamp, red sensitive photomultiplier, and filters for excitation (CS-5-60) and emission (CS-2-64).

***Note**. The modified method requires excitation filter (436FS10) and emission filter (680FS10).*

2. Centrifuge capable of 675 g.
3. Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with ¼" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity glass grinding tube.
4. Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives.
5. Aluminum foil
6. Assorted labware.

2.7.6 Reagents and Standards

1. Acetone, HPLC grade.
2. Hydrochloric acid (HCl), concentrated.
3. Chlorophyll *a* free of chlorophyll *b*. May be commercially obtained.
4. Water. ASTM Type I water is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.
5. HCl Solution (0.1 N). Add 8.5 mL of concentrated HCl to aout 500 mL and dilute to 1 L.
6. Aqueous Acetone Solution (90% acetone/10% water). Carefully measure 100 Ml of water into a 1-L graduated cylinder. Transfer to a 1-L flask or storage bottle. Measure 900 mL of acetone into the graduated cylinder and transfer to the flask or bottle containing the water. Mix, label and store appropriately.
7. Chlorophyll Stock Standard Solution (SSS). The dry standard received from supplier should be stored at -20°C or -70°C. Prepare SSS just prior to use. Tap the ampoule of dry standard until all the dried chlorophyll is in the bottom of the ampoule. In subdued light, carefully break the tip off the ampoule and transfer the entire contents into a 50-mL volumetric flask. Dilute to volume with 90% acetone, label the flask, and wrap in aluminum foil to protect from light. The concentration of the solution must be determined spectrophotometrically using a multiwavelength spectrophotometer. The concentration of all dilutions must be determined spectrophotometrically each time they are made.
8. Chlorophyll *a* Primary Dilution Standard solution (PDS). Add 1 mL of the SSS to a clean 100-mL flask and dilute to volume with the aqueous acetone solution. If exactly 1 mg of pure chlorophyll a was used to prepare the SSS, the concentration of the PDS is 200 µg/L. Prepare fresh just prior to use.

2.7.7 Sample Storage

Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction. Filters can be stored frozen at these temperatures for up to 24 days without significant loss of chlorophyll *a*.

2.7.8 Quality Control

Each laboratory is required to operate a formal quality control (QC) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the continued analysis of laboratory reagent blanks, field duplicates and quality control samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of the data generated.

2.7.8.1 Initial Demonstration of Performance

1. The laboratory must demonstrate performance prior to sample analysis.
2. Linear Dynamic Range (LDR). The LDR is determined by analyzing a minimum of 5 calibration standards ranging in concentration from 0.2 µg chl *a* /L to 200 µg chl *a*/L across all sensitivity settings of the fluorometer. If using an analog fluorometer or a digital fluorometer requiring manual changes in sensitivity settings, normalize responses by dividing the response by the sensitivity multiplier. Perform the linear regression of normalized response v. concentration and obtain the constants *m* and *b*, where *m* is the slope and *b* is the y-intercept. Incrementally analyze standards of higher concentration until the measured fluorescence response, *R*, of a standard no longer yields a calculated concentration, C_C, that is ± 10% of the known concentration, *C*, where $C_C = (R-b)/m$. That concentration defines the upper limit of the LDR for your instrument. If samples have a concentration that is 90% of the upper limit of the LDR, dilute the samples and reanalyze.
3. Instrumental Detection Limit (IDL). Zero the fluorometer with a solution of 90% acetone on the maximum sensitivity setting. Pure chlorophyll *a* in 90% acetone should be serially diluted until it is no longer detected by the fluorometer on a maximum sensitivity setting.
4. Estimated Detection Limit (EDL). Several blank filters are extracted according to the procedure outlined in section 2.7.10. A solution of pure chlorophyll *a* in 90% acetone is serially diluted until it yields a response that is 3X the average response of blank filters.
5. Quality Control Sample (QCS). Verify the calibration standards and acceptable instrument performance with the analysis of a QCS. If the determined value is not within the confidence limits established by project data quality objectives, then the determinative step of this method is unacceptable. The source of the problem must be identified and corrected before performing this method.

2.7.8.2 Assessing Laboratory Performance

Laboratory Reagent Blank (LRB). The laboratory must analyze at least 1 blank filter with each sample batch. The LRB should be the last filter extracted. LRB values that exceed the IDL indicate contamination from the lab environment. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples of filed duplicates must be analyzed after the contamination has been corrected and acceptable LRB values have been obtained.

2.7.9 Calibration and Standardization

Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (section 2.5.6.9). Allow the instrument to warm up for at least 15 minutes. Measure the fluorescence of each standard at sensitivity settings that provide midscale readings. Obtain response factors for chlorophyll *a* for each sensitivity setting as follows:

$$F_S = C_a/R_S$$

where,

F_S = response factor for sensitivity setting, S.

R_S = fluorometer reading for sensitivity setting, S.

C_a = concentration of chlorophyll *a*.

Note. If you are using special narrow bandpass filters for chlorophyll *a* determination, DO NOT acidify. Use the “uncorrected” chl *a* calculation described in section 2.5.11.

2.7.10 Procedure

2.7.10.1 Extraction of Filter Samples

1. If samples are frozen, remove them from the freezer but keep them in the dark. Set up the tissue grinder and have on hand tissues and squirt bottles containing water and acetone. Workspace lighting should be the lowest possible while still being able to read instructions and operate machinery.
2. Remove filter from container and place in glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push filter to the bottom of the tube with a glass rod. With a volumetric volumetric pipette, add 4 mL of aqueous acetone solution to the grinding tube. Grind the filter until it is converted to a slurry. Do not overheat sample by overgrinding.
3. Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipette, rinse the pestle and the grinding tube with 90% acetone. Add the rinse to the centrifuge tube with the filter slurry. Cap the tube and shake it vigorously. Place in the dark before proceeding to the next filter extraction. Thoroughly rinse the pestle, grinding tube and glass rod alternatively with water and acetone ending with acetone.
4. Shake each tube vigorously before placing all of to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 hrs but not to exceed 24 hrs. The tubes should be shaken at least once during the steeping process.
5. After steeping is complete, shake the tubes vigorously and centrifuge samples for 15 min. at 675 g or for 5 min at 1000 g. Samples should be allowed to come to ambient temperature before analysis. Recalibrate the fluorometer if the room temperature has fluctuated ± 3°C from last calibration.

2.7.10.2 Sample Analysis

1. After the fluorometer has warmed up for at least 15 minutes, use 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.
2. Pour or pipette the supernatant of the extracted sample into a sample cuvette. For a cuvette that holds 5 mL of extraction solution, 0.15 mL of the 0.1 N HCl solution should be used.
3. Choose a sensitivity setting that yields a midscale reading. If the concentration of chlorophyll *a* in a sample is ≥ 90% of the upper limit of the LDR, then dilute the sample with 90% acetone solution and reanalyze.
4. Record the fluorescence measurement and sensitivity setting.
5. Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 NHCl using the 0.1 N NCl solution. Use a Pasteur pipette to thoroughly mix the sample be aspirating and dispensing the sample into the cuvette, keeping the pipette tip below the surface of the liquid to avoid aerating the sample.
6. Wait 90 seconds and measure fluorescence again.

***Note**. If you are using special narrow bandpass filters, DO NOT acidify samples. Use the uncorrected chl a calculations described in the following section (section 2.5.11).*

2.7.11 Data Anlaysis and Calculations

2.7.11.1 Uncorrected Chlorophyll a

1. Calculate the chlorophyll *a* concentration in the extract as follows:

$$C_{E,u} = R_b \times F_S$$

where,

$C_{E,u}$ = uncorrected chlorophyll a concentration (µg/L) in the extract solution

R_b = fluorescence response of sample extract before acidification

F_S = fluorescence response factor for sensitivity setting S.

2. Calculate the "uncorrected" concentration of chlorophyll *a* in whole water sample as follows:

$$C_{S,u} = \frac{C_{E,u} \text{ x Extract volume (L) x DF}}{\text{Sample volume (L)}}$$

where,

$C_{S,u}$ = uncorrected chl-*a* concentration (µg/L) in the whole water sample

Extract volume = volume (L) of extraction prepared before any dilutions

DF = dilution factor

Sample volume = volume (L) of whole water sample.

3. LRB and QCS data should be reported with each sample data set.

2.7.11.2 Corrected Chlorophyll a

1. Calculate the chlorophyll *a* concentration in the extract as follows:

$$C_{E,c} = F_s(r/r-1)(R_b - R_a)$$

where,

$C_{E,c}$ = corrected chlorophyll a concentration (µg/L) in the extract solution analyzed

F_s = response factor for the sensitivity setting S

r = the before-to-after acidification ratio of a pure chlorophyll *a* solution

R_b = fluorescence of sample extract before acidification

R_a = fluorescence of sample extract after acidification

2. Calculate the "corrected" concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{S,C} = \frac{C_{E,u} \text{ x Extract volume (L) x DF}}{\text{Sample volume (L)}}$$

where,

$C_{S,C}$ = corrected chlorophyll *a* concentration (µg/L) in the whole water sample

Extract volume = volume (L) of extraction prepared before any dilutions

DF = dilution factor

Sample volume = volume (L) of whole water sample.

3. LRB and QCS data should be reported with each sample data set.

2.7.12 References

USEPA. 1997. Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Matrices – 2nd Edition. EPA No. 600-R-97-072. U.S. Environmental Protection Agency, Office of Research and Development, Washington, DC.

3.0 FECAL INDICATOR

3.1 SCOPE AND APPLICATION

This document describes the application of *Draft* EPA Enterococcus TaqMan qPCR Method for the processing and qPCR analysis of water sample concentrates from coastal marine waters and the freshwaters of the Great Lakes (NCCA 2010) for the purpose of determining water quality by Real-Time Quantitative Polymerase Chain Reaction (qPCR) assays that determine the concentration of the fecal indicator *Enterococcus* bacteria, by measuring the concentration of their DNA in the water sample.

This method facilitates the microbiological determination of water quality of water bodies at remote locations from which collected water samples cannot feasibly be analyzed for the enumeration of viable (culturable) indicator bacteria because they cannot be transported to an analytical laboratory within 6 hours of collection time for analysis by membrane filtration and / or selective media inoculation and incubation (e.g. MPN broth analysis) methods (EPA Method 1600). Instead, water samples to be analyzed by Enterococci levels by qPCR are concentrated by "field" filtration within 6 hours after collection of the samples. The filter concentrates, inserted into sterile sample extraction tubes containing sterile glass beads or ceramic beads are quickly frozen on dry ice until transport to the analytical laboratory by air courier where they are analyzed up to one year after being received. This method extends the window of time available for analysis due to the stability of the Enterococcus cell DNA whose concentration directly correlates with the number of Enterococcus cells.

3.2 SUMMARY OF METHOD

Aliquots of each water sample have previously been filtered aseptically, the filters have been folded inwardly in half four times to form an umbrella or folded in half and rolled up into a cylinder and then inserted into sterile sample extraction tubes containing sterile glass beads or Roche MagNA Lyser Green Beads™ (actually siliconized white ceramic beads in a green capped tube). Extraction tubes containing filter concentrates (retentates) have been stored on dry ice until transport to the analytical laboratory by air courier. The filter retentate vials can also be preserved in -20˚ to -80˚ C freezers after being frozen on dry ice. Filter concentrates in vials will be shipped by air courier on dry ice to the analytical team at EPA New England Regional Laboratory. Filter concentrates received by NERL staff will be subjected to DNA extraction procedures and subsequently analyzed by the *Draft* EPA Enterococcus TaqMan qPCR Method along with modifications to the QA/QC procedures described below. The laboratory methods are summarized in Table 3.4 of Section 3.18. Each filter is subjected to bead-beating in extraction buffer to lyse cells and produce a clarified extract. A sub-sample of the diluted extract is subjected to *Enterococcus* qPCR analysis. This processing can be completed up to 1 year after cell concentration if the sample filter retentates are maintained frozen at -20 to -80˚C.

3.3 DEFINITIONS OF METHOD

Batch Size: The number of samples that will be processed by filter extraction with the same batch (volume) of SAE buffer and analyzed by the same qPCR assay(s) using the same batch of qPCR master mix. A batch is covered for quantitation purposes by the same "batch" calibrator samples, a minimum of three, analyzed during the same week.

Bottle Blank: Analyte-free water is collected into a sample container, of the same lot number as the containers used for collection of the environmental samples. Analysis of this sample is performed to evaluate the level of contamination, if any, introduced into the environmental and control samples from the sample container(s) from a common vendor's lot.

DNA: Deoxyribo-Nucleic Acid, double-stranded genetic molecules containing sequences of the four nucleotide bases, adenine, thymine, guanidine, and cytosine that encode rRNA, mRNA, and tRNA involved in protein synthesis.

Field Filter Blank: A volume of sterile PBS, free of target organisms (i.e. *Enterococcus*) filtered through a sterile filter and processed in parallel with all other samples to serve as a sentinel for detection of reagent contamination or contamination transferred between samples by processing and analysis.

Field Replicates: Samples collected from coastal marine waters and the Great Lakes that are collected at the same sampling site one right after the other with only slight temporal variation. They are not "splits" of the same sample volume.

Filtrate: Sample liquid or buffer rinsate passing through the filter into the vacuum flask.

Laboratory Quality Samples: Mock samples created in the lab such as lab blanks, lab-fortified blanks (LFBs), and Lab-Fortified Matrices (LFMs) used to assure lack of sample contamination and to measure analytical recovery during performance of sample processing and analysis methods.

Performance Testing (PT) / Performance Evaluation Sample (PES): Calibrator samples (filters spiked with *E. faecalis* grown in Brain Heart Infusion Broth) and Laboratory Fortified Blanks (Phosphate Buffered Saline; PBS) spiked with *Enterococcus faecalis* cells from BHI Broth suspension will be assayed by EPA Method 1600 and Draft EPA Enterococcus TaqMan qPCR Method to ascertain method performance. Ball-T Bioballs® which contain a specified number of *E. faecalis* cells may also be acquired to determine the performance of the Relative Quantitation Method. Purified *E. faecalis* DNA acquired from American Type Culture Collection and TIB Mol Biol Inc. is used to test the performance of the Absolute Quantitation Method.

Retentate: The sample residue retained by the filter after the sample is vacuum-filtered. The retentate contains particulates, microbiota, and macrobiota from which the DNA is extracted into buffer by bead-beating for subsequent qPCR analysis.

Rinsate: The volume of phosphate buffered saline (PBS) applied to a sample's filter retentate in order to "wash" any residual fine particles, smaller than the filter's nominal pore size, through the retentate and the filter.

Sample Processing Control (SPC): A surrogate homologue analyte (e.g. Salmon DNA) spiked into each sample to determine the recovery of target analyte and/or detect assay inhibition caused by matrix effects.

Standards: Known amounts or numbers of copies of *Enterococcus* genomic DNA analyzed by the *Enterococcus* qPCR assay to generate a Standard Curve (Log Copy Number vs. Crossing Point Value) in order to determine *Enterococcus* genomic copy numbers in "Unkown" test sample extracts by Absolute Quantitation Method.

3.4 INTERFERENCES

a. Low pH (acidic) water

b. Humic and fulvic acid content

c. Suspended solids (e.g. fecal matter) and particulates (sand, dirt)

d. Excessive algal growth

3.5 HEALTH AND SAFETY WARNINGS

All proper personal protection clothing and equipment (e.g. lab coat, protective eyeware / goggles) must be worn or applied. When working with potential hazardous chemicals (e.g. 95% ethanol) or biological agents (fecally-contaminated water) avoid inhalation, skin contact, eye contact, or ingestion. If skin contact occurs remove clothing immediately and wash / rinse thoroughly. Wash the affected skin areas thoroughly with large amounts of soap and water. If available consult the MSDS for prompt action, and in all cases seek medical attention immediately. If inhalation, eye contact or ingestion occurs, consult the MSDS for prompt action, and in all cases seek medical attention immediately.

3.6 PERSONNEL QUALIFICATIONS

All laboratory personnel shall be trained in advance in the use of equipment and procedures used during the sample extraction and qPCR analysis steps of this SOP. All personnel shall be responsible for complying with all of the quality assurance / quality control requirements that pertain to their organizational / technical function. All personnel shall be responsible for being aware of proper health and safety precautions and emergency procedures.

3.7 EQUIPMENT AND SUPPLIES

Clean powderless latex or vinyl gloves

Goggles or Face Shield

Micropipettors

Cepheid SmartCycler PCR Thermocycler

Roche MagNA Lyser

Roche MagNA Pure LC (automated nucleic acid isolation and purification platform)

High Speed Microfuge

Roche MagNA Lyser Rotor Cooling Block

2-mL tube racks

Cepheid Smart tubes

MagNA Pure LC sample processing cartridges, reagent trays, and pipette tips

Semi-conical, screw cap microcentrifuge tubes (PGC, #506-636 or equivalent) pre-filled with 0.3 $\pm$ 0.02 g Acid-washed glass beads (Sigma, # G-1277 or equivalent). Filled tubes are autoclaved 15-min. Liquid Cycle (Slow Exhaust).

Or

Roche MagNA Lyser Green Bead tubes (Roche Applied Science, #03-358-941-001) sterile, siliconized 3-mm diameter ceramic beads in a siliconized 2-mL microfuge tube.

Permanent marking pens (fine point and regular point) for labeling tubes

Bench Sheets & Printouts of Computer Software Sampling Loading Screen

3.8 REAGENTS AND STANDARDS

a. Qiagen AE Buffer (Qiagen 19077)

b. Salmon DNA (Sigma D1626)

c. Frozen tubes of *Enterococcus faecalis* (ATCC #29212) calibrator cell stock

d. Purified *Enterococcus* faecalis (ATCC #29212D) genomic DNA, 10-µg

e. TaqMan® Environmental PCR Master Mix (ABI #4396838)

f. *Enterococcus* PCR primers and TaqMan® probe (TIB Mol Biol Inc.)

g. Sketa PCR primers and TaqMan® probe (TIB Mol Biol Inc.)

h. Bovine Serum Albumen (BSA; Sigma Cat. #B-4287)

i. MagNA Pure LC DNA Isolation Kit III for Fungi & Bacteria (Roche Biochemical Division)

3.9 PREPARATIONS PRIOR TO DNA EXTRACTION AND ANALYSIS

Determine / Estimate the sample batch size (number of samples) for one-week of sample processing and qPCR analysis. The batch size is the number of sample filter concentrates that will be extracted by bead-beating with the same batch (volume) of SAE buffer and analyzed by the same qPCR assay(s) using the same batch of qPCR master mix. A batch is covered for quantitation purposes by the batch calibrator samples, (a minimum of three) whose 5-fold and 25-fold diluted extracts are analyzed at the outset of the week along with a reagent blank. Fill out a batch sample analysis bench sheet. (See Section 3.18.)

1. Micropipettors are calibrated annually and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration check. Measure three replicate volumes per pipettor and keep log book of their weights on a calibrated balance scale.
2. Preparation of stock Salmon Sperm (SS) DNA: Dissolve Salmon DNA in PCR grade water to a concentration equal to approximately 10 µg/mL. Determine concentration of Salmon testes DNA stock by measuring the OD_{260} in a UV spectrophotometer or by PicoGreen DNA Concentration Determination Protocol utilizing a NanoDrop UV fluorometer. A DNA solution with an OD_{260} of 1.0 has a concentration equal to approximately 50 µg/mL depending on the GC content of the DNA's sequence(s).
3. Dilute Salmon testes DNA stock with AE buffer to make 0.2 µg/mL Salmon DNA Extraction Buffer (SAE). Extraction buffer may be prepared in advance and stored at 4 ºC for a maximum of 1 week.

Note: *Determine the total volume of Salmon DNA Extraction Buffer required for each day or week by multiplying the extraction volume (i.e. 600-µL) times the total number of samples to be analyzed including controls, water samples, and calibrator samples. For example, for 18 samples, prepare enough Salmon/DNA extraction buffer for 24 extraction tubes (18) / 6 = 3, therefore, 3 extra tubes for water sample filtration blanks (method blanks) and 3 extra tubes for calibrator samples). Note that the number of samples is divided by 6 because you should conduct one method blank for every 6 samples analyzed. Additionally, prepare excess volume to allow for accurate dispensing of 600 µL per tube, generally 1 extra tube. Thus, in this example, prepare sufficient Salmon DNA Extraction Buffer for 24 tubes plus one extra. The total volume SAE needed per sample is 600 µL. Hence for the SAE volume for 25 sample tubes is equal to 15,000 µL. Dilute the Salmon DNA working stock 1:50, for a total volume needed (15,000 µL) 50 = 300 µL of 10 µg/mL Salmon DNA working stock. The AE buffer needed is the difference between the total volume and the Salmon testes DNA working stock. For this example, 15,000 µL - 300 µL = 14,700 µL AE buffer needed.*

4. Make stocks of ATCC *Enterococcus faecalis* purified genomic DNA to be diluted to specific concentrations for use as internal standards in individual qPCR runs whose results are used to generate the weekly *Enterococcus* qPCR Standard Curve for quantitation purposes.
5. Use *Enterococcus faecalis* genomic DNA (10-µg) to make a Frozen *E. faecalis* DNA Reference Stock (20-µL) at a concentration of 2.89×10^6 GEQs per µL
6. Dilute 10-µL of the Frozen *E. faecalis* Reference DNA stock 363-fold to a final volume of 3,630 µL AE buffer. Aliquot 20-µL volumes into many 200-µL microfuge tubes and store

frozen at -20 ºC. The net concentration of *Enterococcus* GEQs is 8,000 / µL. Each week perform a series of 10-fold and 4-fold dilutions from one thawed tube of the 8,000 GEQ/µL standard solution to create 800 GEQ/µL, 80 GQ/µL and 20 GEQ/µL standard solutions. The analyst performs *Enterococcus* qPCR upon duplicate 5-µL volumes of each of the four standards yielding a Standard Curve of Log GEQs ENT versus Ct value from which the assays "efficiency" is subsequently calculated in the Relative Quantitation EXCEL Spreadsheet.

7. Make *Enterococcus faecalis* calibrator filter samples:
   i. Assemble calibrator positive control samples by thawing tubes of *E. faecalis* cell stocks, diluting their contents (10-µL) up to 1-mL AE buffer and spotting 10-µL on sterile PC filter previously folded and inserted into a pre-chilled Green Bead tube.
   ii. Spot a sufficient number of calibrator filter samples for the entire study to insure uniform, consistent relative quantitation of study samples. Store the calibrator filter samples in -20ºC freezer and thaw individual calibrators (three per week) for extraction with each week's batch of samples.
   iii. The calibrator sample filters are spotted with 10^4 or 10^5 *Enterococcus faecalis* cells and this number is incorporated into the Relative Quantitation EXCEL spreadsheet.
8. Prior to and after conducting work with cells and / or genomic DNA standards, disinfect and inactivate (render non-amplifiable) DNA in the Sample Extraction Hood, the qPCR Cabinet, and the qPCR Sample Loading Hood with 10% bleach and $\geq$ 15-min. exposure to high intensity germicidal (254 nm) ultraviolet light.

3.10 PROCEDURES FOR PROCESSING AND QPCR ANALYSIS OF SAMPLE CONCENTRATES

3.10.1 Sample Processing (DNA Extraction)

Typically, 100-mL volumes of surface water are filtered according to *Draft* EPA Enterococcus TaqMan qPCR Method for processing and analysis by PCR assays. Due to the limitations of field crew sampling time and the performance limitations of the manually-operated vacuum pumps used in the field sampling operations, two 100-mL or four 50-mL (optional) surface water samples will be filtered depending on difficulty of filtration. Lower volumes ($\leq$ 50-mL) were acceptable if suspended particulates hinder the filtering of the standard 50-mL volume during the National Lakes & Ponds but should not be necessary in the NCCA if the prescribed 0.4 micron pore size Polycarbonate filters (Whatman Nucleopore Cat. #1111007) are correctly employed. If the filtration of 100-mL volumes is not possible after diligent efforts by field crews, lesser volumes may be acceptable but only if equal replicate volumes are filtered. Field crews should make every effort to filter 100-mL of water sample per filter, filtering 50-mL sub-samples at a time. If filtration has slowed to a drip by the end of finishing the first 50-mL sub-sample the field crew should make the decision to filter four filters of 50-mL volumes instead of two filers of 100-mL each. If a sample is highly turbid with extreme amounts of Total Suspended Solids field crew members should filter 25-mL sub-sample aliquots at a time in their attempt to reach the 100-mL or 50-mL target volume. Although every attempt should be made to filter a minimum of 50-mL of sample per filter, if only volumes less than 50-mL can be filtered then at least make sure all four of the replicate filters contain an equivalent volume of sample filtered through them (e.g. 4 x 30-mL).

Filtration of lower sample volumes has necessitated modifications to *Draft* EPA Enterococcus TaqMan qPCR Method which are directed by the Analysis Decision Tree (ADT; Section 3.18.8). In accordance with the ADT, if ≤ 50-mL of a water sample is filtered per filter replicate, then the laboratory analyst extracts two replicate filters in parallel and combines equivalent volumes of the filter extracts to form one composite filter extract. Each individual filter is extracted with only 300-µL of SAE Extraction Buffer instead of the usual prescribed 600-µL volume of SAE buffer. Halving the SAE buffer volume enables the analyst to maintain an equivalent Method Detection Limit and maintain a similar Sample Equivalence Volume (SEQ; i.e. water sample volume per extract volume) in the extract volumes (e.g. 5-µL) of each sample filter concentrate added to the PCR reactions.

1. Pre-chill MagNA Lyser Rotor Cooling Block in -20˚C freezer. Label 1.7-mL sterile microfuge tubes with sample ID number to match them with Green Bead Tubes. Two supernatant recovery tubes and one "5-fold" dilution tube is needed per sample and should be labeled accordingly. The dilution tube shall be filled with 80-µL AE buffer using a micropipettor.
2. To extract sample filters, uncap green bead tube (cold) and add 0.6-mL (600-µL) SAE Buffer (Qiagen AE Buffer spiked with Salmon DNA). Re-cap tubes tightly.
3. Insert Green Bead tubes of samples into MagNA Lyser and bead-beat for 60-sec (1-min) at 5,000 rpm at Room Temperature. Transfer sample tubes to microfuge. Spin tubes at 12,000 rpm for 2-min. Being careful to move filter aside, recover and transfer up to 400-µL of supernatant (sans debris) to new tube with a P-200 or P-1000 micropipettor.
4. Spin the supernatant tubes for 5-min at 14,000 rpm at Room Temperature. Recover ≥350-µL supernatant and transfer to new 1.7-mL tube. When all samples in a batch have been extracted transfer dilute 20-µL of DNA extract (2nd supernatant) five-fold (5X) in 80-µL AE buffer (sans SS-DNA) and store at 4˚C for qPCR assays. (If supernatant, 5X and even 25X sample dilutions possess dark pigment and exhibit severe qPCR inhibition in Sketa assays, consider extracting replicate filters of samples using the Modified MagNA Pure LC DNA Isolation Protocol (see Section 3.18.9).

3.10.2 Sample Analysis by *Enterococcus* qPCR

3.10.2.1 Preparation of qPCR Assay Mix

1. To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach or reagent to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes. Alternatively use commercial products (e.g. DNA Away or DNA ZAP) according to vendor instructions.
2. Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers and probe to create stock solutions of 500 µM primer and 100 µM probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20ºC.
3. Prepare working stocks of *Enterococcus*, and Salmon DNA primer/probe mixes by adding 10 µL of each *Enterococcus* or Salmon DNA primer stock and 4 µL of respective probe stock to 676 µL of PCR grade water, and vortex. Pulse centrifuge to create pellet. Use a micropipettor with aerosol barrier tips for all liquid transfers. Transfer aliquots of working stocks for single day use to separate tubes and store at 4ºC.

4. Using a micropipettor, prepare assay mix of the *Enterococcus*, and Salmon DNA reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in
    a. Table 3.1.
5. Finger vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.
6. Thaw and finger vortex sample extract (dilution) tubes that will be assayed in PCR run. Microfuge a few seconds to coalesce droplets. Finger mix and spin the standards and calibrator samples (dilutions). Temporarily store all samples in 4˚C refrigerators until use in assay or return to long term storage at -20˚C. Discard disposable gloves and put on a new pair.
7. Set 32 Smart tubes in Cepheid Racks in PCR cabinet along with micro-pippetors and expose to germicidal UV lamp for 15-min.
8. Pipette 20-µL of respective Master Mix into each labeled Smart tube. Transfer Smart tubes (racks) from PCR cabinet to disinfected Sample Loading Fume Hood.
9. Using P-10 or P-20 micro-pipettor load each Smart tube with 5- µL volume of respectively designated sample extract (dilution), standard, or buffer blank (SAE). Cap each sample's Smart tube after loading.
10. Check to make sure each Smart tube is properly labeled and identifiable by sample number or I-core position (e.g. A4). Insert loaded Smart tubes into Smart Tube microfuge. Close lid and spin 5-sec. Pop lid to stop. Remove Smart Tubes from microfuge and insert into proper position in SmartCycler.

Enterococcus (Entero1) and Salmon (Sketa) qPCR assays (*Draft* EPA Enterococcus TaqMan qPCR Method) will be performed upon 5-µL aliquots of un-diluted & 5X diluted extracts of sample unknowns, calibrator, field blank, and lab blank. A "No Template Controls" (NTC) shall be analyzed on an ongoing basis to ensure that the Master Mix PCR reagents are not contaminated. To minimize the number of *Enterococcus* qPCR reactions needed to be performed upon samples, Sketa qPCR assays will be performed upon the 5-fold diluted DNA extracts of samples before any *Enterococcus* qPCR assays are run in order to screen samples for the presence and dilution of PCR inhibitors by comparison with the undiluted and 5-fold dilution DNA extract of the calibrator samples and unused portions of SAE buffer. Each sample's lowest dilution DNA extract not exhibiting PCR inhibition in the Sketa qPCR assay will be re-assayed by the *Enterococcus* qPCR assay and it's results will be used for quantitation of *Enterococcus* DNA sequences and CCEs.

Detection of reduced levels of Salmon DNA (higher instrument Ct values) is indicative of technical error during extract dilution or excessive levels of PCR inhibitors or nuclease activity which could impact detection of the *Enterococcus* DNA target sequences in the *Enterococcus* PCR assay. Alternatively, the high Sketa Ct value may be indicative of the occurrence of a technical error during extract dilution. If a test sample's Ct value is less than 3 cycles different than the blank negative control and calibrator samples, indicating only negligible or marginal inhibition (the Sketa Assay is more sensitive to inhibitors than the ENT Assay), an aliquot of its five-fold diluted extract is analyzed in the *Enterococcus* Assay. If an abundance of PCR inhibitors or DNA nucleases are present in a sample extract which are causing a greater increase in an extract's Ct value ($\geq$ 3 cycles increase), then the extract is diluted an additional five-fold (net 25-fold dilution) and re-assayed by both the Sketa and ENT assays. If the inhibition is not ameliorated by the additional dilution, which should restore the Sketa Ct value to

that of the 25-fold diluted calibrator samples' extracts, the following actions are taken by the analyst. First, the analyst re-dilutes the sample's undiluted DNA extract five-fold and re-analyzes the dilution with the Sketa PCR assay to confirm that Ct variance is not due to a dilution error. If the Ct difference is not attributed to a dilution error, replicate sample filters of the "inhibited" samples are subjected to DNA extraction and purification by the MagNA Pure LC automated platform loaded with the Roche DNA Isolation Kit III (Bacteria; Fungi) reagents (see Section 3.18.9).

The EPA Modified MagNA Pure LC extraction process which includes the spiking of the Lysis Binding Buffer with the Salmon (IPC) DNA is more effective, but more costly, than *Draft* EPA Enterococcus TaqMan qPCR Method in neutralizing severe levels of PCR inhibitors and DNA nucleases present in some environmental samples, especially those containing high levels of algae or phytoplankton. The purified DNA extract yielded by MagNA Pure extraction of the few ($\leq$5%) "severely inhibited" samples is subsequently analyzed by the Sketa and *Enterococcus* qPCR assays and the number of *Enterococcus* CCEs per 100-mL determined by the Delta C_T and Delta Delta C_T Relative Quantitation Methods. While the MagNA Pure LC extraction method is not 100% conservative (no partitioning or recovery issues) like *Draft* EPA Enterococcus TaqMan qPCR Method, it typically exhibits DNA recoveries in the range of 25-50%. DNA recoveries and *Enterococcus* CCE concentrations are calculated using only the Delta-Delta Ct Relative Quantitation Method. The relative DNA recoveries are determined by comparison of the Sketa results from purified DNA eluates of each test sample with those of the extracted lab blank and calibrator samples. The absolute DNA recovery is calculated by comparison of the former Sketa results with those of elution buffer spiked with an amount of Salmon DNA equivalent to the amount in the Salmon-spiked Lysis Binding Buffer added to each sample filter lysate during the MagNA Pure LC DNA extraction process.

The "Unknown" and "Control" sample extracts whether processed using the SAE buffer or MagNA Pure LC Kit III reagents are analyzed according to the Cepheid SmartCycler *Enterococcus* and Sketa qPCR protocols described in Appendix A of the Draft EPA Enterococcus TaqMan qPCR Method with C*t* determination made by the software using Manual Determination (equivalent of Fit Points Method of Roche LightCycler) with the fluorescence threshold set at 8.0 units which enables uniform analysis and comparability of all samples' qPCR results.

Sample analysis sequence for SmartCycler:

Example: For analyses on a single 16-position SmartCycler, calibrator samples and water samples are analyzed in separate runs and a maximum of 6 water samples (or 2 replicates of 3 samples) are analyzed per run, as described in Table 2 and Table 3 of Section 3.18 (below).

Enterococcus and Sketa (Salmon DNA = SPC) qPCR results are exported to an EXCEL spreadsheet in which relative quantitation calculations are performed by analysts. The *Draft* EPA Enterococcus TaqMan qPCR Method results are reported in terms (units of measure) of Number of *Enterococcus* Sequences and Number of *Enterococcus* Calibrator Cell Equivalents (CCEs) per 100-mL sample volume. The qPCR results are converted to this standardized unit of measure based on the volume of water sample actually filtered (*e.g.*, 10-mL, 25-mL, or 50-mL).

***Note**: Samples with Enterococcus qPCR results below the Reporting Limits (qPCR results with Ct values below 35.5 cycles) are qualified as "Estimates". The RL varies proportionally to the volume of sample filtered by each sample crew at a specific site. Reporting limits (RL) and*

Method Detection Limits (MDLs) will be higher among samples for which a volume of water <50-mL is filtered.

Enterococcus qPCR results are flagged if some part of the sample collection, hold-time, processing, shipment, storage, sample extraction, or qPCR analysis are compromised and did not meet the requirements of the Sampling and Analysis SOPs.

3.11 STORAGE AND TIMING OF PROCESSING / ANALYSIS OF FILTER CONCENTRATES

When a sufficient number of water sample filter concentrates (filters and retentates) have been received by NERL and qPCR analytical reagents have been obtained the samples will be logged into LIMS. Sample processing and qPCR will commence and results will be entered into the LIMS upon completion of analysis.

3.12 CHAIN OF CUSTODY

Follow the Sample Control Procedures, Field Sampling Form / Enterococci Filtration / Sample Processing Standard Operating Procedures.

Field Sampling forms and NCAA 2010 Sample Tracking EXCEL Spreadsheet shall be consulted to determine if a sample has been properly preserved during collection and transport prior to analysis and that it has passed all criteria permitting its analysis. The qPCR results of samples exceeding established criteria or whose associated field / lab blanks had positive *Enterococcus* qPCR detections of DNA shall be flagged.

3.13 QUALITY CONTROL / QUALITY ASSURANCE

The Data Quality Objectives and the Laboratory QC Procedures are listed and summarized in Tables 5 and 6 of section 3.18 below.

The number of field blanks (dilution buffer only) shipped by field crews during their first visit to subsequently “re-visited” marine and freshwater sampling sites represents a frequency of 5-10% of the total number of samples extracted and analyzed by qPCR. All field blanks (negative controls) will be extracted and analyzed by qPCR for the detection of *Enterococcus*. The blanks will be analyzed in these cases to insure that positive detections in field samples are not due to contamination by sampling crews.

One Lab / Method Blank (LB; sterile filters) will be run per extraction sub-batch in order to insure the sterility (lack of DNA contamination) in the SAE buffer and pipette tips used to process all of the samples. The LB sample will be processed and diluted like all other “Unknown” samples

Up to four replicate filter concentrates (retentates) derived from the field filtration of 100-mL or 50-mL sample volumes of every sample will be received by NERL and stored at -20 to -80˚C. One filter retentate of each sample (and duplicates for 10% of samples) will be extracted to obtain DNA lysates for *Enterococcus* qPCR analysis. The remaining filter concentrates will be archived for possible extraction and analysis at a later time if needed.

Enterococcus and Sketa qPCR analysis will be performed upon 5-µL volumes of the non-diluted and 5-fold diluted (in AE buffer) extracts which will be added to 20-µL qPCR Master Mix

volumes and analyzed in the Cepheid SmartCycler qPCR instrument in accordance with Draft EPA Enterococcus TaqMan qPCR Method.

Duplicate *Enterococcus* and Sketa qPCR assays will be performed upon 10% of the sample extracts (diluted and un-diluted) each week (batch) to determine qPCR assay variance.

3.14 METHOD PERFORMANCE

Method Performance will be determined by the use of Performance Testing (PT) / Performance Evaluation Samples (PES). Calibrator samples (filters spiked with frozen stocks of *E. faecalis* grown in Brain Heart Infusion Broth) and Lab-Fortified Matrices (LFMs; duplicate sample filters spiked with frozen stocks of *E. faecalis* grown in Brain Heart Infusion Broth) will be extracted and assayed by *Draft* EPA Enterococcus TaqMan qPCR Method *Enterococcus* and Sketa qPCR assays in order to ascertain method performance. The LFMs are performed upon several samples (approx. 5% frequency) per batch, typically samples exhibiting non-detection of *Enterococcus,* in order to determine method performance and also to insure that non-detects are not due to poor DNA recovery caused by matrix effects.

3.15 RECORD KEEPING AND DATA MANAGEMENT

Laboratory analysts shall follow the EPA OEME Laboratory Data Management SOP.

Each lab analyst shall record all details pertaining to sample processing and analysis in a designated, bound laboratory notebook.

Pertinent sample collection and analysis data shall be entered into the Laboratory Information Management System (LIMS) and SeaGate Crystal Reports shall be generated as required by the EPA (TOPO).

An EXCEL spreadsheet of sample analysis data and associated calculations used to derive a field sample's or control sample's *Enterococcus* genomic DNA (GEQ) and Cell Equivalent (CEQ) concentration shall be uploaded to the NCCA 2010 database stored on a computer server in Corvallis, Oregon.

3.16 WASTE MANAGEMENT AND POLLUTION PREVENTION

During the sample processing procedures there may be hazardous waste produced. The waste must be handled and disposed of in accordance with federal, state, and municipal regulations.

All recyclable and non-recyclable materials for disposal will be properly sorted for their respective waste streams and placed into proper containers for janitorial staff to collect and process according to EPA guidelines.

All ethanol used shall be consumed by ignition or evaporation. Volumes of ethanol remaining at the end of the project can be stored for later use in a flammable cabinet or disposed of through appropriate hazardous waste disposal vendors.

Reagent ethanol shall be contained in screw cap tubes along with the filter forceps to sterilize the latter and to prevent ethanol spillage during transport between sampling sites.

After the DNA extract is recovered from the sample filter after bead-beating in buffer and centrifugation, the filter and bead-tube will be discarded in autoclave bags and sterilized for 30-min at 121˚C/30 psi to inactivate any potential pathogens that may be associated with the samples.

3.17 REFERENCES

USEPA Region 1 (New England) OEME NERL Standard Operating Procedure for the Collection of Chemical & Biological Ambient Water Samples (ECASOP-Ambient Water Sampling 2; January 31, 2007)

Draft EPA Enterococcus TaqMan qPCR Method for Quantitation of Enterococci in Water and Wastewater by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. December 2006 (12/15/06 a)

USEPA NERL OEME Draft Bench SOP for Real-Time PCR Method Quantifying Enterococci in Recreational Water Samples (August 2006)

3.18 TABLES, DIAGRAMS, FLOWCHARTS, CHECKLISTS, AND VALIDATION DATA

Table 3.1. PCR Assay Mix Composition (according to Draft EPA Enterococcus TaqMan qPCR Method)

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H_2O	1.5 µL
Bovine Serum Albumen (20 mg/mL)	2.5 µL
TaqMan® 2X Environmental Master	12.5 µL
Primer/probe working stock solution	3.5 µL*

Note: *This will give a final concentration of 1 µM of each primer and 80 nM of probe in the reactions. Prepare sufficient quantity of assay mix for the number of samples to be analyzed per day including calibrators and negative controls plus at least two extra samples. It is strongly recommended that preparation of assay mixes be performed each day before handling of DNA samples.*

Table 3.2. Batch Calibrator & Enterococcus Standards PCR Run - 7 Samples

Sample Description*	Quantity Samples	PCR Assay Master Mix	Quantity PCR Reactions
3 Calibrators (5- and/or 25-fold dilution)	3	Salmon DNA (Sketa)	6
3 Calibrators (5- and/or 25-fold dilution)	3	*Enterococcus*	6
4 *Enterococcus faecalis* DNA Standards	4	*Enterococcus*	8
No template control (reagent blank)	1	*Enterococcus*	1

* Diluted equivalently to the water samples

Table 3.3. Sub-Batch Test Sample PCR Run – 26 Samples & 1 Method Blank

Sample Description*	Quantity Samples	PCR Assay Master Mix	Quantity PCR Reactions
Water samples, (5-fold dilution)	26	*Enterococcus*	26
Method blank or Sample PCR Reaction Duplicate, (1- or 5-fold dilution)	1	*Enterococcus*	1
Non-diluted SAE Buffer	1	*Enterococcus*	1
Water samples, (1- or 5-fold dilution)	26	Salmon DNA	26
Method blank or Sample PCR Reaction Duplicate, (1- & 5-fold dilution)	1	Salmon DNA	1

** Use of 5-fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while 25-fold dilutions analyses may unnecessarily sacrifice sensitivity.*

Table 3.4. Laboratory Methods: Fecal Indicator (Enterococci)

Variable or Measurement	QA Class	Expected Range and/or Units	Summary of Method	References
Sample Collection	C	NA	Sterile sample bottle submerged to collect 250-mL sample 6-12" below surface at 1-m from shore	NCCA Field Operations Manual 2010
Sub-sampling	N	NA	2 x 100-mL or 4 x 50-mL sub-samples poured in sterile 50-mL tube after mixing by inversion 25 times.	NCCA Laboratory Methods Manual 2008
Sub-sample (& Buffer Blank) Filtration	N	NA	Up to 100-mL sub-samples filtered through sterile polycarbonate filter. Funnel rinsed with 2 x 10-mL buffer. Filter folded or rolled, then inserted in tube then frozen on dry ice.	NCCA Laboratory Methods Manual 2008
Preservation & Shipment	C	-40Cto+40 C	Batches of sample tubes shipped on dry ice to lab for analysis.	NCCA Laboratory Methods Manual 2008
DNA Extraction (Recovery)	C	10-141%	Bead-beating of filter in buffer containing Extraction Control (SPC) DNA. DNA recovery measured	EPA Draft *Enterococcus* TaqMan qPCR Method
EPA Draft *Enterococcus* TaqMan qPCR Method (*Enterococcus* & Sketa SPC qPCR)	C	<60 (RL) to >100,000 ENT CCEs /100-mL	5-µL aliquots of sample extract are analyzed by ENT & Sketa qPCR assays along with blanks, calibrator samples & standards. Field and lab duplicates are analyzed at 5% frequency. Field blanks analyzed along with test samples.	EPA Draft *Enterococcus* TaqMan qPCR Method; NERL NCCA 2010 qPCR Analytical SOP

C = critical, N = non-critical quality assurance classification.

Table 3.5. Parameter Measurement Data Quality Objectives

Variable or Measurement	QA Class	Expected Range and/or Units	Summary of Method	References
DNA Extraction (Recovery)	C	10-141%	Bead-beating of filter in buffer containing Extraction Control (SPC) DNA. DNA recovery measured	EPA Draft *Enterococcus* TaqMan qPCR Method
Enterococcus & SPC qPCR	C	<60 to >10,000 ENT CEQs /100-mL	5-µL aliquots of sample extract are analyzed by ENT & Sketa qPCR assays along with blanks, calibrator samples & standards. Field and lab duplicates are analyzed at 5% frequency. Field blanks analyzed at end of testing only if significant detections observed.	EPA Draft *Enterococcus* TaqMan qPCR Method NERL NCCA 2008 2009 qPCR Analytical SOP (QAPP)
SPC & ENT DNA sequence numbers of Calibrators & Standards by AQM	RSD = 40%	80%	95%	
ENT CCEs by d*Ct* RQM	RSD = 55%	40%	95%	
ENT CCEs by dd*Ct* RQM	RSD = 55%	50%	95%	

C = critical, N = non-critical quality assurance classification.
*AQM = Absolute Quantitation Method; RQM = Relative Quantitation Method;
SPC = Sample Processing Control (Salmon DNA / Sketa); CCEs = Calibrator Cell Equivalents

Table 3.6. Laboratory QC Procedures: Enterococci DNA Sequences

Check or Sample Description	Frequency	Acceptance Criteria	Corrective Action
SAMPLE PROCESSING			
Re-process sub-samples (duplicates)	10% of all samples completed per laboratory	Percent Similarity ≥70%	If <70%, re-process additional sub-samples
qPCR ANALYSIS			
Duplicate analysis by different biologist within lab	10% of all samples completed per laboratory	Percent Congruence ≤40% RSD	If >30%, determine reason and if cause is systemic, re-analyze all samples in question.
Independent analysis by external laboratory	None	Independent analysis TBD	Determine if independent analysis can be funded and conducted.
Use single stock of *E. faecalis* calibrator	For all qPCR calibrator samples for quantitation	All calibrator sample C*p* (C*t*) must have an RSD ≤ 40%.	If calibrator C*p* (C*t*) values exceed an RSD value of 30% a batch's calibrator samples shall be re-analyzed and replaced with new calibrators to be processed and analyzed if RSD not back within range.
DATA PROCESSING & REVIEW			
100% verification and review of qPCR data	All qPCR amplification traces, raw and processed data sheets	All final data will be checked against raw data, exported data, and calculated data printouts before entry into LIMS and upload to Corvallis, OR database.	Second tier review by contractor and third tier review by EPA.

Purified DNA Extracts

NCCA Batch #_____ **Dates** _____

Batch Sample #	Sample ID #	QA/QC Qual Code	Sample Vol (mL) Filtered	Vol. SAE Buffer Added (µL)	Color of Filter	25X Dilution Needed?	Comments

Figure 3.1. Batch Sample Analysis Bench Sheet for *Draft* EPA Enterococcus TaqMan qPCR Method

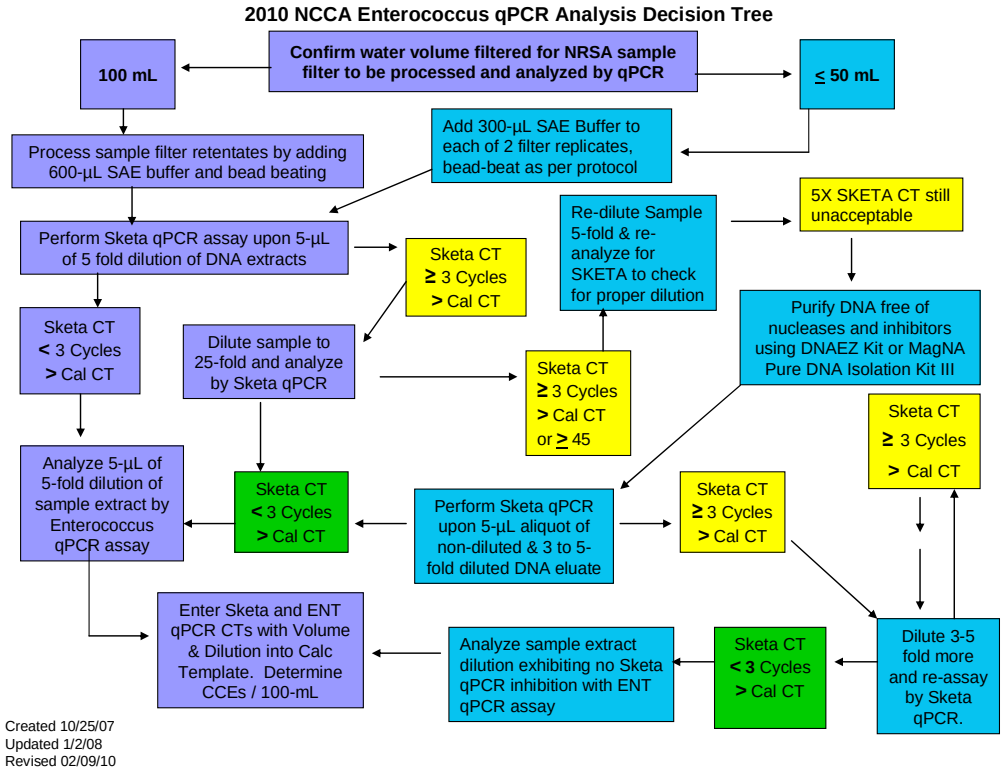

Figure 3.2. *Enterococcus* qPCR Analysis Decision Tree (ADT)

3.18.1 SOP for "Modified" MagNA Pure LC DNA Purification Kit III Protocol

1. Pre-warm the MagNA Pure LC DNA Isolation Kit III Lysis Buffer to 65 ºC in waterbath. Quickly pipette 260-µL of warm Lysis Buffer (un-amended) into each "Green Bead" tube with filter (preserved after filtration temporarily on ice or during long-term storage in freezer). Shake tube 5-10 sec to mix buffer with beads and filter. Let stand at RT until batch of 16 samples (including positive control LFB or LFM and negative control LB samples) have all had Lysis Buffer and had their caps sealed tight. Leave water bath on to use during 30-minute Proteinase K treatment period.
2. Load the 16 samples into MagNA Lyser Rotor Plate and insert into MagNA Lyser. Tighten the three handscrews of the locking mechanism. Close the lid tightly. Set controls to shake for 60-sec at 5,000 rpm. Press the start button.
3. When the shake cycle has ended press the Open Lid Button. Open the lid and unlock the locking mechanism screws. Remove tube plate and set on bench top MagNA Lyser tube ring hub. Remove tubes, insert into tube styrofoam water bath float and cool tubes in ice water for 2-min. or place directly into 24-place microfuge rotor, pre-chilled in freezer.
4. Insert tubes into centrifuge rotor symmetrically in order to balance rotor. Close lid of centrifuge. Set spin parameters for 3,000 rpm for 1-min at 4ºC. Press Start button. Centrifuge to collect drops and foam off of cap down into tube.
5. When centrifuge stops, open lid and remove tubes from rotor. Uncap tubes in order and add 40-µL of Proteinase K (dissolved in Lysis Buffer Elution Buffer). Re-cap tubes and mix lysate by inversion. Do not vortex. Knock beads and filter down from cap into bottom of tube by tapping tubes on bench countertop.

6. Insert tubes into styrofoam floating rack. Incubate tubes 30-min at 65ºC in water bath. Set timer for 15-min. At end of 15-min remove rack from water bath and inverts several times to mix samples and tap beads and filter back down into tube. Re-place rack in 65ºC waterbath for 15-min. for total of 30-min.
7. Repeat steps 3 to 8 to process 16 more samples in parallel for loading MagNA Pure LC sample cartridge with 32 DNA extracts for downstream processing in the robotic platform.
8. After 30-min in 65 ºC waterbath remove tubes from water bath and place in MagNA Lyser Bead Beater for 15 seconds at 5,000 rpm. After 15 seconds of bead-beating, place in ice bath for 5-min to cool.
9. Insert tubes in centrifuge rotor and spin 3-min at 12,000 rpm and 4 ºC to pellet sediment and cell debris. When spinning is complete, open lid of centrifuge and rotor and mark side of outer side of cap where pellet should have formed.
10. Carefully remove rotor from centrifuge and set on bench. Remove tubes one at a time from rotor and use 200-µL pipettor and sterile aerosol-proof tips to transfer approximately 150µL lysate supernatant from tube to wells in MagNA Pure LC Sample Cartridge in pre-designated order.
11. When all 16 sample supernatants transferred to sample cartridge put adhesive film over cartridge to prevent contamination and evaporation. Put sample cartridge in ice water bath or fridge to maintain 4 ºC.
12. Repeat steps 9 to 13 for second batch of 16 samples (lysates). Re-cover sample cartridge with adhesive film for storage. Centrifuge sample cartridge opposite a balance cartridge for 75-sec (1-min, 15-sec) at 2800 rpm in IEC centrifuge (or equivalent) with rotor adaptors for microtiter plates in place. Insert the film-covered sample cartridge in MagNA Pure LC platform.
13. Load the MagNA Pure LC platform with volumes of extraction kit reagents prescribed by MagNA Pure LC computer software for the number of samples being extracted. Before closing the platform lid and starting the extraction process add 1.5-µL of 0.27 mg/mL Salmon DNA Stock per 1mL Lysis Binding Buffer (blue soapy solution) as the Sample Processing Control (SPC). If the amount of Salmon DNA stock to be added is less than 10-µL, dilute the Salmon DNA stock so that a volume $\geq$ 10-µL can be pipetted into the Lysis Binding Buffer. Rinse pipette tip up and down three times in Lysis Binding Buffer.
14. Remove film from top of sample cartridge and re-insert in Roche MagNA Pure LC platform set up with DNA Purification Kit III (Fungi; Bacteria) reagents in tubs, tips, tip holders, and processing / elution cartridges. Close platform lid and after checking off checklist of loaded items (e.g. reagents, tips) lock the lid and start the automated DNA III Extraction Protocol which purifies each sample's DNA and elutes it into 100-µL Elution Buffer.
15. When extraction process is complete, unlock the MagNA Pure LC platform lid and remove the sample eluate cartridge. Cover the cartridge with adhesive film and store at 4 C until qPCR analysis. Store cartridge at $\leq$ -20 ºC for long term preservation.
16. Prepare Elution Buffer Control from 9.3µg/mL Salmon DNA Stock by diluting a small volume to 37.2 pg/1000µL (1-mL). This control sample is only analyzed by the Sketa qPCR assay. The Ct value obtained represents that value expected in Sketa qPCR assays of each MagNA Pure LC purified sample if 100% of the Salmon DNA was recovered and detected. Vortex to mix on low speed briefly prior qPCR analysis. Centrifuge for 1.5-min to coalesce droplets. Remove film to aliquot sub-samples and re-place with new film cover to restore at cool temperatures.

4.0 CONTAMINANTS

PERFORMANCE-BASED METHODOLOGIES

Suggested analytical methods for contaminants in sediment and fish tissue are described in section 4.0 of this manual. However, some laboratories participating in the survey may choose to employ other analytical methods. Laboratories engaged by EPA or the State may use a different analytical method as long as the lab is able to achieve the same performance requirements as the standard methods. Performance data must be submitted to EPA prior to initiating any analyses. Methods performance requirements for this program identify detection limit, precision and accuracy objectives for each indicator. Method performance requirements for contaminants in sediment and fish tissue are shown in Table 4.1

Table 4.1. Laboratory method performance requirements for contaminants in sediment and fish tissue

Inorganic Analytes	MDL Objective – Fish Tissue (wet weight, µg/g (ppm))	MDL Objective – Sediments (dry weight, µg/g (ppm))	Maximum Allowable Accuracy[1]		Maximum Allowable Precision[2]		Completeness Objective[3]
			Tissue	Sediment	Tissue	Sediment	
Aluminum	10.0	1500	35%	20%	30%	30%	95%
Antimony	Not measured	0.2	35%	20%	30%	30%	95%
Arsenic	2.0	1.5	35%	20%	30%	30%	95%
Cadmium	0.2	0.05	35%	20%	30%	30%	95%
Chromium	0.1	5.0	35%	20%	30%	30%	95%
Copper	5.0	5.0	35%	20%	30%	30%	95%
Iron	50.0	500	35%	20%	30%	30%	95%
Lead	0.1	1.0	35%	20%	30%	30%	95%
Manganese	Not measured	1.0	35%	20%	30%	30%	95%
Mercury	0.01	0.01	35%	20%	30%	30%	95%
Nickel	0.5	1.0	35%	20%	30%	30%	95%
Selenium	1.0	0.1	35%	20%	30%	30%	95%
Tin	0.05	0.1	35%	20%	30%	30%	95%
Zinc	50.0	2.0	35%	20%	30%	30%	95%
Organic Analytes	**MDL Objective – Fish Tissue (wet weight, ng/g (ppb))**	**MDL Objective – Sediments (dry weight, ng/g (ppb))**	**Maximum Allowable Accuracy[1]**		**Maximum Allowable Precision[2]**		**Completeness Objective[3]**
			Tissue	**Sediment**	**Tissue**	**Sediment**	
PAHs	NA	10	20%	35%	30%	30%	95%
PCB congeners	2.0	1.0	20%	35%	30%	30%	95%
Chlorinated pesticides/DDTs	2.0	1.0	20%	35%	30%	30%	95%
TOC	Not measured	100	20%	35%	30%	30%	95%

[1] Accuracy (bias) goals are expressed either as absolute difference (± value) or percent deviation from "true" value. [2] Precision goals are expressed as relative percent difference (RPD) or relative standard deviation (RSD) between two or more replicate measurements. [3] Completeness goal is the percentage of expected results that are obtained successfully.

4.1 SAMPLE PREPARATION FOR METALS ANALYSIS

4.1.1 Microwave Assisted Acid Digestion

1. This method is applicable to the microwave assisted acid digestion of siliceous matrices, and organic matrices including biological tissues. This method is applicable for the following elements:

Aluminum	Beryllium	Copper	Mercury	Sodium
Antimony	Cadmium	Iron	Molybdenum	Strontium
Arsenic	Calcium	Lead	Nickel	Thallium
Boron	Chromium	Magnesium	Potassium	Vanadium
Barium	Cobalt	Manganese	Selenium	Zinc

   Other elements and matrices may be analyzed by this method if performance is demonstrated for the analyte of interest, in the matrices of interest, at the concentration levels of interest.

2. This method is a rapid multi-element microwave assisted acid digestion prior to analysis protocol so that decisions can be made about the material. Digests and alternative procedures produced by the method are suitable for analysis by flame atomic absorption spectrometry (FLAA), cold vapor atomic absorption spectrometry (CVAA), graphite furnace atomic absorption spectrometry (GFAA), inductively coupled plasma atomic emission spectrometry (ICPAES), inductively coupled plasma mass spectrometry (ICP-MS) and other analytical elemental analysis techniques where applicable. Due to the rapid advances in microwave technology, consult your manufacturer's recommended instructions for guidance on their microwave digestion system and refer to this manual's "Disclaimer" when conducting analyses using this method.

3. The goal of this method is total sample decomposition and with judicious choice of acid combinations this is achievable for most matrices. Selection of reagents which give the highest recoveries for the target analytes is considered the optimum method condition.

4.1.2 Summary of Method

A representative sample is digested in concentrated nitric acid and usually hydrofluoric acid using microwave heating with a suitable laboratory microwave system. The method has several additional alternative acid and reagent combinations including hydrochloric acid and hydrogen peroxide. The method has provisions for scaling up the sample size to a maximum of 1.0 g. The sample and acid are placed in suitably inert polymeric microwave vessels. The vessel is sealed and heated in the microwave system. The temperature profile is specified to permit specific reactions and incorporates reaching 180 ± 5. After cooling, the vessel contents may be filtered, centrifuged, or allowed to settle and then decanted, diluted to volume, and analyzed by the method found in section 4.3 of this manual.

4.1.3 Interferences

1. Gaseous digestion reaction products, very reactive, or volatile materials that may create high pressures when heated and may cause venting of the vessels with potential loss of sample and analytes. The complete decomposition of either carbonates, or carbon based samples, may cause enough pressure to vent the vessel if the sample size is greater than 0.25 g. Variations of the method due to very reactive materials are specifically addressed in section 4.1.6.2.

2. Most samples will be totally dissolved by this method with judicious choice of the acid combinations. A few refractory sample matrix compounds, (e.g., TiO_2, alumina, other oxides) may not be totally dissolved and in some cases may sequester target analyte elements.
3. The use of several digestion reagents that are necessary to either completely decompose the matrix or to stabilize specific elements may limit the use of specific analytical instrumentation methods. Hydrochloric acid is known to interfere with some instrumental analysis methods such as flame atomic absorption (FLAA) and inductively coupled plasma atomic emission spectrometry (ICP-AES). The presence of hydrochloric acid may be problematic for graphite furnace atomic absorption (GFAA) and inductively coupled plasma mass spectrometry (ICP-MS). Hydrofluoric acid, which is capable of dissolving silicates, may require the removal of excess hydrofluoric acid or the use of specialized non-glass components during instrumental analysis. This method enables the analyst to select other decomposition reagents that may also cause problems with instrumental analyses requiring matrix matching of standards to account for viscosity and chemical differences.

4.1.4 Apparatus and Supplies

4.1.4.1 Microwave

1. The temperature performance requirements necessitate the microwave decomposition system sense the temperature to within ± 2.5°C and automatically adjust the microwave field output power within 2 seconds of sensing. Temperature sensors should be accurate to ± 2°C (including the final reaction temperature of 180°C). Temperature feedback control provides the primary control performance mechanism for the method. Due to the flexibility in the reagents used to achieve total analysis, temperature feedback control is necessary for reproducible microwave heating.

   Alternatively, for a specific set of reagent(s) combination(s), quantity, and specific vessel type, a calibration control mechanism can be developed similar to previous microwave methods. Through calibration of the microwave power, vessel load and heat loss, the reaction temperature profile described in section 4.6.2 can be reproduced. The calibration settings are specific for the number and type of vessel used and for the microwave system in addition to the variation in reagent combinations. Therefore no specific calibration settings are provided in this method. These settings may be developed by using temperature monitoring equipment for each specific set of equipment and reagent combination. They may only be used if not altered as previously described in other methods. In this circumstance, the microwave system provides programmable power which can be programmed to within ± 12 W of the required power.

   Typical systems provide a nominal 600 W to 1200 W of power. Calibration control provides backward compatibility with older laboratory microwave systems without temperature monitoring or feedback control and with lower cost microwave systems for some repetitive analyses. Older lower pressure vessels may not be compatible.
2. The temperature measurement system should be periodically calibrated at an elevated temperature. Pour silicon oil (a high temperature oil into a beaker and adequately stirred to ensure a homogeneous temperature. Place the microwave temperature sensor and a calibrated external temperature measurement sensor into the beaker. Heat the beaker to a constant temperature of 180 ± 5°C. Measure the temperature with both sensors. If the measured temperatures vary by more than 1 - 2°C, the microwave temperature

measurement system needs to be calibrated. Consult the microwave manufacturer's instructions about the specific temperature sensor calibration procedure.

CAUTION: *The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested reagent combinations of unknown samples. These tests may require additional vessel requirements such as increased pressure capabilities.*

3. The microwave unit cavity is corrosion resistant and well ventilated. All electronics are protected against corrosion for safe operation.

CAUTION: *There are many safety and operational recommendations specific to the model and manufacturer of the microwave equipment used in individual laboratories. A listing of these specific suggestions is beyond the scope of this method, and requires the analyst to consult the specific equipment manual, manufacturer, and literature for proper and safe operation of the microwave equipment and vessels.*

4. The method requires essentially microwave transparent and reagent resistant suitably inert polymeric materials (examples are PFA or TFM suitably inert polymeric polymers) to contain acids and samples. For higher pressure capabilities the vessel may be contained within layers of different microwave transparent materials for strength, durability, and safety. The vessels internal volume should be at least 45 mL, capable of withstanding pressures of at least 30 atm (30 bar or 435 psi), and capable of controlled pressure relief. These specifications are to provide an appropriate, safe, and durable reaction vessel of which there are many adequate designs by many suppliers.

CAUTION: *The outer layers of vessels are frequently not as acid or reagent resistant as the liner material and must not be chemically degraded or physically damaged to retain the performance and safety required. Routine examination of the vessel materials may be required to ensure their safe use.*

CAUTION*: The second safety concern relates to the use of sealed containers without pressure relief devices. Temperature is the important variable controlling the reaction. Pressure is needed to attain elevated temperatures, but must be safely contained. However, many digestion vessels constructed from certain suitably inert polymerics may crack, burst, or explode in the unit under certain pressures. Only suitably inert polymeric (e.g., PFA or TFM) containers with pressure relief mechanisms or containers with suitably inert polymeric liners and pressure relief mechanisms are considered acceptable. Users are therefore advised not to use domestic (kitchen) type microwave ovens or to use inappropriate sealed containers without pressure relief for microwave acid digestions by this method. Use of laboratory-grade microwave equipment is required to prevent safety hazards.*

5. A rotating turntable is employed to insure homogeneous distribution of microwave radiation within most systems. The speed of the turntable should be a minimum of 3 rpm.

CAUTION: *Laboratories should not use domestic (kitchen) type microwave ovens for this method. There are several significant safety issues. First, when an acid such as nitric is used to effect sample digestion in microwave units in open vessel(s), or sealed vessels equipment, there is the potential for the acid gas vapor released to corrode the safety devices that prevent the microwave magnetron from shutting off when the door is opened. This can result in operator exposure to microwave energy. Use of a system with isolated and corrosion resistant safety devices prevents this from occurring.*

4.1.4.2 Supplies

1. Volumetric ware, volumetric flasks, graduated cylinders, 50 & 100 mL capacity or equivalent.
2. Filter paper, qualitative or equivalent.
3. Filter funnel, polypropylene, polyethylene or equivalent.
4. Analytical balance, of appropriate capacity, with a ± 0.0001 g or appropriate precision for the weighing of the sample. Optionally, the vessel with sample and reagents may be weighed, with an appropriate precision balance, before and after microwave processing to evaluate the seal integrity in some vessel types.

4.1.5 Reagents

All reagents should be of appropriate purity or high purity (acids for example, should be sub-boiling distilled where possible) to minimize the blank levels due to elemental contamination. All references to water in the method refer to reagent water. Other reagent grades may be used, provided it is first ascertained that the reagent is of sufficient purity to permit its use without lessening the accuracy of the determination. If the purity of a reagent is questionable, analyze the reagent to determine the level of impurities. The reagent blank must be less than the MDL in order to be used.

4.1.6 Procedure

4.1.6.1 General

1. Temperature control of closed vessel microwave instruments provides the main feedback control performance mechanism for the method. Control requires a temperature sensor in one or more vessels during the entire decomposition. The microwave decomposition system should sense the temperature to within ± 2.5 °C and permit adjustment of the microwave output power within 2 seconds.
2. All digestion vessels and volumetric ware must be carefully acid washed and rinsed with reagent water. When switching between high concentration samples and low concentration samples, all digestion vessels (fluoropolymer liners only) should be cleaned by leaching with hot (1:1) hydrochloric acid (greater than 80◦C, but less than boiling) for a minimum of two hours followed with hot (1:1) nitric acid (greater than 80°C, but less than boiling) for a minimum of two hours and rinsed with reagent water and dried in a clean environment. This cleaning procedure should also be used whenever the prior use of the digestion vessels is unknown or cross contamination from vessels is suspected. Polymeric or glass volumetric ware (not used with HF) and storage containers should be cleaned by leaching with more dilute acids (approximately 10% V/V) appropriate for the specific plastics used and then rinsed with reagent water and dried in a clean environment.

4.1.6.2 Sample Digestion

1. Weigh a well-mixed sample to the nearest 0.001 g into an appropriate vessel equipped with a pressure relief mechanism. For biological tissues initially use no more than 0.5 g.
2. Add 9 ± 0.1 mL concentrated nitric acid and 3 ± 0.1 mL concentrated hydrofluoric acid to the vessel in a fume hood. If the approximate silicon dioxide content of the sample is known, the

quantity of hydrofluoric acid may be varied from 0-5 mL for stoichiometric reasons. Samples with higher concentrations of silicon dioxide (>70%) may require higher concentrations of hydrofluoric acid (>3 mL HF). Alternatively samples with lower concentrations of silicon dioxide (< 10% to 0%) may require much less hydrofluoric acid (0.5 mL to 0 mL). Acid digestion reagent combinations used in the analysis of biological samples is as follows:

Sample	HNO_3	HF	HCl
NIST SRM 2704 Oyster Tissue	9	0	0

3. The addition of other reagents with the original acids prior to digestion may permit more complete oxidation of organic sample constituents, address specific decomposition chemistry requirements, or address specific elemental stability and solubility problems.

   The addition of 2 ± 2 mL concentrated hydrochloric acid to the nitric and hydrofluoric acids is appropriate for the stabilization of Ba, and Sb and high concentrations of Fe and Al in solution. The amount of HCl needed will vary depending on the matrix and the concentration of the analytes. The addition of hydrochloric acid may; however, limit the techniques or increase the difficulties of analysis. The addition of hydrogen peroxide (30%) in small or catalytic quantities (such as 0.1 to 2 mL) may aid in the complete oxidation of organic matter. The addition of water (double deionized) may (0 to 5 mL) improve the solubility of minerals and prevent temperature spikes due to exothermic reactions.

***CAUTION**: Only one acid mixture or quantity may be used in a single batch in the microwave to insure consistent reaction conditions between all vessels and monitored conditions. This limitation is due to the current practice of monitoring a representative vessel and applying a uniform microwave field to reproduce these reaction conditions within a group of vessels being simultaneously heated.*

***CAUTION:** Toxic nitrogen oxide(s), hydrogen fluoride, and toxic chlorine (from the addition of hydrochloric acid) fumes are usually produced during digestion. Therefore, all steps involving open or the opening of microwave vessels must be performed in a properly operating fume ventilation system.*

***CAUTION**: The analyst should wear protective gloves and face protection and must not at any time permit a solution containing hydrofluoric acid to come in contact with skin or lungs.*

***CAUTION:** The addition of hydrochloric acid must be from concentrated hydrochloric acid and not from a premixed combination of acids as a buildup of toxic chlorine and possibly other gases will result from a premixed acid solution. This will over pressurize the vessel due to the release of these gases from solution upon heating. The gas effect is greatly lessened by following this suggestion.*

***CAUTION**: When digesting samples containing volatile or easily oxidized organic compounds, initially weigh no more than 0.10 g and observe the reaction before capping the vessel. If a vigorous reaction occurs, allow the reaction to cease before capping the vessel. If no appreciable reaction occurs, a sample weight up to 0.25g can be used.*

***CAUTION:** The addition of hydrogen peroxide should only be done when the reactive components of the sample are known. Hydrogen peroxide may react rapidly and violently on easily oxidizable materials and should not be added if the sample may contain large quantities of easily oxidizable organic constituents.*

4. The analyst should be aware of the potential for a vigorous reaction. If a vigorous reaction occurs upon the initial addition of reagent or the sample is suspected of containing easily

oxidizable materials, allow the sample to predigest in the uncapped digestion vessel. Heat may be added in this step for safety considerations (for example the rapid release of carbon dioxide from carbonates, easily oxidized organic matter, *etc*.). Once the initial reaction has ceased, the sample may continue through the digestion procedure.

5. Seal the vessel according to the manufacturer's directions. Properly place the vessel in the microwave system according to the manufacturer's recommended specifications and connect appropriate temperature and pressure sensors to vessels according to manufacturer's specifications.

6. This method is a performance based method, designed to achieve or approach total decomposition of the sample through achieving specific reaction conditions. The temperature of each sample should rise to 180 ± 5 ºC in approximately 5.5 minutes and remain at 180 ± 5 ºC for 9.5 minutes. The number of samples simultaneously digested is dependent on the analyst. The number may range from 1 to the maximum number of vessels that the microwave units magnetron can heat according to the manufacturer's or literature specifications (the number will depend on the power of the unit, the quantity and combination of reagents, and the heat loss from the vessels). The pressure should peak between 5 and 15 minutes for most samples. If the pressure exceeds the pressure limits of the vessel, the pressure will be reduced by the relief mechanism of the vessel. The total decomposition of some components of a matrix may require or the reaction kinetics is dramatically improved with higher reaction temperatures. If microwave digestion systems and/or vessels are capable of achieving higher temperatures and pressures, the minimum digestion time of 9.5 minutes at a temperature of at least 180 ± 5°C is an appropriate alternative. This change will permit the use of pressure systems if the analysis verifies that 180°C is the minimum temperature maintained by these control systems.

   For reactive substances, the heating profile may be altered for safety purposes. The decomposition is primarily controlled by maintaining the reagents at 180 ± 5°C for 9.5 minutes; therefore the time it takes to heat the samples to 180 ± 5°C is not critical. The samples may be heated at a slower rate to prevent potential uncontrollable exothermic reactions. The time to reach 180 ± 5 ºC may be increased to 10 minutes provided that 180 ± 5 ºC is subsequently maintained for 9.5 minutes. The extreme difference in pressure is due to the gaseous digestion products.

   Calibration control is applicable in reproducing this method provided the power in watts versus time parameters are determined to reproduce the specifications listed. The calibration settings will be specific to the quantity and combination of reagents, quantity of vessels, and heat loss characteristics of the vessels. If calibration control is being used, any vessels containing acids for analytical blank purposes are counted as sample vessels and when fewer than the recommended number of samples are to be digested, the remaining vessels should be filled with the same acid mixture to achieve the full complement of vessels. This provides an energy balance, since the microwave power absorbed is proportional to the total absorbed mass in the cavity. Irradiate each group of vessels using the predetermined calibration settings. (Different vessel types should not be mixed).

   Pressure control for a specific matrix is applicable if instrument conditions are established using temperature control. Because each matrix will have a different reaction profile, performance using temperature control must be developed for every specific matrix type prior to use of the pressure control system.

7. At the end of the microwave program, allow the vessels to cool for a minimum of 5 minutes before removing them from the microwave system. When the vessels have cooled to near

room temperature, determine if the microwave vessels have maintained a seal throughout the digestion. Due to the wide variability of vessel designs, a single procedure is not appropriate. For vessels that are sealed as discrete separate entities, the vessel weight may be taken before and after digestion to evaluate seal integrity. If the weight loss of sample exceeds 1% of the weight of the sample and reagents, then the sample is considered compromised. For vessels with burst disks, a careful visual inspection of the disk may identify compromised vessels. For vessels with resealing pressure relief mechanisms, an auditory or sometimes a physical sign indicates a vessel has vented.

8. Complete the preparation of the sample by carefully uncapping and venting each vessel in a fume hood. Vent the vessels using the procedure recommended by the vessel manufacturer. Transfer the sample to an acid-cleaned bottle. If the digested sample contains particulates which may clog nebulizers or interfere with injection of the sample into the instrument, the sample may be centrifuged, allowed to settle, or filtered.

   Centrifugation at 2,000 - 3,000 rpm for 10 mins is usually sufficient to clear the supernatant.

   Settling: If undissolved material remains such as TiO_2, or other refractory oxides, allow the sample to stand until the supernatant is clear. Allowing a sample to stand overnight will usually accomplish this. If it does not, centrifuge or filter the sample.

   Filtering: If necessary, the filtering apparatus must be thoroughly cleaned and prerinsed with dilute (approximately 10% V/V) nitric acid. Filter the sample through qualitative filter paper into a second acid-cleaned container.

9. If the hydrofluoric acid concentration is a consideration in the analysis technique such as with ICP methods, boric acid may be added to permit the complexation of fluoride to protect the quartz plasma torch. The amount of acid added may be varied, depending on the equipment and the analysis procedure. If this option is used, alterations in the measurement procedure to adjust for the boric acid and any bias it may cause are necessary. This addition will prevent the measurement of boron as one of the elemental constituents in the sample. Alternatively, a hydrofluoric acid resistant ICP torch may be used and the addition of boric acid would be unnecessary for this analytical configuration. All major manufacturers have hydrofluoric resistant components available for the analysis of solutions containing hydrofluoric acid.

CAUTION*: The traditional use of concentrated solutions of boric acid can cause problems by turning the digestion solution cloudy or result in a high salt content solution interfering with some analysis techniques. Dilute solutions of boric acid or other methods of neutralization or reagent elimination are appropriate to avoid problems with HF and glass sample introduction devices of analytical instrumentation. Gentle heating often serves to clear cloudy solutions. Matrix matching of samples and standards will eliminate viscosity differences*.

10. The removal or reduction of the quantity of the hydrochloric and hydrofluoric acids prior to analysis may be desirable. The chemistry and volatility of the analytes of interest should be considered and evaluated when using this alternative. Evaporation to near dryness in a controlled environment with controlled pure gas and neutralizing and collection of exhaust interactions is an alternative where appropriate. This manipulation may be performed in the microwave system, if the system is capable of this function, or external to the microwave system in more common apparatus(s). This option must be tested and validated to determine analyte retention and loss and should be accompanied by equipment validation possibly using the standard addition method and standard reference materials. This alternative may be used to alter either the acid concentration and/or acid composition.

***NOTE**: The final solution typically requires nitric acid to maintain appropriate sample solution acidity and stability of the elements. Commonly, a 2% (v/v) nitric acid concentration is desirable. Waste minimization techniques should be used to capture reagent fumes. This procedure should be tested and validated in the apparatus and on standards before using on unknown samples.*

11. Transfer or decant the sample into volumetric ware and dilute the digest to a known volume. The digest is now ready for analysis for elements of interest using appropriate elemental analysis techniques and/or methods.
12. Sample size may be scaled-up from 0.1, 0.25, or 0.5 g to 1.0 g through a series of 0.2 g sample size increments. Scale-up can produce different reaction conditions and/or produce increasing gaseous reaction products. Increases in sample size may not require alteration of the acid quantity or combination, but other reagents may be added to permit a more complete decomposition and oxidation of organic and other sample constituents where necessary (such as increasing the HF for the complete destruction of silicates). Each step of the scale-up must demonstrate safe operation before continuing.

4.1.7 Calculations

The concentrations determined are to be reported on the basis of the actual weight of the original sample.

4.1.8 Calibration of Microwave Equipment

***NOTE**: If the microwave unit uses temperature feedback control to follow performance specifications of the method, then the calibration procedure will not be necessary.*

1. Calibration is the normalization and reproduction of microwave field strength to permit reagent and energy coupling in a predictable and reproducible manner. It balances reagent heating and heat loss from the vessels and is equipment dependent due to the heat retention and loss characteristics of the specific vessel. Available power is evaluated to permit the microwave field output in watts to be transferred from one microwave system to another. Use of calibration to control this reaction requires balancing output power, coupled energy, and heat loss to reproduce the temperature heating profile in section 4.1.6.2.6. The conditions for each acid mixture and each batch containing the same specified number of vessels must be determined individually. Only identical acid mixtures and vessel models and specified numbers of vessels may be used in a given batch.
2. For cavity type microwave equipment, this is accomplished by measuring the temperature rise in 1 kg of water exposed to microwave radiation for a fixed period of time. The analyst can relate power in watts to the partial power setting of the system. The calibration format required for laboratory microwave systems depends on the type of electronic system used by the manufacturer to provide partial microwave power. Few systems have an accurate and precise linear relationship between percent power settings and absorbed power. Where linear circuits have been utilized, the calibration curve can be determined by a three-point calibration method (sec. 4.1.8.4); otherwise, the analyst must use the multiple point calibration method (sec.4.1.8.3).
3. The multiple point calibration involves the measurement of absorbed power over a large range of power settings. Typically, for a 600 W unit, the following power settings are measured; 100, 99, 98, 97, 95, 90, 80, 70, 60, 50, and 40% using the procedure described

in section 4.1.8.5. This data is clustered about the customary working power ranges. Nonlinearity has been encountered at the upper end of the calibration. If the system's electronics are known to have nonlinear deviations in any region of proportional power control, it will be necessary to make a set of measurements that bracket the power to be used. The final calibration point should be at the partial power setting that will be used in the test. This setting should be checked periodically to evaluate the integrity of the calibration. If a significant change is detected (±10 W) then the entire calibration should be reevaluated.

4. The three-point calibration involves the measurement of absorbed power at three different power settings. Measure the power at 100% and 50% using the procedure described in section 4.1.8.5. From the 2-point line calculate the power setting corresponding to the required power in watts specified in the procedure. Measure the absorbed power at that partial power setting. If the measured absorbed power does not correspond to the specified power within ±10 W, use the multiple point calibration in 4.9.3. This point should also be used to periodically verify the integrity of the calibration.

5. Equilibrate a large volume of water to room temperature (23 ± 2 ºC). One kg of reagent water is weighed (1,000.0 g + 0.1 g) into a suitably inert polymeric beaker or a beaker made of some other material that does not significantly absorb microwave energy (glass absorbs microwave energy and is not recommended). The initial temperature of the water should be 23 ± 2 ºC measured to ± 0.05 ºC. The covered beaker is circulated continuously (in the normal sample path) through the microwave field for 2 minutes at the desired partial power setting with the system's exhaust fan on maximum (as it will be during normal operation). The beaker is removed and the water vigorously stirred. Use a magnetic stirring bar inserted immediately after microwave irradiation, and record the maximum temperature within the first 30 seconds to ± 0.05 ºC. Use a new sample for each additional measurement. If the water is reused, both the water and the beaker must have returned to 23 ± 2 ºC. Three measurements at each power setting should be made.

   The absorbed power is determined by the following relationship:

$$P = \frac{K\ Cp\ m\ \Delta T}{t}$$

   where:

   P = the apparent power absorbed by the sample in watts (W, W = joule sec^{-1})

   K = the conversion factor for thermochemical calories_sec^{-1} to watts (which is 4.184)

   Cp = the heat capacity, thermal capacity, or specific heat (cal g^{-1} ºC^{-1}) of water

   m = the mass of the water sample in grams (g)

   ΔT = the final temperature minus the initial temperature (ºC)

   t = the time in seconds (s)

   Using the experimental conditions of 2 minutes and 1 kg of distilled water (heat capacity at 25 ºC is 0.9997 cal g^{-1} ºC^{-1}) the calibration equation simplifies to:

$$P = 34.86\ \Delta T$$

***NOTE**: Stable line voltage is necessary for accurate and reproducible calibration and operation. The line voltage should be within manufacturer's specification, and during measurement and operation should not vary by more than ±5 V. Electronic components in most microwave units are matched to the system's function and output. When any part of the high voltage circuit, power source, or control components in the system have been serviced or replaced,*

it will be necessary to recheck the system's calibration. If the power output has changed significantly (±10 W) then the entire calibration should be reevaluated.

4.1.9 Quality Control

1. All quality control data must be maintained and available for reference or inspection for a period determined by all involved parties based on program or project requirements. This method is restricted to use by, or under supervision of, experienced analysts.
2. Duplicate samples should be processed on a routine basis. A duplicate sample is a sample brought through the whole sample preparation and analytical process. A duplicate sample should be processed with each analytical batch or every 20 samples, whichever is the greater number. A duplicate sample should be prepared for each matrix type.
3. Spiked samples and/or standard reference materials should be included with each group of samples processed or every 20 samples, whichever is the greater number. A spiked sample should also be included whenever a new sample matrix is being analyzed.
4. Blank samples should be prepared using the same reagents and quantities used in sample preparation, placed in vessels of the same type, and processed with the samples.

4.2 METALS IN FISH TISSUE AND SEDIMENT

4.2.1 Inductively Coupled Plasma – Mass Spectrometry

The sensitivity and optimum and linear ranges for each element will vary with the wavelength, spectrometer, matrix, and operating conditions. Background correction is required for trace element determination. Background emission must be measured adjacent to analyte lines on samples during analysis. The position selected for background-intensity measurement, on either or both sides of the analytical line, will be determined by complexity of the spectrum adjacent to the analyte line. The position used should be as free as possible from spectral interference and should reflect the same change in background intensity as occurs at the analyte wavelength measured. Background correction is not required in cases of line broadening where background correction measurement would actually degrade the analytical result. The possibility of additional interferences identified in section 4.2.1.2 should also be recognized and appropriate corrections made; tests for their presence are described in sections 4.2.1.5.5 and 4.2.1.5.6. Alternatively, users may choose multivariate calibration methods. In this case, point selections for background correction are superfluous since whole spectral regions are processed.

4.2.1.1 Summary of Method

This method describes multi-elemental determination of analytes by ICP-MS in environmental samples (Figure 4.1). The method measures ions produced by a radio-frequency inductively coupled plasma. Analyte species originating in a liquid are nebulized (See Appendix A for methodology) and the resulting aerosol is transported by argon gas into the plasma torch. The ions produced by high temperatures are entrained in the plasma gas and extracted through a differentially pumped vacuum interface and separated on the basis of their mass-to-charge ratio by a mass spectrometer. The ions transmitted through the mass spectrometer are quantified by a channel electron multiplier or Faraday detector and the ion information is processed by the instrument's data handling system. Interferences must be assessed and valid corrections applied or the data qualified to indicate problems. Interference correction must include

compensation for background ions contributed by the plasma gas, reagents, and constituents of the sample matrix.

4.2.1.2 Interferences

1. Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All these materials must be demonstrated to be free from interferences under the conditions of the analysis by analyzing method blanks. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be necessary.
2. Interferences must be assessed and valid corrections applied or the data qualified to indicate problems. Interference correction must include compensation for background ions contributed by the plasma gas, reagents, and constituents of the sample matrix.

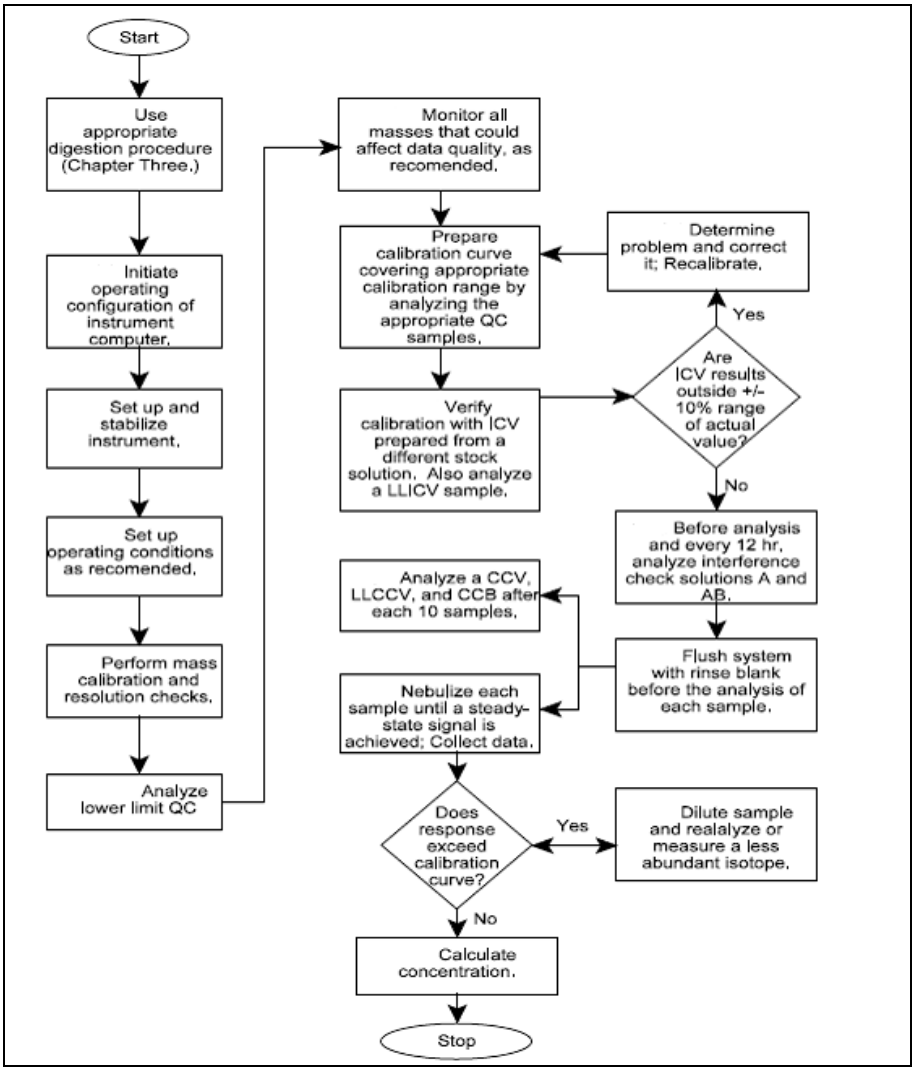

Figure 4.1. Inductively Coupled Plasma- Mass Spectrometry

3. Isobaric elemental interferences in ICP-MS are caused by isotopes of different elements forming atomic ions with the same nominal mass-to-charge ratio (m/z). A data system must

be used to correct for these interferences. This involves determining the signal for another isotope of the interfering element and subtracting the appropriate signal from the analyte isotope signal. Since commercial ICP-MS instruments nominally provide unit resolution at 10% of the peak height, very high ion currents at adjacent masses can also contribute to ion signals at the mass of interest. Although this type of interference is uncommon, it is not easily corrected, and samples exhibiting a significant problem of this type could require resolution improvement, matrix separation, or analysis using another verified and documented isotope, or use of another method.

Isobaric molecular and doubly-charged ion interferences in ICP-MS are caused by ions consisting of more than one atom or charge, respectively. Most isobaric interferences that could affect ICP-MS determinations have been identified. Examples include $^{75}ArCl^+$ ion on the ^{75}As signal and MoO^+ ions on the cadmium isotopes. While the approach used to correct for molecular isobaric interferences is demonstrated below using the natural isotope abundances from the literature, the most precise coefficients for an instrument can be determined from the ratio of the net isotope signals observed for a standard solution at a concentration providing suitable (<1%) counting statistics.

Because the ^{35}Cl natural abundance of 75.77% is 3.13 times the ^{37}Cl abundance of 24.23%, the chloride correction for arsenic can be calculated (approximately) where the $^{38}Ar^{37}Cl^+$ contribution at m/z 75 is a negligible 0.06% of the $^{40}Ar^{35}Cl^+$ signal.

Corrected arsenic signal (using natural isotopes abundances for coefficient approximations) = (m/z 75 signal) - (3.13) (m/z 77 signal) + (2.73) (m/z 82 signal),

where the final term adjusts for any selenium contribution at 77 m/z.

Note. *Arsenic values can be biased high by this type of equation when the net signal at m/z 82 is caused by ions other than $_{82}Se^+$, (e.g., $^{81}BrH^+$ from bromine wastes).*

Corrected cadmium signal (using natural isotopes abundances for coefficient approximations) = (m/z 114 signal) - (0.027)(m/z 118 signal) - (1.63)(m/z 108 signal)

where last 2 terms adjust for any $^{114}Sn^+$ or $^{114}MoO_+$ contributions at m/z 114.

Note. *Cadmium values will be biased low by this type of equation when $^{92}ZrO^+$ ions contribute at m/z 108, but use of m/z 111 for Cd is even subject to direct ($^{94}ZrOH^+$) and indirect ($^{90}ZrO^+$) additive interferences when Zr is present.*

The accuracy of these types of equations is based upon the constancy of the observed isotopic ratios for the interfering species. Corrections that presume a constant fraction of a molecular ion relative to the parent ion have not been found to be reliable, *e.g.*, oxide levels can vary with operating conditions. If a correction for an oxide ion is based upon the ratio of parent-to-oxide ion intensities, the correction must be adjusted for the degree of oxide formation by the use of an appropriate oxide internal standard previously demonstrated to form a similar level of oxide as the interferent. The use of aerosol desolvation and/or mixed gas plasmas have been shown to greatly reduce molecular interferences. These techniques can be used provided that the lower limits of quantitation, accuracy, and precision requirements for analysis of the samples can be met.

Also, solid phase chelation may be used to eliminate isobaric interferences from element and molecular sources. An on-line method has been demonstrated for environmental waters such as sea water, drinking water and acid decomposed samples. The method also provides a way for preconcentration to enhance quantitation limits simultaneously with elimination of isobaric interferences. The method relies on chelating resins such as

imminodiacetate or other appropriate resins and selectively concentrates the elements of interest while eliminating interfering elements from the sample matrix. By eliminating the elements that are direct isobaric interferences or those that form isobaric interfering molecular masses, the mass region is simplified and these interferences cannot occur. The method has been proven effective for the certification of standard reference materials and validated using SRMs. The method has the potential to be used on-line or off-line as an effective sample preparation method specifically designed to address interference problems.

4. Physical interferences are associated with the sample nebulization and transport processes as well as with ion-transmission efficiencies. Nebulization and transport processes can be affected if a matrix component causes a change in surface tension or viscosity. Changes in matrix composition can cause significant signal suppression or enhancement. Dissolved solids can deposit on the nebulizer tip of a pneumatic nebulizer and on the interface skimmers (reducing the orifice size and the instrument performance). Total solid levels below 0.2% (2,000 mg/L) are recommended to minimize solid deposition. An internal standard can be used to correct for physical interferences, if it is carefully matched to the analyte so that the two elements are similarly affected by matrix changes. When intolerable physical interferences are present in a sample, a significant suppression of the internal standard signals (to < 30% of the signals in the calibrations standard) will be observed. Dilution of the sample fivefold (1+4) will usually eliminate the problem (see sec. 4.2.1.5.4).
5. Memory interferences or carry-over can occur when there is large concentration differences between samples or standards which are analyzed sequentially. Sample deposition on the sampler and skimmer cones, spray chamber design, and the type of nebulizer affect the extent of observed memory interferences. The rinse period between samples must be long enough to eliminate significant memory interference.

4.2.1.3 Equipment and Supplies

1. Inductively coupled plasma-mass spectrometer – the system must be capable of providing resolution, better than or equal to 1.0 amu at 10% peak height is required. The system must have a mass range from at least 6 to 240 amu and a data system that allows corrections for isobaric interferences and the application of the internal standard technique. Use of a mass-flow controller for the nebulizer argon and a peristaltic pump for the sample solution is recommended.
2. Argon gas supply -- High-purity grade (99.99%)
3. Only polyethylene or fluorocarbon (TFE or PFA) containers are recommended for use in this method.

4.2.1.4 Reagents and Standards

1. Reagent- or trace metals-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.
2. Acids used in the preparation of standards and for sample processing must be of high purity. Redistilled acids are recommended because of the high sensitivity of ICP-MS. Nitric acid at less than 2% (v/v) is required for ICP-MS to minimize damage to the interface and to

minimize isobaric molecular-ion interferences with the analytes. Concentration of antimony between 50-500 µg/l require 1% (v/v) HCl for stability. Consequently, accuracy of analytes requiring significant chloride molecular ion corrections (such as As) will degrade.

3. Reagent water -- All references to water in the method refer to distilled water, unless otherwise specified. Reagent water must be free of interferences.

4. Standard stock solutions for each analyte may be purchased or prepared from ultra-high purity grade chemicals or metals (99.99 or greater purity). Preapartion procedures are outlined in Table 4.2. Recommended internal standards are ^{6}Li, ^{45}Sc, ^{89}Y, ^{103}Rh, ^{115}In, ^{159}Tb, ^{165}Ho, ^{74}Ge, and ^{209}Bi. The lithium internal standard should have an enriched abundance of ^{6}Li, so interference from lithium native to the sample is minimized. Other elements may need to be used as internal standards when samples contain significant native amounts of the recommended internal standards.

Table 4.2. Recommended Stock Solution Preparation Procedures.[1]

Element	Stock Solution	Directions
Bismuth	1 ml = 100 µg of Bi	Dissolve 0.1115 g of Bi_2O_3 in a minimum amount of dilute HNO_3. Add 10 ml of conc. HNO_3 and dilute to 1,000 ml with reagent water.
Geranium	1 ml= 100 µg of Ge	Dissolve 0.2954 g of $GeCl_4$ in a minimum amount of dilute HNO_3. Add 10 ml of conc. HNO_3 and dilute to 1,000 ml with reagent water.
Holmium	1 ml = 100 µg of Ho	Dissolve 0.1757 g of $Ho_2(CO_3)_2$5H2O in 10 ml of reagent water and 10 ml of HNO_3. After dissolution is complete, warm the solution to degas. Add 10 ml conc. of HNO_3 and dilute to 1,000 ml with reagent water.
Indium	1 ml = 100 µg of In	Dissolve 0.1000 g of indium metal in 10 ml of conc. HNO_3. Dilute to 1,000 ml with reagent water.
Lithium	1 ml = 100 µg of 6Li	Dissolve 0.6312 g of 95-atom-% ^{6}Li, Li_2CO_3 in 10 ml of reagent water and 10 ml of HNO_3. After dissolution is complete, warm the solution to degas. Add 10 ml conc. of HNO_3 and dilute to 1,000 ml with reagent water.
Rhodium	1 ml = 100 µg of Rh	Dissolve 0.3593 g of ammonium hexachlororhodate (III) $(NH_4)_3RhCl_6$ in 10 ml reagent water. Add 100 ml of conc. HCl and dilute to 1,000 ml with reagent water.
Scandium	1 ml = 100 µg of Sc	Dissolve 0.15343 g of Sc_2O_3 in 10 ml (1+1) of hot HNO_3. Add 5 ml of conc. HNO_3 and dilute to 1,000 ml with reagent water.
Terbium	1 ml = 100 µg of Tb	Dissolve 0.1828 g of $Tb_2(CO_3)_3$5H2O in 10 m (1+1) of HNO_3. After dissolution is complete, warm the solution to degas. Add 5 ml of conc. HNO_3 and dilute to 1,000 ml with reagent water.
Yttrium	1 ml = 100 µg of Y	Dissolve 0.2316 g of $Y_2(CO_3)_3$3H2O in 10 ml (1+1) of HNO_3. Add 5 ml conc. of HNO_3 and dilute to 1,000 ml with reagent water.
Titanium (interference)	1 ml = 100 µg of Ti	Dissolve 0.4133 g of $(NH_4)_2TiF_6$ in reagent water. Add 2 drops of conc. HF and dilute to 1,000 ml with reagent water.
Molybdenum (interference)	1 ml = 100 µg of Mo	Dissolve 0.2043 g of $(NH_4)_2MoO_4$ in reagent water. Dilute to 1,000 ml with reagent water.

5. Mixed calibration standard solutions are prepared by diluting the stock-standard solutions to levels in the linear range for the instrument in a solvent consisting of 1% (v/v) HNO_3 in reagent water. The calibration standard solutions must contain a suitable concentration of

1 Concentrations are calculated based upon the weight of pure metal added, or with the use of the element fraction and the weight of the metal salt added. The weight of the analyte is expressed to four significant figures for consistency with the weights below because rounding to two decimal places can contribute up to 4% error for some of the compounds.

an appropriate internal standard for each analyte. Internal standards may be added on-line at the time of analysis using a second channel of the peristaltic pump and an appropriate mixing manifold. Generally, an internal standard should be no more than 50 amu removed from the analyte.

6. Prior to preparing the mixed standards, each stock solution must be analyzed separately to determine possible spectral interferences or the presence of impurities. Care must be taken when preparing the mixed standards to ensure that the elements are compatible and stable together. Transfer the mixed standard solutions to freshly acid-cleaned FEP fluorocarbon or previously unused polyethylene or polypropylene bottles for storage. For all intermediate and working standards, especially low level standards (*i.e*., <1 ppm), stability must be demonstrated prior to use. Fresh mixed standards must be prepared as needed with the realization that concentrations can change on aging.

   Three types of blanks are required for analysis. The calibration blank is used to establish the calibration curve. The method blank is used to monitor for possible contamination resulting from either the reagents (acids) or the equipment used during sample processing including filtration. The rinse blank is used to flush the system between all samples and standards.

   The calibration blank consists of the same concentration(s) of the same acid(s) used to prepare the final dilution of the calibrating solutions of the analytes (often 1% HNO_3 (v/v) in reagent water) along with the selected concentrations of internal standards such that there is an appropriate internal standard element for each of the analytes. Use HCl for antimony.

   The method blank must contain all of the reagents in the same volumes as used in the processing of the samples. The method blank must be carried through the complete procedure and contain the same acid concentration in the final solution as the sample solution used for analysis.

   The rinse blank consists of 1 to 2% of HNO_3 (v/v) in reagent water. Prepare a sufficient quantity to flush the system between standards and samples.

7. The interference check solution (ICS) is prepared to contain known concentrations of interfering elements that will demonstrate the magnitude of interferences and provide an adequate test of any corrections. Chloride in the ICS provides a means to evaluate software corrections for chloride-related interferences such as $^{35}Cl^{16}O^+$ on $^{51}V^+$ and $^{40}Ar^{35}Cl^+$ on $^{75}As^+$. Iron is used to demonstrate adequate resolution of the spectrometer for the determination of manganese. Molybdenum serves to indicate oxide effects on cadmium isotopes. The other components are present to evaluate the ability of the measurement system to correct for various molecular-ion isobaric interferences. The ICS is used to verify that the interference levels are corrected by the data system within quality control limits.

   These solutions can be obtained commercially or prepared from ultra-pure reagents by the procedure outlined in Table 4.3.

Table 4.3. Interference Check Solution Preparation Procedures.

ICS solution	Directions
Mixed solution I	Add 13.903 g of $Al(NO_3)_3\,9H_2O$, 2.498 g of $CaCO_3$ (dried at 180 EC for 1 hr before weighing), 1.000 g of Fe, 1.658 g of MgO, 2.305 g of Na_2CO_3, and 1.767 g of K_2CO_3 to 25 ml of reagent water. Slowly add 40 ml of (1+1) HNO_3. After dissolution is complete, warm the solution to degas. Cool and dilute to 1,000 ml with reagent water.
Mixed solution II	Slowly add 7.444 g of 85 % H_3PO_4, 6.373 g of 96% H_2SO_4, 40.024 g of 37% HCl, and 10.664 g of citric acid $C_6O_7H_8$ to 100 ml of reagent water. Dilute to 1,000 ml with reagent

	water.
Mixed solution III	Add 1.00 ml each of 100-µg/ml As, Cd, Se, Cr, Cu, Mn, Ni, and Zn stock solutions to about 50 ml of reagent water. Add 2.0 ml of concentrated HNO_3, and dilute to 100.0 ml with reagent water.
Working solution A	Add 10.0 ml of mixed ICS solution I, 2.0 ml each of 100-µg/ml titanium stock solution and molybdenum stock solution, and 5.0 ml of mixed ICS solution II. Dilute to 100 ml with reagent water. ICS solution A must be prepared fresh weekly.
Working solution AB	Adding 10.0 ml of mixed ICS solution I, 2.0 ml each of 100-µg/ml titanium stock solution and molybdenum stock solution, 5.0 ml of mixed ICS solution II and 2.0 ml of mixed ICS solution III. Dilute to 100 ml with reagent water. ICS solution AB must be prepared fresh weekly.

The final ICS solution concentrations in Table 4.4 are intended to evaluate corrections for known interferences.

Table 4.4. Recommended Interference Check Sample Components and Concentrations.

Solution Component	Solution A (mg/L)	Solution AB (mg/L)
Al	100.0	100.0
As	0.0	0.100
Cd	0.0	0.100
Cr	0.0	0.200
Cu	0.0	0.200
Fe	250.0	250.0
Mn	0.0	0.200
Ni	0.0	0.200
Se	0.0	0.100
Zn	0.0	0.100

8. The initial calibration verification (ICV) standard may be purchased or prepared by combining compatible elements from a standard source different from that of the calibration standard, and at concentration near the midpoint of the calibration curve.
9. The continuing calibration verification (CCV) standard should be prepared in the same acid matrix using the same standards used for calibration, at a concentration near the mid-point of the calibration curve.
10. Mass spectrometer tuning solution. A solution containing elements representing all of the mass regions of interest must be prepared to verify that the resolution and mass calibration of the instrument are within the required specifications. This solution is also used to verify that the instrument has reached thermal stability.

4.2.1.5 Quality Control

1. Refer to the QAPP for additional guidance on quality assurance and quality control protocols. When inconsistencies exist between QC guidelines, method specific QC criteria take precedence. Each lab must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit. The laboratory should also maintain records to document the

quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.

2. Instrument detection limits (IDLs) are useful means to evaluate the instrument noise level and response changes over time for each analyte from a series of reagent blank analyses to obtain a calculated concentration. They are not to be confused with the lower limit of quantitation, nor should they be used in establishing this limit. It may be helpful to compare the calculated IDLs to the established lower limit of quantitation (see section 4.3.1.6).

   IDLs in µg/L can be estimated by calculating the average of the standard deviations of three runs on three non-consecutive days from the analysis of a reagent blank solution with seven consecutive measurements per day. Each measurement should be performed as though it were a separate analytical sample (*i.e*., each measurement must be followed by a rinse and/or any other procedure normally performed between the analysis of separate samples). IDLs should be determined at least every three months or at a project-specific designated frequency and kept with the instrument log book.

3. Each laboratory must demonstrate initial proficiency. Each laboratory must demonstrate initial proficiency with each sample preparation and determinative method combination it utilizes by generating data of acceptable accuracy and precision for target analytes in a clean matrix. If an autosampler is used to perform sample dilutions, the laboratory should verify that those dilutions are of equivalent or better accuracy than is achieved by an experienced analyst performing manual dilutions.

4. Dilute and reanalyze samples that exceed the linear dynamic range or use an alternate, less sensitive calibration for which quality control data are already established.

5. The intensities of all internal standards must be monitored for every analysis. If the intensity of any internal standard in a sample falls below 70% of the intensity of that internal standard in the initial calibration standard, a significant matrix effect must be suspected. Under these conditions, the established lower limit of quantitation has degraded and the correction ability of the internal standardization technique becomes questionable. The following procedure is followed: Make sure the instrument has not drifted by observing the internal standard intensities in the nearest clean matrix (calibration blank). If the low internal standard intensities are also seen in the nearest calibration blank, terminate the analysis, correct the problem, recalibrate, verify the new calibration, and reanalyze the affected samples. If drift has not occurred, matrix effects need to be removed by dilution of the affected sample. The sample must be diluted fivefold (1+4) and reanalyzed with the addition of appropriate amounts of internal standards. If the first dilution does not eliminate the problem, this procedure must be repeated until the internal-standard intensities rise to the minimum 70% limit. Reported results must be corrected for all dilutions.

6. To obtain analyte data of known quality, it is necessary to measure more than the analytes of interest in order to apply corrections or to determine whether interference corrections are necessary. For example, tungsten oxide moleculars can be very difficult to distinguish from mercury isotopes. If the concentrations of interference sources (such as C, Cl, Mo, Zr, W) are such that, at the correction factor, the analyte is less than the limit of quantification and the concentration of interferents are insignificant, then the data may go uncorrected. Note that monitoring the interference sources does not necessarily require monitoring the interferant itself, but that a molecular species may be monitored to indicate the presence of the interferent. When correction equations are used, all QC criteria must also be met. Extensive QC for interference corrections is required at all times. The monitored masses must include those elements whose hydrogen, oxygen, hydroxyl, chlorine, nitrogen, carbon

and sulfur molecular ions could impact the analytes of interest. Unsuspected interferences may be detected by adding pure major matrix components to a sample to observe any impact on the analyte signals. When an interference source is present, the sample elements impacted must be flagged to indicate (a) the percentage interference correction applied to the data or (b) an uncorrected interference by virtue of the elemental equation used for quantitation. The isotope proportions for an element or molecular-ion cluster provide information useful for quality assurance.

***Note**: Only isobaric elemental, molecular, and doubly charged interference corrections which use the observed isotopic-response ratios or parent-to-oxide ratios (provided an oxide internal standard is used) for each instrument system are acceptable corrections for use.*

7. For each batch of samples processed, at least one method blank must be carried throughout the entire sample preparation and analytical process. If the method blank does not contain target analytes at a level that interferes with the project-specific DQOs, then the method blank would be considered acceptable. In the absence of project-specific DQOs, if the blank is less than 10% of the lower limit of quantitation check sample concentration, less than 10% of the regulatory limit, or less than 10% of the lowest sample concentration for each analyte in a given preparation batch, whichever is greater, then the method blank is considered acceptable. If the method blank cannot be considered acceptable, the method blank should be re-run once, and if still unacceptable, then all samples after the last acceptable method blank should be reprepared and reanalyzed along with the other appropriate batch QC samples. If the method blank exceeds the criteria, but the samples are all either below the reporting level or below the applicable action level or other DQOs, then the sample data may be used despite the contamination of the method blank.

8. For each batch of samples processed, at least one laboratory control sample (LCS) must be carried throughout the entire sample preparation and analytical process. The laboratory control samples should be spiked with each analyte of interest at the project-specific action level or, when lacking project specific action levels, at approximately mid-point of the linear dynamic range. Acceptance criteria should either be defined in the project-specific planning documents or set at a laboratory derived limit developed through the use of historical analyses. In the absence of project-specific or historical data generated criteria, this limit should be set at ± 20% of the spiked value. Acceptance limits derived from historical data should be no wider that ± 20%. If the laboratory control sample is not acceptable, then the laboratory control sample should be re-run once and, if still unacceptable, all samples after the last acceptable laboratory control sample should be reprepared and reanalyzed.

   Concurrent analyses of standard reference materials (SRMs) containing known amounts of analytes in the media of interest are recommended and may be used as an LCS. For solid SRMs, 80 -120% accuracy may not be achievable and the manufacturer's established acceptance criterion should be used for soil SRMs.

9. Documenting the effect of the matrix, for a given preparation batch consisting of similar sample characteristics, should include the analysis of at least one matrix spike and one duplicate unspiked sample (MS/Dup) or one matrix spike/matrix spike duplicate (MS/MSD) pair. The decision on whether to prepare and analyze duplicate samples or a matrix spike/matrix spike duplicate must be based on a knowledge of the samples in the sample batch or as noted in the project-specific planning documents. If samples are expected to contain target analytes, then laboratories may use one matrix spike and a duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, laboratories should use a matrix spike and matrix spike duplicate pair.

For each batch of samples processed, at least one MS/Dup or MS/MSD sample set should be carried throughout the entire sample preparation and analytical. MS/MSDs are intralaboratory split samples spiked with identical concentrations of each analyte of interest. The spiking occurs prior to sample preparation and analysis. An MS/Dup or MS/MSD is used to document the bias and precision of a method in a given sample matrix.

MS/MSD samples should be spiked at the same level, and with the same spiking material, as the corresponding laboratory control sample that is at the project-specific action level or, when lacking project-specific action levels, at approximately mid-point of the linear dynamic range. Acceptance criteria should either be defined in the project-specific planning documents or set at a laboratory-derived limit developed through the use of historical analyses per matrix type analyzed. In the absence of project-specific or historical data generated criteria, these limits should be set at ± 25% of the spiked value for accuracy and 20 relative percent difference (RPD) for precision. Acceptance limits derived from historical data should be no wider that ± 25% for accuracy and 20% for precision. If the bias and precision indicators are outside the laboratory control limits, if the percent recovery is less than 75% or greater than 125%, or if the relative percent difference is greater than 20%, then the interference test discussed in Sec. 9.9 should be conducted.

The relative percent difference between spiked matrix duplicate or unspiked duplicate determinations is to be calculated as follows:

$$RPD = \frac{D_1 - D_2}{(D_1 + D_2)/2} \times 100$$

where:

RPD = relative percent difference.

D_1 = first sample value.

D_2 = second sample value (spiked or unspiked duplicate)

The spiked sample or spiked duplicate sample recovery should be within ± 25% of the actual value, or within the documented historical acceptance limits for each matrix.

10. If less than acceptable accuracy and precision data are generated, additional quality control tests below are recommended prior to reporting concentration data for the elements in this method. At a minimum, these tests should be performed with each batch of samples prepared/analyzed with corresponding unacceptable data quality results. These tests will then serve to ensure that neither positive nor negative interferences are affecting the measurement of any of the elements or distorting the accuracy of the reported values. If matrix effects are confirmed, the laboratory should consult with the data user when feasible for possible corrective actions which may include the use of alternative or modified test procedures so that the analysis is not impacted by the same interference.

Post digestion spike addition. If the MS/MSD recoveries are unacceptable, the same sample from which the MS/MSD aliquots were prepared should also be spiked with a post digestion spike. Otherwise, another sample from the same preparation should be used as an alternative. An analyte spike is added to a portion of a prepared sample, or its dilution, and should be recovered to within 80% to 120% of the known value. The spike addition should produce a minimum level of 10 times and a maximum of 100 times the lower limit of quantitation. If this spike fails, then the dilution test should be run on this sample. If both the MS/MSD and the post digestion spike fail, then matrix effects are confirmed.

Dilution test. If the analyte concentration is sufficiently high (minimally, a factor of 10 above the lower limit of quantitation after dilution), an analysis of a 1:5 dilution should agree within ±10% of the original determination. If not, a chemical or physical interference effect should be suspected.

4.2.1.6 Calibration and Standardization

1. Set up the instrument with proper operating parameters established as detailed below. The instrument should be allowed to become thermally stable before beginning (usually requiring at least 30 min of operation prior to calibration).
2. Conduct mass calibration and resolution checks in the mass regions of interest. The mass calibration and resolution parameters are required criteria which must be met prior to any samples being analyzed. If the mass calibration differs more than 0.1 amu from the true value, then the mass calibration must be adjusted to the correct value. The resolution must also be verified to be less than 0.9 amu full width at 10% peak height.

   Sensitivity, instrumental detection limit, precision, linear dynamic range, and interference corrections need to be established for each individual target analyte on each particular instrument. All measurements (both target analytes and constituents which interfere with the target analytes) need to be within the instrument linear range where the correction equations are valid.
3. The lower limits of quantitation should be established for all isotope masses utilized for each type of matrix analyzed and for each preparation method used and for each instrument. These limits are considered the lowest reliable laboratory reporting concentrations and should be established from the lower limit of quantitation check sample and then confirmed using either the lowest calibration point or from a low-level calibration check standard.

   The lower limit of quantitation check (LLQC) sample should be analyzed after establishing the lower laboratory reporting limits and on an as needed basis to demonstrate the desired detection capability. Ideally, this check sample and the low-level calibration verification standard will be prepared at the same concentrations with the only difference being the LLQC sample is carried through the entire preparation and analytical procedure. Lower limits of quantitation are verified when all analytes in the LLQC sample are detected within ± 30% of their true value. This check should be used to both establish and confirm the lowest quantitation limit.

   The lower limits of quantitation determination using reagent water represents a best case situation and does not represent possible matrix effects of real-world samples. For the application of lower limits of quantitation on a project-specific basis with established data quality objectives, low-level matrix specific spike studies may provide data users with a more reliable indication of the actual method sensitivity and minimum detection capabilities.
4. All masses which could affect data quality should be monitored to determine potential effects from matrix components on the analyte peaks.
5. Determine calibration curve.
6. All analyses require that a calibration curve be prepared to cover the appropriate concentration range based on the intended application and prior to establishing the linear dynamic range. Usually, this means the preparation of a calibration blank and mixed calibration standard solutions, the highest of which would not exceed the anticipated linear dynamic range of the instrument. Check the instrument standardization by analyzing appropriate QC samples as follows.

Individual or mixed calibration standards should be prepared from known primary stock standards every six months to one year as needed based on the concentration stability as confirmed from the ICV analyses. The analysis of the ICV, which is prepared from a source independent of the calibration standards, is necessary to verify the instrument performance once the system has been calibrated for the desired target analytes. It is recommended that the ICV solution be obtained commercially as a certified traceable reference material such that an expiration date can be assigned. Alternately the ICV solution can be prepared from an independent source on an as needed basis depending on the ability to meet the calibration verification criteria. If the ICV analysis is outside of the acceptance criteria, at a minimum the calibration standards must be prepared fresh and the instrument recalibrated prior to beginning sample analyses. Consideration should also be given to preparing fresh ICV standards if the new calibration cannot be verified using the existing ICV standard.

Note: *This method describes the use of both a low-level and mid-level ICV standard analysis. For purposes of verifying the initial calibration, only the mid-level ICV needs to be prepared from a source other than the calibration standards.*

The calibration standards should be prepared using the same type of acid or combination of acids and at similar concentrations as will result in the samples following processing. The response of the calibration blank should be less than the response of the typical laboratory lower limit of quantitation for each desired target analyte. Additionally, if the calibration blank response or continuing calibration blank verification is used to calculate a theoretical concentration, this value should be less than the level of acceptable blank contamination as specified in the approved quality assurance project planning documents. If this is not the case, the reason for the out-of-control condition must be found and corrected, and the sample analyses may not proceed or the previous ten samples need to be reanalyzed.

For the initial and daily instrument operation, calibrate the system according to the instrument manufacturer's guidelines using the mixed calibration standards as noted in section 4.2.1.4.5. The calibration curve should be prepared daily with a minimum of a calibration blank and a single standard at the appropriate concentration to effectively outline the desired quantitation range. Flush the system with the rinse blank between each standard solution. Use the average of at least three integrations for both calibration and sample analyses. The resulting curve should then be verified with mid-level and low-level initial calibration verification standards as outlined below.

Alternatively, the calibration curve can be prepared daily with a minimum of a calibration blank and three non-zero standards that effectively bracket the desired sample concentration range. If low-level as compared to mid- or high-level sample concentrations are expected, the calibration standards should be prepared at the appropriate concentrations in order to demonstrate the instrument linearity within the anticipated sample concentration range. For all multi-point calibration scenarios, the lowest non-zero standard concentration should be considered the lower limit of quantitation.

Note*. Regardless of whether the instrument is calibrated using only a minimum number of standards or with a multi-point curve, the upper limit of the quantitation range may exceed the highest concentration calibration point and can be defined as the "linear dynamic" range, while the lower limit can be identified as the "lower limit of quantitation limit" (LLQL) and will be either the concentration of the lowest calibration standard (for multi-point curves) or the concentration of the low level ICV/CCV check standard. Results reported outside these limits would not be recommended unless they are qualified as estimated.*

To be considered acceptable, the calibration curve should have a correlation coefficient greater than or equal to 0.998. When using a multipoint calibration curve approach, every effort should be made to attain an acceptable correlation coefficient based on a linear response for each desired target analyte. If the recommended linear response cannot be attained using a minimum of three non-zero calibration standards, consideration should be given to adding more standards, particularly at the lower concentrations, in order to better define the linear range and the lower limit of quantitation. Conversely, the extreme upper and lower calibration points may be removed from the multi-point curve as long as three non-zero points remain such that the linear range is narrowed and the non-linear upper and/or lower portions are removed. As with the single point calibration option, the multi-point calibration should be verified with both a mid- and low-level ICV standard analysis using the same 90 - 110% and 70 - 130% acceptance criteria, respectively.

Many instrument software packages allow multi-point calibration curves to be "forced" through zero. It is acceptable to use this feature, provided that the resulting calibration meets the acceptance criteria, and can be verified by acceptable QC results. Forcing a regression through zero should NOT be used as a rationale for reporting results below the calibration range defined by the lowest standard in the calibration curve.

After initial calibration, the calibration curve should be verified by use of an initial calibration verification (ICV) standard analysis. At a minimum, the ICV standard should be prepared from an independent (second source) material at or near the midrange of the calibration curve. The acceptance criteria for this mid-range ICV standard should be ±10% of its true value. Additionally, a low-level initial calibration verification (LLICV) standard should be prepared, using the same source as the calibration standards, at a concentration expected to be the lower limit of quantitation. The suggested acceptance criteria for the LLICV is ±30% of its true value. Quantitative sample analyses should not proceed for those analytes that fail the second source standard initial calibration verification, with the exception that analyses may continue for those analytes that fail the criteria with an understanding these results should be qualified and would be considered estimated values. Once the calibration acceptance criteria is met, either the lowest calibration standard or the LLICV concentration can be used to demonstrate the lower limit of quantitation and sample results should not be quantitated below this lowest standard. In some cases depending on the stated project data quality objectives, it may be appropriate to report these results as estimated, however, they should be qualified by noting the results are below the lower limit of quantitation. Therefore, the quantitation limit cannot be reported lower than either the LLICV standard used for the single point calibration option or the low calibration and/or verification standard used during initial multi-point calibration. If the calibration curve cannot be verified within these specified limits for the mid-range ICV and LLICV analyses, the cause needs to be determined and the instrument recalibrated before samples are analyzed. The analysis data for the initial calibration verification analyses should be kept on file with the sample analysis data.

Both single and multi-point calibration curves must be verified at the end of each analysis batch and after every 10 samples by a continuing calibration verification (CCV) standard and a continuing calibration blank (CCB). The CCV must be made from the same material as the initial calibration standards at or near the mid-range concentration. For the curve to be considered valid the acceptance criteria for the CCV standard must be ±10% of its true value and the CCB must contain target analytes less than the established lower limit of quantitation for the target analyte. If the calibration cannot be verified within the specified limits, the sample analysis must be discontinued, the cause determined and the instrument

recalibrated. All samples following the last acceptable CCV/CCB must be reanalyzed. The analysis data for the CCV/CCB must be kept on file with the sample analysis data.

The low level continuing calibration verification (LLCCV) standard should also be analyzed at the end of each analysis batch. A more frequent LLCCV analysis, *i.e*., every 10 samples may be necessary if low-level sample concentrations are anticipated and the system stability at low end of the calibration is questionable. In addition, the analysis of a LLCCV on a more frequent basis will minimize the number of samples for re-analysis should the LLCCV fail if only run at the end of the analysis batch. The LLCCV standard should be made from the same source as the initial calibration standards at the established lower limit of quantitation as reported by the laboratory. The acceptance criteria for the LLCCV standard should be ± 30% of its true value. If the calibration cannot be verified within these specified limits, the analysis of samples containing the affected analytes at similar concentrations cannot continue until the cause is determined and the LLCCV standard successfully analyzed. The instrument may need to be recalibrated or the lower limit of quantitation adjusted to a concentration that will ensure a compliant LLCCV analysis. The analysis data for the LLCCV standard should be kept on file with the sample analysis data.

7. Verify the magnitude of elemental and molecular-ion isobaric interferences and adequacy of any corrections at the beginning of an analytical run or once every 12 hr, whichever is more frequent. Do this by analyzing the interference check solutions A and AB. The analyst should be aware that precipitation from solution AB may occur with some elements.

Note *Analysts have noted improved performance in calibration stability if the instrument is exposed to the interference check solution after cleaning sampler and skimmer cones. Improved performance is also realized if the instrument is allowed to rinse for 5 or 10 min before the calibration blank is run.*

8. The linear dynamic range is established when the system is first setup, or whenever significant instrument components have been replaced or repaired, and on an as needed basis only after the system has been successfully calibrated using either the single or multi-point standard calibration approach. The upper limit of the linear dynamic range needs to be established for each wavelength utilized by determining the signal responses from a minimum of three, preferably five, different concentration standards across the range. The ranges which may be used for the analysis of samples should be judged by the analyst from the resulting data. The data, calculations and rationale for the choice of range made should be documented and kept on file. A standard at the upper limit should be prepared, analyzed and quantitated against the normal calibration curve. The calculated value should be within ±10% of the true value. New upper range limits should be determined whenever there is a significant change in instrument response. At a minimum, the range should be checked every 6 months. The analyst must be aware that if an analyte that is present above its upper range limit is used to apply a spectral correction, the correction may not be valid and those analytes where the spectral correction has been applied may be inaccurately reported.

Note*. Some metals may exhibit non-linear response curves due to ionization and self-absorption effects. These curves may be used if the instrument allows it, however the effective range must be checked and the second order curve fit should have a correlation coefficient of 0.998 or better. Third order fits are not acceptable. These non-linear response curves should be revalidated and/or recalculated on a daily basis using the same calibration verification QC checks as a linear calibration curve. Since these curves are much more sensitive to changes in operating conditions than the linear lines, they should be checked whenever there have been moderate equipment changes. Under these calibration*

conditions, quantitation is not acceptable above or below the calibration standards. Additionally, a non-linear curve should be further verified by calculating the actual recovery of each calibration standard used in the curve. The acceptance criteria for the calibration standard recovery should be ±10% of its true value for all standards except the lowest concentration. A recovery of ±30% of its true value should be achieved for the lowest concentration standard.

9. The analyst should (1) verify that the instrument configuration and operating conditions satisfy the project-specific analytical requirements and (2) maintain quality control data that demonstrate and confirm the instrument performance for the reported analytical results.

4.2.1.7 Procedures

1. All samples must be acid digested prior to analysis. Preliminary treatment of matrices is necessary because of the complexity and variability of sample matrices.
2. Initiate appropriate operating configuration of the instrument's computer and set up the instrument with the proper operating parameters according to the instrument manufacturer's instructions.
3. Allow at least 30 min for the instrument to equilibrate before analyzing any samples. This must be verified by an analysis of the tuning solution at least four integrations with relative standard deviations of 5% for the analytes contained in the tuning solution.

Note. *The instrument should have features to protect it from high ion currents. If not, precautions must be taken to protect the detector from high ion currents. A channel electron multiplier or active film multiplier suffers from fatigue after being exposed to high ion currents. This fatigue can last from several seconds to hours depending on the extent of exposure. During this time period, response factors are constantly changing, which invalidates the calibration curve, causes instability, and invalidates sample analyses.*

4. Flush the system with the rinse blank solution until the signal levels return to the DQO or method's levels of quantitation (usually about 30 sec) before the analysis of each sample. Nebulize each sample until a steady-state signal is achieved (usually about 30 sec) prior to collecting data. Flow-injection systems may be used as long as they can meet the performance criteria of this method.
5. Regardless of whether the initial calibration is performed using a single high standard and the calibration blank or the multi-point option, the laboratory should analyze an LLCCV (section 4.3.1.6.5). For all analytes and determinations, the laboratory must analyze an ICV and LLICV immediately following daily calibration. It is recommended that a CCV, LLCCV, and CCB be analyzed after every ten samples and at the end of the analysis batch.
6. Dilute and reanalyze samples that are more concentrated than the linear range for an analyte (or species needed for a correction) or measure an alternate but less-abundant isotope. The linearity at the alternate mass must be confirmed by appropriate calibration. Alternatively apply solid phase chelation chromatography to eliminate the matrix as described in section 4.3.1.2.3.

4.2.1.8 Data Analysis and Calculations

1. The quantitative values must be reported in appropriate units, such as micrograms per liter (µg/L) for aqueous samples and milligrams per kilogram (mg/kg) for solid samples. If

dilutions were performed, the appropriate corrections must be applied to the sample values. All results should be reported with up to three significant figures.

2. Calculate results for solids on a dry-weight basis as follows:

   (1) A separate determination of percent solids must be performed.

   (2) The concentrations determined in the digest are to be reported on the basis of the dry weight of the sample.

   Concentration (dry weight)(mg/kg) = (C x V) / (W x S)

   where:

   C = Digest Concentration (mg/L)

   V = Final volume in liters after sample preparation

   W = Weight in kg of wet sample

   S = % Solids / 100

   Calculations must include appropriate interference, internal-standard normalization, and the summation of signals at 206, 207, and 208 m/z for lead (to compensate for any differences in the abundances of these isotopes between samples and standards).

3. Results must be reported in units commensurate with their intended use and all dilutions must be taken into account when computing final results.

4.2.2 Inductively Coupled Plasma – Atomic Emission Spectrometry

The sensitivity and the optimum and linear ranges for each element will vary with the wavelength, spectrometer, matrix, and operating conditions.

Background correction is required for trace element determination. Background emission must be measured adjacent to analyte lines on samples during analysis. The position selected for the background-intensity measurement, on either or both sides of the analytical line, will be determined by the complexity of the spectrum adjacent to the analyte line. The position used should be as free as possible from spectral interference and should reflect the same change in background intensity as occurs at the analyte wavelength measured. Background correction is not required in cases of line broadening where a background correction measurement would actually degrade the analytical result.

The possibility of additional interferences identified in section 4.2.2.2 should also be recognized and appropriate corrections made; tests for their presence are described in sections 4.2.6.5 and 4.2.6.6. Users may instead choose multivariate calibration methods; if used, point selections for background correction are superfluous since whole spectral regions are processed.

4.2.2.1 Summary of Method

This method describes multielemental determinations by ICP-AES using sequential or simultaneous optical systems and axial or radial viewing of the plasma (Figure 4.2). The instrument measures characteristic emission spectra by optical spectrometry. Samples are nebulized (Appendix A) and the resulting aerosol is transported to the plasma torch. Element-specific emission spectra are produced by a radio-frequency inductively coupled plasma. The spectra are dispersed by a grating spectrometer, and the intensities of the emission lines are monitored by photosensitive devices.

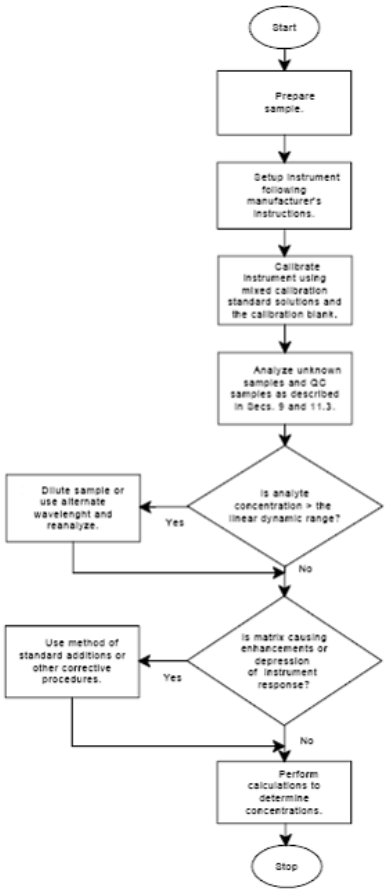

Figure 4.2. Inductively Coupled Plasma-Atomic Emission Spectrometry

4.2.2.2 Interferences

Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/ or interferences to sample analysis. All materials must be confirmed free from interferences under the conditions of the analysis by analyzing method blanks. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be necessary.

Background and Stray Light

1. Compensation for background emission and stray light can usually be conducted by subtracting the background emission determined by measurements adjacent to the analyte wavelength peak. Spectral scans of samples or single element solutions in the analyte regions may indicate when alternate wavelengths are desirable because of severe spectral interference. These scans will also show whether the most appropriate estimate of the background emission is provided by an interpolation from measurements on both sides of the wavelength peak or by measured emission on only one side. The locations selected for the measurement of background intensity will be determined by the complexity of the spectrum adjacent to the wavelength peak. The locations used for routine measurement must be free of off-line spectral interference (interelement or molecular) or adequately corrected to reflect the same change in background intensity as occurs at the wavelength peak. For multivariate methods using whole spectral regions, background scans should be

included in the correction algorithm. Off-line spectral interferences are handled by including spectra on interfering species in the algorithm.

2. To determine the appropriate location for off-line background correction, the analyst must scan the area on either side adjacent to the wavelength and record the apparent emission intensity from all other method analytes. This spectral information must be documented and kept on file. The location selected for background correction must be either free of off-line interelement spectral interference or a computer routine must be used for automatic correction on all determinations. If a wavelength other than the recommended wavelength is used, the analyst must determine and document both the overlapping and nearby spectral interference effects from all method analytes and common elements and provide for their automatic correction on all analyses. Tests to determine spectral interference must be done using analyte concentrations that will adequately describe the interference. Normally, 100 mg/L single-element solutions are sufficient. However, for analytes such as iron that may be found in the sample at high concentration, a more appropriate test would be to use a concentration near the upper limit of the analytical range.

3. Spectral overlaps may be avoided by using an alternate wavelength or can be compensated for by equations that correct for interelement contributions. Instruments that use equations for interelement correction require that the interfering elements be analyzed at the same time as the element of interest. When operative and uncorrected, interferences will produce false positive or positively biased determinations. Analysts may apply interelement correction equations determined on their instruments with tested concentration ranges to compensate (off-line or on-line) for the effects of interfering elements. For multivariate calibration methods using whole spectral regions, spectral interferences are handled by including spectra of the interfering elements in the algorithm.

Note. *When using interelement correction equations, the interference may be expressed as analyte concentration equivalents (i.e., false positive analyte concentrations) arising from 100 mg/L of the interference element. It should be noted that the interference effects must be evaluated for each individual instrument, since the intensities will vary.*

4. Interelement corrections will vary for the same emission line among instruments because of differences in resolution, as determined by the grating, the entrance and exit slit widths, and by the order of dispersion. Interelement corrections will also vary depending upon the choice of background correction points. Selecting a background correction point where an interfering emission line may appear should be avoided when practical. Interelement corrections that constitute a major portion of an emission signal may not yield accurate data. Users should continuously note that some samples may contain uncommon elements that could contribute spectral interferences.

5. Interference effects must be evaluated for each individual instrument, whether configured as a sequential or simultaneous instrument. For each instrument, intensities will vary not only with optical resolution but also with operating conditions (e.g., power, viewing height, argon flow rate). When using the recommended wavelengths, the analyst is required to determine and document for each wavelength the effect from referenced interferences as well as any other suspected interferences that may be specific to the instrument or matrix. The analyst is encouraged to utilize a computer routine for automatic correction on all analyses.

6. Users of sequential instruments must verify the absence of spectral interference by scanning over a range of 0.5 nm centered on the wavelength of interest for several samples. The range for lead, for example, would be from 220.6 to 220.1 nm. This procedure must be repeated whenever a new matrix is to be analyzed and when a new calibration curve using

different instrumental conditions is to be prepared. Samples that show an elevated background emission across the range may be background corrected by applying a correction factor equal to the emission adjacent to the line or at two points on either side of the line and interpolating between them. An alternate wavelength that does not exhibit a background shift or spectral overlap may also be used.

7. If the correction routine is operating properly, the determined apparent analyte(s) concentration from analysis of each interference solution should fall within a specific concentration range around the calibration blank. The concentration range is calculated by multiplying the concentration of the interfering element by the value of the correction factor being tested and dividing by 10. If after the subtraction of the calibration blank the apparent analyte concentration falls outside of this range, in either a positive or negative direction, a change in the correction factor of more than 10% should be suspected. The cause of the change should be determined and corrected and the correction factor updated. The interference check solutions should be analyzed more than once to confirm a change has occurred. Adequate rinse time between solutions and before analysis of the calibration blank will assist in the confirmation.

8. When interelement corrections are applied, their accuracy should be verified daily, by analyzing spectral interference check solutions. The correction factors or multivariate correction matrices tested on a daily basis must be within the 20% criteria for five consecutive days. All interelement spectral correction factors or multivariate correction matrices must be verified and updated every six months or when an instrumentation change occurs, such as one in the torch, nebulizer, injector, or plasma conditions. Standard solutions should be inspected to ensure that there is no contamination that may be perceived as a spectral interference.

9. When interelement corrections are not used, verification of absence of interferences is required.One method to verify the absence of interferences is to use a computer software routine for comparing the determinative data to established limits for notifying the analyst when an interfering element is detected in the sample at a concentration that will produce either an apparent false positive concentration (*i.e*., greater than the analyte instrument detection limit), or a false negative analyte concentration (*i.e*., less than the lower control limit of the calibration blank defined for a 99% confidence interval).

   Another way to verify the absence of interferences is to analyze an interference check solution which contains similar concentrations of the major components of the samples (>10 mg/L) on a continuing basis to verify the absence of effects at the wavelengths selected. These data must be kept on file with the sample analysis data. If the check solution confirms an operative interference that is ∃20% of the analyte concentration, the analyte must be determined using (1) analytical and background correction wavelengths (or spectral regions) free of the interference, (2) by an alternative wavelength, or (3) by another documented test procedure.

Physical Interferences

Physical Interferences are effects associated with the sample nebulization and transport processes. Changes in viscosity and surface tension can cause significant inaccuracies, especially in samples containing high dissolved solids or high acid concentrations. If physical interferences are present, they must be reduced by diluting the sample, by using a peristaltic pump, by using an internal standard, or by using a high solids nebulizer. The test described in section 4.3.2.6.8 will help determine if a physical interference is present.

***Note**. One problem that can occur with high dissolved solids is salt buildup at the tip of the nebulizer, affecting aerosol flow rate and causing instrumental drift. The problem can be controlled by wetting the argon prior to nebulization, by using a tip washer, by using a high solids nebulizer, or by diluting the sample. Also, it has been reported that better control of the argon flow rate, especially to the nebulizer, improves instrument performance. This may be accomplished with the use of mass flow controllers.*

Chemical interferences

Chemical interferences include molecular compound formation, ionization effects, and solute vaporization effects. Normally, these effects are not significant with the ICP technique, but if observed, can be minimized by careful selection of operating conditions (incident power, observation position, and so forth), by buffering of the sample, by matrix matching, and by standard addition procedures. Chemical interferences are highly dependent on matrix type and the specific analyte element.

***Note.** An alternative to using the method of standard additions is to use the internal standard technique, which involves adding one or more elements that are both not found in the samples and verified to not cause an interelement spectral interference to the samples, standards, and blanks. Yttrium or scandium is often used. The concentration should be sufficient for optimum precision, but not so high as to alter the salt concentration of the matrix. The element intensity is used by the instrument as an internal standard to ratio the analyte intensity signals for both calibration and quantitation. This technique is very useful in overcoming matrix interferences, especially in high solids matrices.*

Memory interferences

Memory interferences result when analytes in a previous sample contribute to the signals measured in a new sample. Memory effects can result from sample deposition on the uptake tubing to the nebulizer and from the build up of sample material in the plasma torch and spray chamber. The site where these effects occur is dependent on the element and can be minimized by flushing the system with a rinse blank between samples. The possibility of memory interferences should be recognized within an analytical run and suitable rinse times should be used to reduce them.

The rinse times necessary for a particular element must be estimated prior to analysis. This may be achieved by aspirating a standard containing elements at a concentration ten times the usual amount or at the top of the linear dynamic range. The aspiration time for this sample should be the same as a normal sample analysis period, followed by analysis of the rinse blank at designated intervals. Note the length of time necessary for reducing analyte signals to "equal to" or "less than" the lower limit of quantitation. Until the required rinse time is established, the rinse period should be at least 60 sec. between samples and standards. If memory interference is suspected, the sample must be reanalyzed after a rinse period of sufficient length. Alternate rinse times may be established by the analyst based upon the project-specific DQOs.

High Salt Concentrations

Users are advised that high salt concentrations can cause analyte signal suppressions and confuse interference tests. If the instrument does not display negative values, fortify the interference check solution with the elements of interest at 0.5 to 1 mg/L and measure the added standard concentration accordingly. Concentrations should be within 20% of the true spiked concentration or dilution of the samples will be necessary. In the absence of a measurable analyte, overcorrection could go undetected if a negative value is reported as zero.

4.2.2.3 Equipment and Supplies

1. Inductively coupled argon plasma emission spectrometer
    a. Computer-controlled emission spectrometer with background correction.
    b. Radio-frequency generator compliant with FCC regulations.
    c. Optional mass flow controller for argon nebulizer gas supply.
    d. Optional peristaltic pump.
    e. Optional autosampler.
2. Argon gas supply -- high purity.
3. Volumetric flasks of suitable precision and accuracy.
4. Volumetric pipets of suitable precision and accuracy.

4.2.2.4 Reagents

Reagent- or trace metals-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. If the purity of a reagent is in question, analyze for contamination. If the concentration of the contamination is less than the lower limit of quantitation, then the reagent is acceptable.

1. Hydrochloric acid (conc), HCl.
2. Hydrochloric acid HCl (1:1) - Add 500 ml concentrated HCl to 400 ml water and dilute to 1L.
3. Nitric acid (conc), HNO_3.
4. Nitric acid, HNO_3 (1:1) -- Add 500 ml concentrated HNO_3 to 400 ml water and dilute to 1 L.
5. Reagent water-- Reagent water must be free of interferences.

4.2.2.5 Standards

1. Standard stock solutions may be purchased or prepared from ultra-high purity grade chemicals or metals (99.99% pure or greater). With several exceptions specifically noted, all salts must be dried for 1 hr at 105°C. Preparation procedures are contained in Table 4.5.

CAUTION: Many metal salts are extremely toxic if inhaled or swallowed. Wash hands thoroughly after handling.

<u>Mixed Calibration Standard Solutions</u>

Prepare mixed calibration standard solutions (Table 4.6) by combining appropriate volumes of the stock solutions above in volumetric flasks. Add the appropriate types and volumes of acids so that the standards are matrix-matched with the sample digestates. Prior to preparing the mixed standards, each stock solution should be analyzed separately to determine possible spectral interference or the presence of impurities. Care should be taken when preparing the mixed standards to ensure that the elements are compatible and stable together.

Transfer the mixed standard solutions to FEP fluorocarbon or previously unused polyethylene or polypropylene bottles for storage. For all intermediate and working standards, especially low level standards (*i.e.*, <1 ppm), stability must be demonstrated prior to use. Freshly-mixed standards should be prepared, as needed, realizing that concentration can change with age.

Table 4.5. Typical Stock Solution Preparation Procedures

Element	Solution	Directions
Aluminum	1 ml = 1000 µg of Al	Dissolve 1.000 g of aluminum metal, accurately weighed to at least four significant figures, in an acid mixture of 4.0 ml of HCl (1:1) and 1.0 ml of concentrated $HN0_3$ in a beaker. Slowly warm the beaker to dissolve the metal. When dissolution is complete, transfer solution quantitatively to a 1000-ml volumetric flask, add an additional 10.0 ml of HCl (1:1) and dilute to volume with reagent water
Antimony	1 m = 1000 µg of Sb	Dissolve 2.6673 g of $K(SbO)C_4H_4O_6$ (element fraction Sb = 0.3749), accurately weighed to at least four significant figures, in reagent water, add 10 ml of HCl (1:1), and dilute to volume in a 1000-ml volumetric flask with reagent water.
Arsenic	1 ml = 1000 µg of As	Dissolve 1.3203 g of As_2O_3 (element fraction As = 0.7574), accurately weighed to at least four significant figures, in 100 ml of reagent water containing 0.4 g of NaOH. Acidify the solution with 2 mL of concentrated HNO3 and dilute to volume in a 1000-ml volumetric flask with reagent water.
Cadmium	1 ml = 1000 µg of Cd	Dissolve 1.1423 g of CdO (element fraction Cd = 0.8754), accurately weighed to at least four significant figures, in a minimum amount of (1:1) HNO_3. Heat to increase the rate of dissolution. Add 10.0 ml of concentrated HNO_3 and dilute to volume in a 1000-ml volumetric flask with reagent water.
Chromium	1 ml = 1000 µg of Cr	Dissolve 1.9231 g of CrO_3 (element fraction Cr = 0.5200), accurately weighed to at least four significant figures, in reagent water. When dissolution is complete, acidify with 10 ml of concentrated HNO_3 and dilute to volume in a 1000-ml volumetric flask with reagent water.
Copper	1 ml = 1000 µg of Cu	Dissolve 1.2564 g of CuO (element fraction Cu = 0.7989), accurately weighed to at least four significant figures, in a minimum amount of (1:1) HNO_3. Add 10.0 ml of concentrated HNO_3 and dilute to volume in a 1000-ml volumetric flask with reagent water.
Iron	1 ml = 1000 µg of Fe	Dissolve 1.4298 g of Fe_2O_3 (element fraction Fe = 0.6994), accurately weighed to at least 4 significant figures, in a warm mixture of 20 ml HCl (1:1) and 2 ml of concentrated HNO_3. Cool, add an additional 5.0 ml of concentrated HNO_3, and dilute to volume in a 1000-ml volumetric flask with reagent water.
Lead	1 ml = 1000 µg of Pb	Dissolve 1.5985 g of $Pb(NO_3)_2$ (element fraction Pb = 0.6256), accurately weighed to at least four significant figures, in a minimum amount of (1:1) HNO_3. Add 10 ml (1:1) HNO_3 and dilute to volume in a 1000-ml volumetric flask with reagent water.
Manganese	1 ml = 1000 µg of Mn	Dissolve 1.00 g of manganese metal, accurately weighed to at least four significant figures, in acid mixture (10 ml of concentrated HCl and 1 ml of concentrated HNO_3) and dilute to volume in a 1000-ml volumetric flask with reagent water.
Nickel	1 ml = 1000 µg of Ni	Dissolve 1.000 g of nickel metal, accurately weighed to at least four significant figures, in 10.0 ml of hot concentrated HNO_3, cool, and dilute to volume in a 1000-ml volumetric flask with reagent water.
Selenium	1 ml = 1000 µg of Se	Do not dry. Dissolve 1.6332 g of H_2SeO_3 (element fraction Se = 0.6123), accurately weighed to at least four significant figures, in reagent water and dilute to volume in a 1000-ml volumetric flask with reagent water.
Tin	1 ml = 1000 µg of Sn	Dissolve 1.000 g of Sn shot, accurately weighed to at least 4 significant figures, in 200 ml of HCl (1:1) with heating to dissolve the metal. Let solution cool and dilute with HCl (1:1) in a 1000-ml volumetric flask.
Zinc	1 mL = 1000 µg of Zn	Dissolve 1.2447g of ZnO (element fraction Zn=0.8034), accurately weighed to at least 4 significant figures, in a minimum amount of dilute HNO_3. Add 10.0 ml of concentrated HNO_3 and dilute to volume in a 1000-ml volumetric flask with reagent water.

***Note**. Concentrations are calculated based upon the weight of pure metal added, or with the use of the element fraction and the weight of the metal salt added. The weight of the analyte is expressed to four significant figures for consistency with the weights below because rounding to two decimal places can contribute up to 4% error for some of the compounds.*

Table 4.6. Mixed Standard Solutions

Solution	Elements
I	Cd, Mn, Pb, Se and Zn
II	Cu and Fe
III	As
IV	Al, Cr and Ni
VI	P, Sn

Blanks

Two types of blanks are required for the analysis. The calibration blank is used in establishing the analytical curve and the method blank is used to identify possible contamination resulting from either the reagents (acids) or the equipment used during sample processing including filtration.

The calibration blank is prepared by acidifying reagent water to the same concentrations of the acids found in the standards and samples. Prepare a sufficient quantity to flush the system between standards and samples. The calibration blank will also be used for all initial (ICB) and continuing calibration blank (CCB) determinations.

The method blank must contain all of the reagents in the same volumes as used in the processing of the samples. The method blank must be carried through the complete procedure and contain the same acid concentration in the final solution as the sample solution used for analysis (section 4.2.2.6.4).

Initial Calibration Standard

The initial calibration verification (ICV) standard may be purchased or prepared by the analyst (or a purchased second source reference material) by combining compatible elements from a standard source different from that of the calibration standard and at concentration near the midpoint of the calibration curve (section 4.2.2.7.7).

Continuing Calibration Standard

The continuing calibration verification (CCV) standard should be prepared in the same acid matrix using the same standards used for calibration, at a concentration near the mid-point of the calibration curve (section 4.2.2.7.7).

Interference Check Solution

The interference check solution is prepared to contain known concentrations of interfering elements that will provide an adequate test of the correction factors. Spike the sample with the elements of interest, particularly those with known interferences at 0.5 to 1 mg/L. In the absence of measurable analyte, overcorrection could go undetected because a negative value could be reported as zero. If the particular instrument will display overcorrection as a negative number, this spiking procedure will not be necessary.

4.2.2.6 Quality Control

1. Refer to the QAPP for additional guidance on quality assurance and quality control protocols. When inconsistencies exist between QC guidelines, method specific QC criteria take precedence. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.

2. Instrument detection limits (IDLs) are useful means to evaluate the instrument noise level and response changes over time for each analyte from a series of reagent blank analyses to obtain a calculated concentration. They are not to be confused with the lower limit of quantitation, nor should they be used in establishing this limit. It may be helpful to compare the calculated IDLs to the established lower limit of quantitation (section 4.2.2.4).

   IDLs in µg/L can be estimated by calculating the average of the standard deviations of three runs on three non-consecutive days from the analysis of a reagent blank solution with seven consecutive measurements per day. Each measurement should be performed as though it were a separate analytical sample (*i.e*., each measurement must be followed by a rinse and/or any other procedure normally performed between the analysis of separate samples). IDLs should be determined at least every three months or at a project-specific designated frequency and kept with the instrument log book.

3. Each laboratory must demonstrate initial proficiency with each sample.

Note. *If an autosampler is used to perform sample dilutions, the laboratory should verify that those dilutions are of equivalent or better accuracy than is achieved by an experienced analyst performing manual dilutions.*

4. Dilute and reanalyze samples that exceed the linear dynamic range or use an alternate, less sensitive calibration for which quality control data are already established.

5. For each batch of samples processed, at least one method blank must be carried throughout the entire sample preparation and analytical process. If the method blank does not contain target analytes at a level that interferes with the project-specific DQOs, then the method blank would be considered acceptable. In the absence of project-specific DQOs, if the blank is less than 10% of the lower limit of quantitation check sample concentration, less than 10% of the regulatory limit, or less than 10% of the lowest sample concentration for each analyte in a given preparation batch, whichever is greater, then the method blank is considered acceptable. If the method blank cannot be considered acceptable, the method blank should be re-run once, and if still unacceptable, then all samples after the last acceptable method blank should be reprepared and reanalyzed along with the other appropriate batch QC samples. If the method blank exceeds the criteria, but the samples are all either below the reporting level or below the applicable action level or other DQOs, then the sample data may be used despite the contamination of the method blank.

6. For each batch of samples processed, at least one laboratory control sample (LCS) must be carried throughout the entire sample preparation and analytical process. The laboratory control samples should be spiked with each analyte of interest at the project-specific action level or, when lacking project specific action levels, at approximately mid-point of the linear dynamic range. Acceptance criteria should either be defined in the project-specific planning documents or set at a laboratory derived limit developed through the use of historical analyses. In the absence of project-specific or historical data generated criteria, this limit should be set at ± 20% of the spiked value. Acceptance limits derived from historical data should be no wider that ± 20%. If the laboratory control sample is not acceptable, then the laboratory control sample should be re-run once and, if still unacceptable, all samples after the last acceptable laboratory control sample should be reprepared and reanalyzed.

7. Concurrent analyses of standard reference materials (SRMs) containing known amounts of analytes in the media of interest are recommended and may be used as an LCS. For solid SRMs, 80 -120% accuracy may not be achievable and the manufacturer’s established acceptance criterion should be used for soil SRMs.

8. Documenting the effect of the matrix, for a given preparation batch consisting of similar sample characteristics, should include the analysis of at least one matrix spike and one duplicate unspiked sample (MS/Dup) or one matrix spike/matrix spike duplicate (MS/MSD) pair. The decision on whether to prepare and analyze duplicate samples or a matrix spike/matrix spike duplicate must be based on knowledge of the samples in the sample batch or as noted in the project-specific planning documents. If samples are expected to contain target analytes, then laboratories may use one matrix spike and a duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, laboratories should use a matrix spike and matrix spike duplicate pair.

   For each batch of samples processed, at least one MS/Dup or MS/MSD sample set should be carried throughout the entire sample preparation and analytical. MS/MSDs are intralaboratory split samples spiked with identical concentrations of each analyte of interest. The spiking occurs prior to sample preparation and analysis. An MS/Dup or MS/MSD is used to document the bias and precision of a method in a given sample matrix.

   MS/MSD samples should be spiked at the same level, and with the same spiking material, as the corresponding laboratory control sample that is at the project-specific action level or, when lacking project-specific action levels, at approximately mid-point of the linear dynamic range. Acceptance criteria should either be defined in the project-specific planning documents or set at a laboratory-derived limit developed through the use of historical analyses per matrix type analyzed. In the absence of project-specific or historical data generated criteria, these limits should be set at ± 25% of the spiked value for accuracy and 20 relative percent difference (RPD) for precision. Acceptance limits derived from historical data should be no wider that ± 25% for accuracy and 20% for precision. If the bias and precision indicators are outside the laboratory control limits, if the percent recovery is less than 75% or greater than 125%, or if the relative percent difference is greater than 20%, then the interference test discussed in Sec. 4.2.2.9.9 should be conducted.

   The relative percent difference between spiked matrix duplicate or unspiked duplicate determinations is to be calculated as follows:

$$RPD = \frac{D_1 - D_2}{(D_1 + D_2)/2} \times 100$$

   where:

   RPD = relative percent difference.

   D_1 = first sample value.

   D_2 = second sample value (spiked or unspiked duplicate)

   The spiked sample or spiked duplicate sample recovery should be within ± 25% of the actual value, or within the documented historical acceptance limits for each matrix.

9. If less than acceptable accuracy and precision data are generated, additional quality control tests below are recommended prior to reporting concentration data for the elements in this method. At a minimum, these tests should be performed with each batch of samples prepared/analyzed with corresponding unacceptable data quality results. These tests will then serve to ensure that neither positive nor negative interferences are affecting the measurement of any of the elements or distorting the accuracy of the reported values. If matrix effects are confirmed, the laboratory should consult with the data user when feasible for possible corrective actions which may include the use of alternative or modified test procedures so that the analysis is not impacted by the same interference.

Post digestion spike addition. If the MS/MSD recoveries are unacceptable, the same sample from which the MS/MSD aliquots were prepared should also be spiked with a post digestion spike. Otherwise, another sample from the same preparation should be used as an alternative. An analyte spike is added to a portion of a prepared sample, or its dilution, and should be recovered to within 80% to 120% of the known value. The spike addition should produce a minimum level of 10 times and a maximum of 100 times the lower limit of quantitation. If this spike fails, then the dilution test should be run on this sample. If both the MS/MSD and the post digestion spike fail, then matrix effects are confirmed.

Dilution test. If the analyte concentration is sufficiently high (minimally, a factor of 10 above the lower limit of quantitation after dilution), an analysis of a 1:5 dilution should agree within ± 10% of the original determination. If not, then a chemical or physical interference effect should be suspected.

***Note**. If spectral overlap is suspected, then the use of computerized compensation, an alternate wavelength, or comparison with an alternate method is recommended.*

4.2.2.7 Calibration and Standardization

1. Set up the instrument with proper operating parameters established as detailed below. The instrument should be allowed to become thermally stable before beginning (usually requiring at least 30 min of operation prior to calibration).
2. Sensitivity, instrumental detection limit, precision, linear dynamic range, and interference corrections need to be established for each individual target analyte on each particular instrument. All measurements (both target analytes and constituents which interfere with the target analytes) need to be within the instrument linear range where the correction equations are valid.
3. The lower limits of quantitation should be established for all isotope masses utilized for each type of matrix analyzed and for each preparation method used and for each instrument. These limits are considered the lowest reliable laboratory reporting concentrations and should be established from the lower limit of quantitation check sample and then confirmed using either the lowest calibration point or from a low-level calibration check standard.

   Lower limit of quantitation check sample:

   The lower limit of quantitation check (LLQC) sample should be analyzed after establishing the lower laboratory reporting limits and on an as needed basis to demonstrate the desired detection capability. Ideally, this check sample and the low-level calibration verification standard will be prepared at the same concentrations with the only difference being the LLQC sample is carried through the entire preparation and analytical procedure. Lower limits of quantitation are verified when all analytes in the LLQC sample are detected within ± 30% of their true value. This check should be used to both establish and confirm the lowest quantitation limit.

   The lower limits of quantitation determination using reagent water represents a best case situation and does not represent possible matrix effects of real-world samples. For the application of lower limits of quantitation on a project-specific basis with established data quality objectives, low-level matrix specific spike studies may provide data users with a more reliable indication of the actual method sensitivity and minimum detection capabilities.

4. Specific recommended wavelengths are listed in Table 4.7 below. Other wavelengths may be substituted (*e.g*., in the case of an interference) if they provide the needed sensitivity and are treated with the same corrective techniques for spectral interference.

Table 4.7. Recommended Wavelengths and Estimated Instrumental Detection Limits

Element	Wavelength (nm)	Estimated Detection Limit (µm)
Aluminum	308.215	30
Antimony	206.833	21
Arsenic	193.696	35
Cadmium	226.502	2.3
Chromium	267.716	4.7
Copper	324.754	3.6
Iron	259.940	4.1
Lead	220.353	28.0
Manganese	257.610	0.93
Nickel	231.604 (2^{nd} order)	10
Selenium	196.026	50
Tin	189.980 (2^{nd} order)	17
Zinc	213.856 (2^{nd} order)	1.2

For radial viewed plasma, operating conditions for aqueous solutions usually vary from:

1100 to 1200 watts forward power,

14 to 18 mm viewing height,

15 to 19 L/min argon coolant flow,

0.6 to 1.5 L/min argon nebulizer flow,

1 to 1.8 mL/min sample pumping rate with a 1 minute preflush time and measurement time near 1 sec per wavelength peak for sequential instruments and a rinse time of 10 sec per replicate with a 1 sec per replicate read time for simultaneous instruments.

For axial viewed plasma, the conditions will usually vary from:

1100 to 1500 watts forward power,

15 to 19 L/min argon coolant flow,

0.6 to 1.5 L/min argon nebulizer flow,

1 to 1.8 mL/min sample pumping rate with a 1 minute preflush time and measurement time near 1 sec per wavelength peak for sequential instruments and a rinse time of 10 sec per replicate with a 1 sec per replicate read time for simultaneous instruments.

One recommended way to achieve repeatable interference correction factors is to adjust the argon aerosol flow to reproduce the Cu/Mn intensity ratio at 324.754 nm and 257.610 nm respectively. This can be performed before daily calibration and after the instrument warm-up period.

5. Plasma optimization

The plasma operating conditions need to be optimized prior to use of the instrument. The purpose of plasma optimization is to provide a maximum signal to background ratio for some of the least sensitive elements in the analytical array. The use of a mass flow controller to regulate the nebulizer gas flow or source optimization software greatly facilitates the procedure. This routine is not required on a daily basis, it is only required when first setting up a new instrument, or following a change in operating conditions. The following procedure is recommended or follow the manufacturer's recommendations.

Ignite the radial plasma and select an appropriate incident radio frequency (RF) power. Allow the instrument to become thermally stable before beginning, about 30 to 60 minutes of operation. While aspirating a 1000 µg/L solution of yttrium, follow the instrument manufacturer's instructions and adjust the aerosol carrier gas flow rate through the nebulizer so a definitive blue emission region of the plasma extends approximately from 5 to 20 mm above the top of the load coil. Record the nebulizer gas flow rate or pressure setting for future reference. The yttrium solution can also be used for coarse optical alignment of the torch by observing the overlay of the blue light over the entrance slit to the optical system.

After establishing the nebulizer gas flow rate, determine the solution uptake rate of the nebulizer in mL/min by aspirating a known volume of a calibration blank for a period of at least three minutes. Divide the volume aspirated by the time in minutes and record the uptake rate. Set the peristaltic pump to deliver that rate in a steady even flow.

Profile the instrument to align it optically as it will be used during analysis. The following procedure is written for vertical optimization in the radial mode, but it also can be used for horizontal optimization. Aspirate a solution containing 10 µg/L of several selected elements. As, Se, Tl, and Pb are the least sensitive of the elements and most in need of optimization. Other elements may be used, based on project-specific protocols. (Cr, Cu, and Mn also have been used with success.) Collect intensity data at the wavelength peak for each analyte at 1 mm intervals from 14 to 18 mm above the load coil. (This region of the plasma is referred to as the analytical zone.) Repeat the process using the calibration blank. Determine the net signal to blank intensity ratio for each analyte for each viewing height setting. Choose the height for viewing the plasma that provides the best net intensity ratios for the elements analyzed or the highest intensity ratio for the least sensitive element. For optimization in the axial mode, follow the instrument manufacturer's instructions.

The instrument operating conditions finally selected as being optimum should provide the most appropriate instrument responses that correlate to the desired target analyte sensitivity while meeting the minimum quality control criteria noted in this method or as specified in the project-specific planning documents.

If the instrument operating conditions, such as incident power or nebulizer gas flow rate, are changed, or if a new torch injector tube with a different orifice internal diameter is installed, then the plasma and viewing height should be re-optimized.

After completing the initial optimization of operating conditions, and before analyzing samples, the laboratory should establish and initially verify an interelement spectral interference correction routine to be used during sample analysis with interference check standards that closely match the anticipated properties of the expected sample matrices, *i.e*., for saltwater type matrices the interference check standard should contain components that match the salinities of the proposed sample matrix. A general description of spectral

interferences and the analytical requirements for background correction, in particular, are discussed in section 4.2.2.2.

Before daily calibration, and after the instrument warm-up period, the nebulizer gas flow rate should be reset to the determined optimized flow. If a mass flow controller is being used, it should be set to the recorded optimized flow rate. In order to maintain valid spectral interelement correction routines, the nebulizer gas flow rate should be the same (<2% change) from day to day.

6. For operation with organic solvents, the use of the auxiliary argon inlet is recommended, as is the use of solvent-resistant tubing, increased plasma (coolant) argon flow, decreased nebulizer flow, and increased RF power, to obtain stable operation and precise measurements.

7. Determine calibration curve.

   All analyses require that a calibration curve be prepared to cover the appropriate concentration range based on the intended application and prior to establishing the linear dynamic range. Usually, this means the preparation of a calibration blank and mixed calibration standard solutions, the highest of which would not exceed the anticipated linear dynamic range of the instrument. Check the instrument standardization by analyzing appropriate QC samples as follows.

   Individual or mixed calibration standards should be prepared from known primary stock standards every six months to one year as needed based on the concentration stability as confirmed from the ICV analyses. The analysis of the ICV, which is prepared from a source independent of the calibration standards, is necessary to verify the instrument performance once the system has been calibrated for the desired target analytes. It is recommended that the ICV solution be obtained commercially as a certified traceable reference material such that an expiration date can be assigned. Alternately the ICV solution can be prepared from an independent source on an as needed basis depending on the ability to meet the calibration verification criteria. If the ICV analysis is outside of the acceptance criteria, at a minimum the calibration standards must be prepared fresh and the instrument recalibrated prior to beginning sample analyses. Consideration should also be given to preparing fresh ICV standards if the new calibration cannot be verified using the existing ICV standard.

Note: *This method describes the use of both a low-level and mid-level ICV standard analysis. For purposes of verifying the initial calibration, only the mid-level ICV needs to be prepared from a source other than the calibration standards.*

   The calibration standards should be prepared using the same type of acid or combination of acids and at similar concentrations as will result in the samples following processing.

   The response of the calibration blank should be less than the response of the typical laboratory lower limit of quantitation for each desired target analyte. Additionally, if the calibration blank response or continuing calibration blank verification is used to calculate a theoretical concentration, this value should be less than the level of acceptable blank contamination as specified in the approved quality assurance project planning documents. If this is not the case, the reason for the out-of-control condition must be found and corrected, and the sample analyses may not proceed or the previous ten samples need to be reanalyzed.

For the initial and daily instrument operation, calibrate the system according to the instrument manufacturer's guidelines using the mixed calibration standards as noted in section 4.2.2.5.3. The calibration curve should be prepared daily with a minimum of a calibration blank and a single standard at the appropriate concentration to effectively outline the desired quantitation range. Flush the system with the rinse blank between each standard solution. Use the average of at least three integrations for both calibration and sample analyses. The resulting curve should then be verified with mid-level and low-level initial calibration verification standards as outlined in section 4.2.2.7.8.

Alternatively, the calibration curve can be prepared daily with a minimum of a calibration blank and three non-zero standards that effectively bracket the desired sample concentration range. If low-level as compared to mid- or high-level sample concentrations are expected, the calibration standards should be prepared at the appropriate concentrations in order to demonstrate the instrument linearity within the anticipated sample concentration range. For all multi-point calibration scenarios, the lowest non-zero standard concentration should be considered the lower limit of quantitation.

***Note**. Regardless of whether the instrument is calibrated using only a minimum number of standards or with a multi-point curve, the upper limit of the quantitation range may exceed the highest concentration calibration point and can be defined as the "linear dynamic" range, while the lower limit can be identified as the "lower limit of quantitation limit" (LLQL) and will be either the concentration of the lowest calibration standard (for multi-point curves) or the concentration of the low level ICV/CCV check standard. Results reported outside these limits would not be recommended unless they are qualified as estimated. See Sec. 4.2.2.10.4.4 for recommendations on how to determine the linear dynamic range, while the guidance in this section and section 4.2.2.7.8 provide options for defining the lower limit of quantitation.*

To be considered acceptable, the calibration curve should have a correlation coefficient greater than or equal to 0.998. When using a multipoint calibration curve approach, every effort should be made to attain an acceptable correlation coefficient based on a linear response for each desired target analyte. If the recommended linear response cannot be attained using a minimum of three non-zero calibration standards, consideration should be given to adding more standards, particularly at the lower concentrations, in order to better define the linear range and the lower limit of quantitation. Conversely, the extreme upper and lower calibration points may be removed from the multi-point curve as long as three non-zero points remain such that the linear range is narrowed and the non-linear upper and/or lower portions are removed. As with the single point calibration option, the multi-point calibration should be verified with both a mid- and low-level ICV standard analysis using the same 90 - 110% and 70 - 130% acceptance criteria, respectively.

Many instrument software packages allow multi-point calibration curves to be "forced" through zero. It is acceptable to use this feature, provided that the resulting calibration meets the acceptance criteria, and can be verified by acceptable QC results. Forcing a regression through zero should NOT be used as a rationale for reporting results below the calibration range defined by the lowest standard in the calibration curve.

After initial calibration, the calibration curve should be verified by use of an initial calibration verification (ICV) standard analysis. At a minimum, the ICV standard should be prepared from an independent (second source) material at or near the midrange of the calibration curve. The acceptance criteria for this mid-range ICV standard should be ±10% of its true value. Additionally, a low-level initial calibration verification (LLICV) standard should be

prepared, using the same source as the calibration standards, at a concentration expected to be the lower limit of quantitation. The suggested acceptance criteria for the LLICV is ±30% of its true value. Quantitative sample analyses should not proceed for those analytes that fail the second source standard initial calibration verification, with the exception that analyses may continue for those analytes that fail the criteria with an understanding these results should be qualified and would be considered estimated values. Once the calibration acceptance criteria is met, either the lowest calibration standard or the LLICV concentration can be used to demonstrate the lower limit of quantitation and sample results should not be quantitated below this lowest standard. In some cases depending on project data quality objectives, it may be appropriate to report these results, as estimated; however, they should be qualified by noting the results are below the lower limit of quantitation. Therefore, the quantitation limit cannot be reported lower than either the LLICV standard used for the single point calibration option or the low calibration and/or verification standard used during initial multi-point calibration. If the calibration curve cannot be verified within these specified limits for the mid-range ICV and LLICV analyses, the cause needs to be determined and the instrument recalibrated before samples are analyzed. The analysis data for the initial calibration verification analyses should be kept on file with the sample analysis data.

Both the single and multi-point calibration curves should be verified at the end of each analysis batch and after every 10 samples by use of a continuing calibration verification (CCV) standard and a continuing calibration blank (CCB). The CCV should be made from the same material as the initial calibration standards at or near the mid-range concentration. For the curve to be considered valid the acceptance criteria for the CCV standard should be ±10% of its true value and the CCB should contain target analytes less than the established lower limit of quantitation for any desired target analyte. If the calibration cannot be verified within the specified limits, the sample analysis must be discontinued, the cause determined and the instrument recalibrated. All samples following the last acceptable CCV/CCB must be reanalyzed. The analysis data for the CCV/CCB should be kept on file with the sample analysis data.

The low level continuing calibration verification (LLCCV) standard should also be analyzed at the end of each analysis batch. A more frequent LLCCV analysis, *i.e*., every 10 samples may be necessary if low-level sample concentrations are anticipated and the system stability at low end of the calibration is questionable. In addition, the analysis of a LLCCV on a more frequent basis will minimize the number of samples for re-analysis should the LLCCV fail if only run at the end of the analysis batch. The LLCCV standard should be made from the same source as the initial calibration standards at the established lower limit of quantitation as reported by the laboratory. The acceptance criteria for the LLCCV standard should be ± 30% of its true value. If the calibration cannot be verified within these specified limits, the analysis of samples containing the affected analytes at similar concentrations cannot continue until the cause is determined and the LLCCV standard successfully analyzed. The instrument may need to be recalibrated or the lower limit of quantitation adjusted to a concentration that will ensure a compliant LLCCV analysis. The analysis data for the LLCCV standard should be kept on file with the sample analysis data.

8. The linear dynamic range is established when the system is first setup, or whenever significant instrument components have been replaced or repaired, and on an as needed basis only after the system has been successfully calibrated using either the single or multi-point standard calibration approach. The upper limit of the linear dynamic range needs to be established for each wavelength utilized by determining the signal responses from a minimum of three, preferably five, different concentration standards across the range. The

ranges which may be used for the analysis of samples should be judged by the analyst from the resulting data. The data, calculations and rationale for the choice of range made should be documented and kept on file. A standard at the upper limit should be prepared, analyzed and quantitated against the normal calibration curve. The calculated value should be within 10% (±10%) of the true value. New upper range limits should be determined whenever there is a significant change in instrument response. At a minimum, the range should be checked every six months. The analyst should be aware that if an analyte that is present above its upper range limit is used to apply a spectral correction, the correction may not be valid and those analytes where the spectral correction has been applied may be inaccurately reported.

***Note**. Some metals may exhibit non-linear response curves due to ionization and self-absorption effects. These curves may be used if the instrument allows it, however the effective range must be checked and the second order curve fit should have a correlation coefficient of 0.998 or better. Third order fits are not acceptable. These non-linear response curves should be revalidated and/or recalculated on a daily basis using the same calibration verification QC checks as a linear calibration curve. Since these curves are much more sensitive to changes in operating conditions than the linear lines, they should be checked whenever there have been moderate equipment changes. Under these calibration conditions, quantitation is not acceptable above or below the calibration standards. Additionally, a non-linear curve should be further verified by calculating the actual recovery of each calibration standard used in the curve. The acceptance criteria for the calibration standard recovery should be ±10% of its true value for all standards except the lowest concentration. A recovery of ±30% of its true value should be achieved for the lowest concentration standard.*

9. The analyst should verify that the instrument configuration and operating conditions satisfy the project-specific analytical requirements and maintain quality control data that demonstrate and confirm the instrument performance for the reported analytical results.

4.2.2.8 Procedure

1. Preliminary acid digestion of matrices is required. All associated QC samples (*i.e*., method blank, LCS and MS/MSD) must undergo the same procedures. Samples which are not digested must either use an internal standard or be matrix-matched with the standards.
2. Profile and calibrate the instrument according to the instrument manufacturer's recommended procedures, using the typical mixed calibration standard solutions described in section 4.2.2.5.2. Flush the system with the calibration blank (Sec 4.2.2.5.3) between each standard or as the manufacturer recommends. (Use the average intensity of multiple exposures for both standardization and sample analysis to reduce random error.) The calibration curve should be prepared as detailed in section 4.2.2.7.7.
3. Regardless of whether the initial calibration is performed using a single high standard and the calibration blank or the multi-point option, the laboratory should analyze an LLCCV. For all analytes and determinations, the laboratory must analyze an ICV and LLICV immediately following daily calibration. It is recommended that a CCV, LLCCV, and CCB be analyzed after every ten samples and at the end of the analysis batch (section 4.2.2.7.7).
4. Rinse the system with the calibration blank solution before the analysis of each sample. The rinse time will be one minute. Each laboratory may establish a reduction in this rinse time through a suitable demonstration. Analyze the samples and record the results.

4.2.2.9 Data Analysis and Calculations

1. The quantitative values must be reported in appropriate units, such as micrograms per liter (µg/L) for aqueous samples and milligrams per kilogram (mg/kg) for solid samples. If dilutions were performed, the appropriate corrections must be applied to the sample values. All results should be reported with up to three significant figures.

2. Calculate results for solids on a dry-weight basis as follows:

   (1) A separate determination of percent solids must be performed.

   (2) The concentrations determined in the digest are to be reported on the basis of the dry weight of the sample.

   $$\text{Concentration (dry weight)(mg/kg)} = \frac{(C \times V)}{(W \times S)}$$

   where:

   C = Digest Concentration (mg/L)

   V = Final volume in liters after sample preparation

   W = Weight in kg of wet sample

   S = % Solids / 100

   Calculations must include appropriate interference, internal-standard normalization, and the summation of signals at 206, 207, and 208 m/z for lead (to compensate for any differences in the abundances of these isotopes between samples and standards).

3. Results must be reported in units commensurate with their intended use and all dilutions must be taken into account when computing final results.

4.3 MERCURY IN FISH TISSUE AND SEDIMENTS

4.3.1 Scope of Application

1. This method may be used for both saltwater and freshwater samples.
2. This procedure measures total mercury (organic v. inorganic) in fish tissue and sediments.
3. The range of the method is 0.2 - 5 µg/g. The range may be extended beyond the normal range by increasing or decreasing sample size or through instrument and recorder control.

4.3.2 Summary of Method

1. A weighed portion of the sample is digested in aqua regia for 2 minutes at 95°C, followed by oxidation with potassium permanganate. Mercury in the digested sample is then measured by the conventional cold vapor technique.
2. An alternate digestion involving the use of an autoclave is described in section 4.3.8.3.

4.3.3 Sample Handling and Preservation

1. Because of the extreme sensitivity of the procedure and the omnipresence of mercury, care must be taken to avoid extraneous contamination. Sampling devices and sample jars should be ascertained to be free of mercury; the sample should not be exposed to any condition in the laboratory that may result in contact or air-borne mercury contamination.
2. While the sample may be analyzed without drying, it has been found to be more convenient to analyze a dry sample. Moisture may be driven off in a drying oven at a temperature of 60°C. No mercury losses have been observed by using this drying step. The dry sample should be pulverized and thoroughly mixed before the aliquot is weighed.

4.3.4 Interferences

1. The same types of interferences that may occur in water samples are also possible with fish tissue and sediments, *i.e*., sulfides, high copper, high chlorides, *etc*.
2. Volatile materials which absorb at 253.7 nm will cause a positive interference. In order to remove any interfering volatile materials, the dead air space in the BOD bottle should be purged before the addition of stannous sulfate.

4.3.5 Apparatus

1. Atomic Absorption Spectrophotometer. Any atomic absorption unit having an open sample presentation area in which to mount the absorption cell is suitable. Instrument settings recommended by the particular manufacturer should be followed.
2. Mercury Hollow Cathode Lamp. Westinghouse WL-22847, argon filled, or equivalent.
3. Any multi-range variable speed recorder that is compatible with the UV detection system.
4. Standard spectrophotometer cells 10 cm long, having quartz end windows may be used. Suitable cells may be constructed from plexiglass tubing, 1" O.D. X 4-1/2". The ends are ground perpendicular to the longitudinal axis and quartz windows (1" diameter X 1/16" thickness) are cemented in place. Gas inlet and outlet ports (also of plexiglass but 1/4" O.D.) are attached approximately 1/2" from each end. The cell is strapped to a burner for support and aligned in the light beam to give the maximum transmittance.

***Note**. Two 2" X 2" cards with one inch diameter holes may be placed over each end of the cell to assist in positioning the cell for maximum transmittance.*

5. Air Pump. Any peristaltic pump capable of delivering 1 liter of air per minute may be used. A Masterflex pump with electronic speed control has been found to be satisfactory. Regulated compressed air can be used in an open one-pass system.
6. Flowmeter. Flowmeter must be capable of measuring an air flow of 1 liter per minute.
7. Aeration Tubing. Tygon tubing is used for passage of the mercury vapor from the sample bottle to the absorption cell and return. Straight glass tubing terminating in a coarse porous frit is used for sparging air into the sample.
8. Drying Tube. 6" X 3/4" diameter tube containing 20 g of magnesium perchlorate.

***Note.** In place of the magnesium perchlorate drying tube, a small reading lamp with 60W bulb may be used to prevent condensation of moisture inside the cell. The lamp is positioned to shine on the absorption cell maintaining the air temperature in the cell about 10°C above ambient.*

4.3.6 Reagents

1. Aqua Regia. Prepare immediately before use by carefully adding three volumes of conc. HCl to one volume of conc. HNO_3.
2. Sulfuric Acid, 0.5 N. Dilute 14.0 ml of conc. sulfuric acid to 1 liter.
3. Stannous Sulfate. Add 25 g stannous sulfate to 250 ml of the 0.5 N sulfuric acid. This mixture is a suspension and should be stirred continuously during use.
4. Sodium Chloride-Hydroxylamine Sulfate Solution. Dissolve 12 g of sodium chloride and 12 g of hydroxylamine sulfate in distilled water and dilute to 100 ml.

***Note**. A 10% solution of stannous chloride may be substituted for the stannous sulfate and hydroxylamine hydrochloride may be used in place of hydroxylamine sulfate in the sodium chloride-hydroxylamine sulfate solution.*

5. Potassium Permanganate. 5% solution, w/v. Dissolve 5 g of potassium permanganate in 100 ml of distilled water.
6. Stock Mercury Solution. Dissolve 0.1354 g of mercuric chloride in 75 ml of distilled water. Add 10 ml of conc. nitric acid and adjust the volume to 100.0 ml. 1.0 ml = 1.0 mg Hg.
7. Working Mercury Solution. Make successive dilutions of the stock mercury solution to obtain a working standard containing 0.1 µg/ml. This working standard and the dilution of the stock mercury solutions should be prepared fresh daily. Acidity of the working standard should be maintained at 0.15% nitric acid. This acid should be added to the flask as needed before the addition of the aliquot.

4.3.7 Calibration

1. Transfer 0, 0..5, 1.0, 2.0, 5.0 and 10 ml aliquots of the working mercury solution containing 0 to 1.0 µg of mercury to a series of 300 ml BOD bottles. Add enough distilled water to each bottle to make a total volume of 10 ml. Add 5 ml of aqua regia and heat for 2 minutes in a water bath at 95°C.
2. Allow sample to cool; add 50 ml distilled water and 15 ml of $KMnO_4$ solution to each bottle and return to water bath for 30 mins. Cool and add 6 ml of sodium chloride-hydroxylamine sulfate solution to reduce the excess permanganate. Add 50 ml distilled water.

3. Treating each bottle individually, add 5 ml of stannous sulfate solution and immediately attach the bottle to the aeration apparatus. At this point, the sample is allowed to stand quietly without manual agitation. The circulating pump, which has previously been adjusted to rate of 1 liter per minute, is allowed to run continuously. The absorbance, as exhibited either on the spectrophotometer or the recorder, will increase and reach maximum within 30 seconds. As soon as the recorder pen levels off, approximately 1 minute, open the bypass value and continue the aeration until the absorbance returns to its minimum value. Close the bypass value, remove the fritted tubing from the BOD bottle and continue the aeration. Proceed with the standards and construct a standard curve by plotting peak height versus micrograms of mercury.

Note. Because of the toxic nature of mercury vapor precaution must be taken to avoid its inhalation. Therefore, a bypass has been included in the system to either vent the mercury vapor into an exhaust hood or pass the vapor through some absorbing media, such as:

a) equal volumes of 0.1 N $KmnO_4$ and 10% H_2SO_4
b) 0.25% iodine in a 3% KI solution.
Specially treated charcoal that will absorb mercury vapor is also commercially available.

4.3.8 Procedure

1. Weigh triplicate 0.2 g portions of dry sample and place in bottom of a BOD bottle. Add 5 ml of distilled water and 5 ml of aqua regia. Heat for 2 mins in a water bath at 95°C. Cool, add 50 ml distilled water and 15 ml potassium permanganate solution to each sample bottle. Mix and place in the water bath for 30 mins at 95°C. Cool and add 6 ml of sodium chloride-hydroxylamine sulfate to reduce the excess permanganate. Add 55 ml of distilled water.
2. Treating each bottle individually, add 5 ml of stannous sulfate and immediately attach the bottle to the aeration apparatus. Continue as described under section 4.2.7.3 above.
3. An alternate digestion procedure employing an autoclave may also be used. In this method 5 ml of conc. H_2SO_4 and 2 ml of conc. HNO_3 are added to the 0.2 g of sample. Add 5 ml of saturated $KMnO_4$ solution and the bottle covered with a piece of aluminum foil. The samples are autoclaved at 121°C and 15 lbs. for 15 minutes. Cool, make up to a volume of 100 ml with distilled water and add 6 ml of sodium chloride hydroxylamine sulfate solution to reduce the excess permanganate. Purge the dead air space and continue as described under section 4.3.7.3.

4.3.9 Calculation

1. Measure the peak height of the unknown from the chart and read the mercury value from the standard curve.
2. Calculate the mercury concentration in the sample by the formula:

$$\mu g\ Hg/g = \frac{\mu g\ Hg \text{ in the aliquot}}{\text{wt. of the aliquot (g)}}$$

3. Report mercury concentrations as follows:
   Below 0.1 µg/g, <0.1; between 0.1 and 1 µg/g, to the nearest 0.01 µg;
   Between 1 and 10 µg/g, to nearest 0.1µg;
   Above 10 µg/g, to nearest µg.

4.4 SAMPLE PREPARATION FOR ORGANIC COMPOUNDS IN FISH TISSUE AND SEDIMENTS

4.4.1 Ultrasonic Extraction

The extraction procedure is divided into two sections, based on the expected concentration of organics in the sample (Figure 4.3). The low concentration method (individual organic components of less than or equal to 20 mg/kg) uses a larger sample size and a more rigorous extraction procedure (lower concentrations are more difficult to extract). The medium/high concentration method (individual organic components of greater than 20 mg/kg) is much simpler and therefore faster.

4.4.2 Apparatus and Materials

1. Apparatus for grinding dry waste samples.
2. Ultrasonic preparation - A horn-type device equipped with a titanium tip, or a device that will give equivalent performance, shall be used.

   Ultrasonic Disrupter - The disrupter must have a minimum power wattage of 300 watts, with pulsing capability. A device designed to reduce the cavitation sound is recommended. Follow the manufacturer's instructions for preparing the disrupter for extraction of samples with low and medium/high concentration.

   Use a 3/4" horn for the low concentration method and a 1/8" tapered microtip attached to a 1/2" horn for the medium/high concentration method.
3. Sonabox - Recommended with above disrupters for decreasing cavitation sound
4. Drying oven - capable of maintaining 105°C.
5. Desiccator.
6. Vacuum or pressure filtration apparatus.
7. Kuderna-Danish (K-D) apparatus.

   Concentrator tube - 10-ml, graduated
   Evaporation flask - 500-ml
   Snyder column - Three-ball macro
   Snyder column - Two-ball micro
   Springs - 1/2 inch
8. The following glassware is recommended for the purpose of solvent recovery during the concentration procedures requiring the use of Kuderna-Danish evaporative concentrators. Incorporation of this apparatus may be required by State or local municipality regulations that govern air emissions of volatile organics.

   Solvent vapor recovery system

   Boiling chips - Solvent-extracted, approximately 10/40 mesh

   Water bath - Heated, with concentric ring cover, capable of temperature control (± 5°C). The batch should be used in a hood.

   Balance - Top-loading, capable of accurately weighing to the nearest 0.01 g.

   Vials - 2-ml, for GC autosampler, with PTFE-lined screw caps or crimp tops.
   Glass scintillation vials - 20-ml, with PTFE-lined screw caps.

   Spatula - Stainless steel or PTFE

Syringe - 5-ml

Drying column - 20-mm ID Pyrex chromatographic column with Pyrex glass wool at the bottom.

Note. Fritted glass discs are difficult to decontaminate after highly contaminated extracts have been passed through. Columns without frits may be purchased. Use a small pad of Pyrex glass wool to retain the adsorbent. Prewash the glass wool pad with 50 ml of acetone followed by 50 ml of elution solvent prior to packing the column with adsorbent.

4.4.3 Reagents

1. Reagent-grade or pesticide-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to specifications of the Committee on Analytical Reagents of the American Chemical Society. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers.
2. Organic-free reagent water.
3. Sodium sulfate (granular, anhydrous), Na_2SO_4 . Purify by heating at 400°C for 4 hours in a shallow tray, or by precleaning the sodium sulfate with methylene chloride. If the sodium sulfate is precleaned with methylene chloride, a method blank must be analyzed, demonstrating that there is no interference from the sodium sulfate.
4. Samples must be extracted using a solvent that gives optimum, reproducible recovery of the analytes of interest from the sample matrix. Solvents must be pesticide grade in quality or equivalent, and each lot of solvent should be determined to be free of phthalates.

   Semivolatile organics may be extracted with acetone/methylene chloride (1:1, v/v), CH_3COCH_3/CH_2Cl_2 or acetone/hexane (1:1, v/v), CH_3COCH_3/C_6H_{14}.

   Organochlorine pesticides may be extracted with acetone/hexane (1:1, v/v), CH_3COCH_3/C_6H_{14}, or acetone/methylene chloride (1:1,v/v), CH_3COCH_3/CH_2Cl_2

   Exchange solvents - All solvents must be pesticide quality or equivalent.

   Hexane, C_6H_{14} — Acetonitrile, CH_3CN
   Propanol, $(CH_3)_2CHOH$ — Methanol, CH_3OH
   Cyclohexane, C_6H_{12}

5. Solvents used in the extraction and cleanup procedures include *n*-hexane, diethyl ether, methylene chloride, acetone, ethylacetate, and isooctane (2,2,4-trimethylpentane) and the solvents must be exchanged to *n*-hexane or isooctane prior to analysis. Therefore, the use of *n*-hexane and isooctane will be required in this procedure.

4.4.4 Procedure

1. Decant and discard any water layer on a sample. Mix sample thoroughly, especially composited samples. Discard any foreign objects such as sticks, leaves, and rocks.
2. Determination of percent dry weight. When sample results are to be calculated on a dry weight basis, a second portion of sample should be weighed out at the same time as the portion used for analytical determination.

***Caution**. The drying oven should be contained in a hood or vented. Significant laboratory contamination may result from drying a heavily contaminated sample*.

Immediately after weighing the sample for extraction, weigh 5-10 g of the sample into a recorded crucible. Dry this aliquot overnight at 105°C. Allow to cool in a desiccator before weighing. Calculate the % dry weight as follows:

$$\% \text{ dry weight} = \frac{\text{g of dry sample}}{\text{g of sample}} \times 100$$

4.4.4.1 Extraction Method for Samples Expected to Contain Low Concentrations of Organics and Pesticides (less than or equal to 20 mg/kg)

The following steps should be performed rapidly to avoid loss of the more volatile extractables.

Weigh approximately 30 g of sample into a 400-ml beaker. Record weight to the nearest 0.1 g.

***Note**. Nonporous or wet samples (e.g., clay type) that do not have a free-flowing sandy texture must be mixed with 60 g of anhydrous sodium sulfate, using a spatula. If required, more sodium sulfate may be added. After addition of sodium sulfate, the sample should be free flowing.*

Add 1.0 ml of the surrogate standard solution to all samples, spiked samples, QC samples, and blanks. For the sample in each batch selected for spiking, add 1.0ml of matrix spiking solution.

***Note**. If gel permeation cleanup will be used, the analyst should either add twice the volume of the surrogate spiking solution (and matrix spiking solution, where applicable), or concentrate the final extract to half the normal volume, to compensate for the half of the extract that is lost due to loading of the GPC column.*

Immediately add 100 ml of the appropriate/recommended extraction solvent or solvent mixture.

Place the bottom surface of the tip of the 3/4 inch disrupter horn about 1/2 inch below the surface of the solvent, but above the tissue or sediment layer. Be sure the horn is properly tuned according to the manufacturer's instructions.

Extract ultrasonically for 3 minutes, with output control knob set at 10 (full power) and with mode switch on Pulse (pulsing energy rather than continuous energy) and percent-duty cycle knob set at 50% (energy on 50% of time and off 50% of time). Do not use microtip probe.

Decant the extract and filter it through Whatman No. 41 filter paper in a Buchner funnel that is attached to a clean 500-ml filtration flask. Alternatively, decant the extract into a centrifuge bottle and centrifuge at low speed to remove particles.

Repeat the extraction two or more times with two additional 100 ml portions of solvent. Decant off the solvent after each ultrasonic extraction. On the final ultrasonic extraction, pour the entire sample into the Buchner funnel and rinse with extraction solvent. Apply a vacuum to the filtration flask, and collect the solvent extract. Continue filtration until all visible solvent is removed from the funnel, but do not attempt to completely dry the sample, as the continued application of a vacuum may result in the loss of some analytes.

Alternatively, if centrifugation is used, transfer the entire sample to the centrifuge bottle. Centrifuge at low speed, and then decant the solvent from the bottle.

Assemble a Kuderna-Danish (K-D) concentrator (if necessary) by attaching a 10-ml concentrator tube to a 500-mL evaporator flask. Attach the solvent vapor recovery glassware (condenser and collection device) to the Snyder column of the Kuderna-Danish apparatus following manufacturer's instructions. Transfer filtered extract to a 500-ml evaporator flask.

Add 1 - 2 clean boiling chips to the evaporation flask, and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 ml methylene chloride to the top. Place the K-D apparatus on a hot water bath (80 - 90°C) so that the concentrator tube is partially immersed in the hot water and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature, as required, to complete the concentration in 10 - 15 min. At the proper rate of distillation the balls of the column will actively chatter, but the chambers will not flood with condensed solvent. When the apparent volume of liquid reaches 1 ml, remove the K-D apparatus and allow it to drain and cool for at least 10 min.

If a solvent exchange is required, momentarily remove the Snyder column, add 50 ml of the exchange solvent and a new boiling chip, and re-attach the Snyder column. Concentrate the extract as described below, raising the temperature of the water bath, if necessary, to maintain proper distillation. When the apparent volume again reaches 1-2 ml, remove the K-D apparatus and allow it to drain and cool for at least 10 minutes.

Remove the Snyder column and rinse the flask and its lower joints into the concentrator tube with 1-2 ml of methylene chloride or exchange solvent. The extract may be further concentrated by using the technique outlined below or adjusted to 10.0 ml with the solvent last used. If further concentration is indicated, either micro Snyder column technique or nitrogen blowdown technique may be used to adjust the extract to the final volume required.

Micro Snyder column technique

Add a clean boiling chip and attach a two-ball micro Snyder column to the concentrator tube. Prewet the column by adding approximately 0.5 ml of methylene chloride, or exchange solvent through the top. Place the apparatus in the hot water bath. Adjust the vertical position and the water temperature, as required, to complete the concentration in 5-10 minutes. At the proper rate of distillation the balls of the column will actively chatter, but the chambers will not flood. When the liquid reaches an apparent volume of approximately 0.5 ml, remove the apparatus from the water bath, allow to drain and cool for at least 10 minutes. Remove the micro Snyder column and rinse its lower joint with approximately 0.2 ml of appropriate solvent and add to the concentrator tube. Adjust the final volume to the volume required for cleanup.

Nitrogen blowdown technique

Place the concentrator tube in a warm water bath (approximately 35°C) and evaporate the solvent volume to the required level using a gentle stream of clean, dry nitrogen (filtered through a column of activated carbon). The internal wall of the tube must be rinsed down several times with the appropriate solvent during the operation. During evaporation, the solvent level in the tube must be positioned to prevent water from condensing into the sample (*i.e.*, the solvent level should be below the level of the water bath). Under normal operating conditions, the extract should not be allowed to become dry. When the volume of solvent is reduced below 1 ml, semivolatile analytes may be lost.

Caution. *Do not use plasticized tubing between the carbon trap and the sample, since it may introduce contaminants.*

4.4.4.2 Extraction Method for Samples Expected to Contain High Concentrations of Organics (greater than 20 mg/kg).

Transfer approximately 2 g of sample to a 20-ml vial. Wipe the mouth of the vial with a tissue to remove any sample material. Record the exact weight of sample taken. Cap the vial before proceeding with the next sample to avoid any cross contamination.

Add 2 g of anhydrous sodium sulfate to sample in the 20-ml vial and mix well.

Surrogates are added to all samples, spikes, and blanks. Add 1.0 ml of surrogate spiking solution to sample mixture. For the sample in each analytical batch selected for spiking, add 1.0 ml of the matrix spiking standard. If gel permeation cleanup is to be used, the analyst should either add twice the volume of the surrogate spiking solution (and matrix spiking solution, where applicable), or concentrate the final extract to half the normal volume, to compensate for the half of the extract that is lost due to loading of the GPC column.

Immediately add whatever volume of solvent is necessary to bring the final volume to 10.0 ml considering the added volume of surrogates and matrix spikes. Disrupt the sample with the 1/8 in. tapered microtip ultrasonic probe for 2 minutes at output control setting 5 and with mode switch on pulse and percent duty cycle at 50%. For nonpolar compounds (*i.e*., organochlorine pesticides, PCBs), use hexane or appropriate solvent. For other semivolatile organics, use methylene chloride.

Loosely pack a disposable Pasteur pipette with 2 to 3 cm of glass wool. Filter the sample extract through the glass wool and collect the extract in a suitable container. The entire 10 ml of extraction solvent cannot be recovered from the sample. Therefore, the analyst should collect a volume appropriate for the sensitivity of the determinative method.

4.4.5 Extract Cleanup

1. The specific cleanup procedure used will depend on the nature of the sample to be analyzed and the data quality objectives for the measurements. General guidance for sample extract cleanup is provided in this section.
2. For biological (*i.e*., fish tissue), or samples containing high molecular weight materials, use of GPC -- pesticide option is recommended. Frequently an adsorption chromatographic cleanup (alumina, silica gel, or Florisil®) may also be necessary following the GPC cleanup.
3. Alumina may be used to remove phthalate esters.
4. Florisil® may be used to separate organochlorine pesticides from aliphatic compounds, aromatics, and nitrogen-containing compounds.
5. Silica gel may be used to separate single component organochlorine pesticides from some interferants.
6. Sulfur, which may be present in certain samples, interferes with the electron capture gas chromatography of certain pesticides and should be removed with either copper or tetrabutylammonium sulfite. The mixture is shaken and the extract is removed from the sulfur cleanup reagent.

4.4.6 Sample Handling

If analysis of the extract will not be performed immediately, stopper the concentrator tube and refrigerate. If the extract will be stored longer than 2 days, it should be transferred to a vial with a PTFE-lined cap and labeled appropriately.

4.5 ORGANOCHLORINE PESTICIDES IN FISH TISSUE AND SEDIMENTS

4.5.1 Scope and Application

1. This method may be used for both saltwater and freshwater fish tissue and sediments
2. This method is used to determine concentrations of organochlorine pesticides in extracts from solid and liquid matrices, using fused-silica, open tubular, capillary columns with electron capture detectors or electrolytic conductivity detectors. The following 20 indicator compounds (Table 4.8) are determined using either single- or dual-column analysis system:

Table 4.8. Indicator List of Organchlorine Pesticides

Compound	Chemical Abstract Service (CAS) Registry No.
Aldrin	309-00-2
γ-BHC (Lindane)	58-89-9
α-Chlordane	5103-71-9
2,4'-DDD	53-19-0
4,4'-DDD	72-54-8
2,4'-DDE	3424-82-6
4,4'-DDE	72-55-9
2,4'-DDT	789-02-6
4,4'-DDT	50-29-3
Dieldrin	60-57-1
Endosulfan I	959-98-8
Endosulfan II	33213-65-9
Endosulfan sulfate	1031-07-8
Endrin	72-20-8
Heptachlor	76-44-8
Heptachlor epoxide	1024-57-3
Hexachlorobenzene	118-74-1
Mirex	2385-85-5
Toxaphene	8001-35-2
trans-Nonachlor	39765-80-5

3. This method no longer includes Polychlorinated biphenyls (PCBs) as in the list of target analytes. A separate method for the analysis of PCBs is included in Section 4.6.

4.5.2 Summary of Method

A measured volume or weight of liquid or solid sample is extracted using an ultrasonic extraction technique. The ultrasonic process ensures intimate contact of the sample matrix with the extraction solvent. Cleanup steps are applied to the extract, depending on the nature of the matrix interferences and the target analytes. Suggested cleanups include alumina, Florisil, silica gel, gel permeation chromatography, and sulfur. Finally the extract is analyzed by injecting a measured aliquot into a gas chromatograph equipped with either a narrow-bore or wide-bore fused-silica capillary column and either an electron capture detector (GC/ECD), or an electrolytic conductivity detector (GC/ELCD).

4.5.3 Interferences

1. Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All materials must be demonstrated to be free from interferences under conditions of analysis by analyzing method blanks. Specific selection of reagents and purification of solvents by distillation in all-glass systems is necessary.

   Cross-contamination of clean glassware routinely occurs when plastics are handled during extraction steps, especially when solvent-wetted surfaces are handled. Glassware must be scrupulously cleaned. Clean all glassware as soon as possible after use by rinsing with the last solvent used. This should be followed by detergent washing with hot water, and rinses with tap water and organic-free reagent water. Drain the glassware and dry it in an oven at 130 °C for several hours, or rinse with methanol and drain. Store dry glassware in a clean environment. Other appropriate glassware cleaning procedures may be employed.

2. Interferences co-extracted from the samples will vary considerably from sample to sample. While general cleanup techniques are referenced or provided as part of this method, unique samples may require additional cleanup approaches to achieve desired degrees of discrimination and quantitation.

3. Interferences by phthalate esters introduced during sample preparation can pose a major problem in pesticide determinations. Interferences from phthalate esters can best be minimized by avoiding contact with any plastic materials and checking all solvents and reagents for phthalate contamination. This includes common flexible plastics contain varying amounts of phthalate esters which are easily extracted or leached from such materials during laboratory operations. Exhaustive cleanup of solvents, reagents and glassware may be necessary to eliminate background phthalate ester contamination.

4. The presence of sulfur will result in broad peaks that interfere with the detection of early-eluting organochlorine pesticides. Sulfur contamination should be expected with tissue or sediment samples. Sulfur should be removed prior to anlaysis.

5. Waxes, lipids, and other high molecular weight materials can be removed by gel permeation chromatography (GPC) cleanup.

6. Other halogenated pesticides or industrial chemicals may interfere with the analysis of pesticides. Certain coeluting organophosphorus pesticides may be eliminated using GPC -- pesticide option. Coeluting chlorophenols may be eliminated by using silica gel, Florisil, or alumina. Polychlorinated biphenyls (PCBs) also may interfere with the analysis of the organochlorine pesticides. The problem may be most severe for the analysis of multicomponent analytes such as chlordane, toxaphene, and Strobane.

7. Coelution among the many target analytes in this method can cause interference problems. The following target analytes may coelute on the GC columns listed, when using the single-column analysis scheme:

DB 608	Trifluralin/diallate isomers PCNB/dichlone/Isodrin
DB 1701	Captafol/mirex Methoxychlor/endosulfan sulfate

8. The following compounds may coelute using the dual-column analysis scheme. In general, the DB-5 column resolves fewer compounds than the DB-1701.

DB-5	Permethrin/heptachlor epoxide

	Endosulfan I/*cis*-chlordane
	Perthane/endrin
	Endosulfan II/chloropropylate/chlorobenzilate
	4,4'-DDT/endosulfan sulfate
	Methoxychlor/dicofol
DB-1701	Chlorothalonil/β-BHC
	δ-BHC/DCPA/permethrin
	cis-Chlordane/*trans*-nonachlor

Nitrofen, dichlone, carbophenothion, and dichloran exhibit extensive peak tailing on both columns. Simazine and atrazine give poor responses on the ECD detector. Triazine compounds should be analyzed using anitrogen-phosphorus detector (NPD) option.

4.5.4 Equipment and Supplies

1. Gas chromatograph (GC) -- An analytical system complete with gas chromatograph suitable for on-column and split-splitless injection and all necessary accessories including syringes, analytical columns, gases, electron capture detectors (ECD), and recorder/integrator or data system. Electrolytic conductivity detectors (ELCD) may also be employed if appropriate for project needs. If the dual-column option is employed, the gas chromatograph must be equipped with two detectors.
2. GC columns. The single-column approach involves one analysis to determine that a compound is present, followed by a second analysis to confirm the identity of the compound (section 4.5.11) The single-column approach may employ either narrow-bore (#0.32-mm ID) columns or wide-bore (0.53-mm ID) columns. The dual-column approach generally employs a single injection that is split between two columns that are mounted in a single gas chromatograph. The dual-column approach generally employs wide-bore (0.53-mm ID) columns, but columns of other diameters may be employed if the analyst can demonstrate and document acceptable performance for the intended application. A third alternative is to employ dual columns mounted in a single GC, but with each column connected to a separate injector and a separate detector.

***Note**. The listing of these columns in this method is not intended to exclude the use of other columns that are available or that may be developed. Laboratories may use these columns or other columns provided that the laboratories document method performance data (e.g., chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.*

Narrow-bore columns for single-column analysis (use both columns to confirm compound identifications unless another confirmation technique such as GC/MS is employed). Narrow-bore columns should be installed in split/splitless (Grob-type) injectors.

30-m x 0.25-mm or 0.32-mm ID fused-silica capillary column chemically bonded with SE-54 (DB-5 or equivalent), 1-µm film thickness.

30-m x 0.25-mm ID fused-silica capillary column chemically bonded with 35 percent phenyl methylpolysiloxane (DB-608, SPB-608, or equivalent), 2.5 µm coating thickness, 1-µm film thickness.

Wide-bore columns for single-column analysis (use two of the three columns listed to confirm compound identifications unless another confirmation technique such as GC/MS is

employed). Wide-bore columns should be installed in 1/4-inch injectors, with deactivated liners designed specifically for use with these columns.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 35 percent phenyl methylpolysiloxane (DB-608, SPB-608, RTx-35, or equivalent), 0.5-µm or 0.83-µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 50 percent phenyl methylpolysiloxane (DB-1701, or equivalent), 1.0- µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 95 percent dimethyl - 5 percent diphenyl polysiloxane (DB-5, SPB-5, RTx-5, or equivalent), 1.5-µm film thickness.

Wide-bore columns for dual-column analysis -- The two pairs of recommended columns are listed below.

Column pair 1:

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54 (DB-5, SPB-5, RTx-5, or equivalent), 1.5-µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 50 percent phenyl methylpolysiloxane (DB-1701, or equivalent), 1.0-µm film thickness.

Column pair 1 is mounted in a press-fit Y-shaped glass 3-way union or a Y-shaped fused-silica connector, or equivalent.

***Note**. When connecting columns to a press-fit Y-shaped connector, a better seal may be achieved by first soaking the ends of the capillary columns in alcohol for about 10 sec to soften the polyimide coating.*

Column pair 2:

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54 (DB-5, SPB-5, RTx-5, or equivalent), 0.83-µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 50 percent phenyl methylpolysiloxane (DB-1701, or equivalent), 1.0-µm film thickness.

Column pair 2 is mounted in an 8-inch deactivated glass injection tee.

3. Column rinsing kit -- Bonded-phase column rinse kit
4. Volumetric flasks, 10-ml and 25-ml, for preparation of standards.

4.5.5 Reagents and Standards

1. Reagent-grade or pesticide-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to specifications of the Committee on Analytical Reagents of the American Chemical Society. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers.
2. Organic-free reagent water.
3. Solvents used for the preparation of GC standards include acetone, $(CH_3)2CO$ and toluene, $C_6H_5CH_3$. All solvent lots must be pesticide grade in quality or equivalent and should be determined to be free of phthalates.

Note. *Store the standard solutions (stock, composite, calibration, internal, and surrogate) at ≤6°C in polytetrafluoroethylene (PTFE)-sealed containers, in the dark. When a lot of standards is prepared, aliquots of that lot should be stored in individual small vials. All stock standard solutions must be replaced after one year, or sooner if routine QC (see section 5.7.7) indicates a problem. All other standard solutions must be replaced after six months, or sooner, if routine QC indicates a problem.*

4. Stock standard solutions

   Stock standard solutions (1000 mg/L) may be prepared from pure standard materials or purchased as certified solutions. Prepare stock standard solutions by accurately weighing 0.0100 g of pure compound. Dissolve the compound in isooctane or hexane and dilute to volume in a 10-ml volumetric flask. If compound purity is 96 percent or greater, the weight can be used without correction to calculate the concentration of the stock standard solution.

Note*. β-BHC, dieldrin, and some other standards may not be adequately soluble in isooctane. A small amount of acetone or toluene should be used to dissolve these compounds during the preparation of the stock standard solutions.*

5. Composite stock standard

   Prepared from individual stock solutions. For composite stock standards containing less than 25 components, take exactly 1 ml of each individual stock solution at a concentration of 1000 mg/L, add solvent, and mix the solutions in a 25-ml volumetric flask. This composite solution can be further diluted to obtain the desired concentrations. For composite stock standards containing more than 25 components, use volumetric flasks of the appropriate volume (*e.g*., 50-ml, 100-ml), and follow the procedure described above.

6. Calibration standards

   Calibration standards should be prepared at a minimum of five different concentrations by dilution of the composite stock standard with isooctane or hexane. The concentrations should correspond to the expected range of concentrations found in real samples and should bracket the linear range of the detector.

   Although all single component analytes can be resolved on a new 35 percent phenyl methyl silicone column (e.g., DB-608), two calibration mixtures should be prepared for the single component analytes of this method. This procedure is established to minimize potential resolution and quantitation problems on confirmation columns or on older 35 percent phenyl methyl silicone (e.g. DB-608) columns and to allow determination of endrin and DDT breakdown for instrument quality control (section 4.6.7).

   Separate calibration standards are necessary for each multi-component target analyte (*e.g*., toxaphene and chlordane). The analysts should evaluate the specific toxaphene standard carefully. Some toxaphene components, particularly the more heavily chlorinated components, are subject to dechlorination reactions. As a result, standards from different vendors may exhibit marked differences which could lead to possible false negative results or to large differences in quantitative results.

7. Internal standard (optional)

   Pentachloronitrobenzene is suggested as an internal standard for the single-column analysis, when it is not considered to be a target analyte. 1-Bromo-2-nitrobenzene may also be used. Prepare a solution of 5000 mg/L (5000 ng/µl) of pentachloronitrobenzene or 1-bromo-2-nitrobenzene. Spike 10 µl of this solution into each 1 ml of sample extract.

Bromo-2-nitrobenzene is suggested as an internal standard for the dual-column analysis. Prepare a solution of 5000 mg/L (5000 ng/µl) of 1-bromo-2-nitrobenzene. Spike 10 µl of this solution into each 1 ml of sample extract.

8. Surrogate standards. The performance of the method should be monitored using surrogate compounds. Surrogate standards are added to all samples, method blanks, matrix spikes, and calibration standards. The following compounds are recommended as possible surrogates. Other surrogates may be used, provided that the analyst can demonstrate and document performance appropriate for the data quality needs of the particular application.

9. Decachlorobiphenyl and tetrachloro-*m*-xylene have been found to be a useful pair of surrogates for both the single-column and dual-column configurations.

   Chloro-3-nitrobenzotrifluoride may also be useful as a surrogate if the chromatographic conditions of the dual-column configuration cannot be adjusted to preclude coelution of a target analyte with either of the surrogates. However, this compound elutes early in the chromatographic run and may be subject to other interference problems. A recommended concentration for this surrogate is 500 ng/µ. Use a spiking volume of 100 µl for a 1-L aqueous sample. Other surrogate concentrations may be used, as appropriate for the intended application.

   Store surrogate spiking solutions at ≤6 °C in PTFE-sealed containers in the dark.

4.5.6 Gas Chromotography Specifications

1. This method allows the analyst to choose between a single-column and a dual-column configuration in the injector port. The list of columns in this method is not intended to exclude the use of other columns that are available or that may be developed. Wide-bore or narrow-bore columns may be used with either option. Laboratories may use these or other capillary columns or columns of other dimensions, provided that the laboratories document method performance data (*e.g.*, chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.

2. Single-column analysis

   This capillary GC/ECD method allows the analyst the option of using 0.25 or 0.32-mm ID capillary columns (narrow-bore) or 0.53-mm ID capillary columns (wide-bore). Narrow-bore columns generally provide greater chromatographic resolution than wide-bore columns, although narrow-bore columns have a lower sample capacity. As a result, narrow-bore columns may be more suitable for relatively clean samples or for extracts that have been prepared with one or more of the clean-up options referenced in the method. Wide-bore columns (0.53-mm ID) may be more suitable for more complex environmental and waste matrices. However, the choice of the appropriate column diameter is left to professional judgement of the analyst.

   Each laboratory must determine retention times and retention time windows for their specific application of the method.

3. Dual-column analysis

   The dual-column/dual-detector approach recommends the use of two 30-m x 0.53-mm ID fused-silica open-tubular columns of different polarities, thus of different selectivities toward the target analytes. The columns are connected to an injection tee and separate electron capture detectors or to both separate injectors and separate detectors. However, the choice of the appropriate column dimensions is left to the professional judgement of the analyst.

Each laboratory must determine retention times and retention time windows for their specific application of the method.

4.5.7 Quality Control and Assurance

1. Refer to the QAPP for guidance on quality assurance (QA) and quality control (QC) protocols. When inconsistencies exist between QC guidelines, method-specific QC criteria take precedence over both technique-specific criteria and technique-specific QC criteria take precedence over the criteria in the QAPP. Each laboratory should maintain a formal quality assurance program. Each lab must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.
2. Include a calibration standard after each group of 20 samples, (however it is recommended that a calibration standard be included after every 10 samples to minimize the number of repeat injections) in the analysis sequence as a calibration check. Thus, injections of method blank extracts, matrix spike samples, and other non-standards are counted in the total. Solvent blanks, injected as a check on cross-contamination, need not be counted in the total. The response factors for the calibration verification standard must be within ±20% of the initial calibration (Section 4.5.9). When this calibration verification standard falls out of this acceptance window, the laboratory should stop analyses and take corrective action.

   Whenever quantitation is accomplished using an internal standard, internal standards must be evaluated for acceptance. The measured area of the internal standard must be no more than 50 percent different from the average area calculated during initial calibration. When the internal standard peak area is outside the limit, all samples that fall outside the QC criteria must be reanalyzed. The retention times of the internal standards must also be evaluated. A retention time shift of >30 sec necessitates reanalysis of the affected sample.

   DDT and endrin are easily degraded in the injection port. Breakdown occurs when the injection port liner is contaminated with high boiling residue from sample injection or when the injector contains metal fittings. Check for degradation problems by injecting a standard containing only 4,4'-DDT and endrin. Presence of 4,4'-DDE, 4,4'-DDD, endrin ketone or endrin indicates breakdown. If degradation of either DDT or endrin exceeds 15%, take corrective action before proceeding with calibration. Unless otherwise specified in an approved project plan, this test should be performed even when DDT and endrin are not target analytes for a given project, as a test of the inertness of the analytical system.

   Calculate percent breakdown as follows:

$$\%\ \text{breakdown of DDT} = \frac{\text{sum of degradation peak areas (DDD + DDE)}}{\text{sum of all peak areas (DDT + DDE + DDD)}} \times 100$$

$$\%\ \text{breakdown of endrin} = \frac{\text{sum of degradation peak areas (aldehyde + ketone)}}{\text{sum of all peak areas (endrin + aldehyde + ketone)}} \times 100$$

   The breakdown of DDT and endrin should be measured before samples are analyzed and at the beginning of each 12-hr shift. Injector maintenance and recalibration should be completed if the breakdown is greater than 15% for either compound.

Whenever silica gel or Florisil® cleanups are used, the analyst must demonstrate that the fractionation scheme is reproducible. Batch to batch variation in the composition of the silica gel or Florisil® or overloading the column may cause a change in the distribution patterns of the organochlorine pesticides. When compounds are found in two fractions, add the concentrations found in the fractions, and correct for any additional dilution.

3. Initial demonstration of proficiency. Each laboratory must demonstrate initial proficiency with each sample preparation and determinative method combination it utilizes, by generating data of acceptable accuracy and precision for target analytes in a clean matrix. If an autosampler is used to perform sample dilutions, before using the autosampler to dilute samples, the laboratory should satisfy itself that those dilutions are of equivalent or better accuracy than is achieved by an experienced analyst performing manual dilutions.

   It is suggested that the QC reference sample concentrate contain each analyte of interest at 10 mg/l in the concentrate. A 1-ml spike of this concentrate into 1 L of reagent water will yield a sample concentration of10 µg/l. If this method is to be used for analysis of chlordane or toxaphene only, the QC reference sample concentrate should contain the most representative multicomponent mixture at a suggested concentration of 50 mg/L in acetone. Other concentrations may be used, as appropriate for the intended application.

   Calculate the average recovery and the standard deviation of the recoveries of the analytes in each of the four QC reference samples.

4. Initially, before processing any samples, the analyst should demonstrate that all parts of the equipment in contact with the sample and reagents are interference-free. This is accomplished through the analysis of a method blank. As a continuing check, each time samples are extracted, cleaned up, and analyzed, and when there is a change in reagents, a method blank should be prepared and analyzed for the compounds of interest as a safeguard against chronic laboratory contamination. If a peak is observed within the retention time window of any analyte that would prevent the determination of that analyte, determine the source and eliminate it, if possible, before processing the samples. The blanks should be carried through all stages of sample preparation and analysis. When new reagents or chemicals are received, the laboratory should monitor the preparation and/or analysis blanks associated with samples for any signs of contamination. It is not necessary to test every new batch of reagents or chemicals prior to sample preparation if the source shows no prior problems. However, if reagents are changed during a preparation batch, separate blanks need to be prepared for each set of reagents.

5. Sample quality control for preparation and analysis. The laboratory must also have procedures for documenting the effect of the matrix on method performance (precision, accuracy. method sensitivity). At a minimum, this should include the analysis of QC samples including a method blank, a matrix spike, a duplicate, and a laboratory control sample (LCS) in each analytical batch and the addition of surrogates to each field sample and QC sample when surrogates are used. Any method blanks, matrix spike samples, and replicate samples should be subjected to the same analytical procedures (section 5.7.9) as those used on actual samples.

   Documenting the effect of the matrix should include the analysis of at least one matrix spike and one duplicate unspiked sample or one matrix spike/matrix spike duplicate pair. The decision on whether to prepare and analyze duplicate samples or a matrix spike/matrix spike duplicate must be based on knowledge of the samples in the sample batch. If samples are expected to contain target analytes, then laboratories may use a matrix spike and a

duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, the laboratories should use a matrix spike and matrix spike duplicate pair.

A laboratory control sample (LCS) should be included with each analytical batch. The LCS consists of an aliquot of a clean (control) matrix similar to the sample matrix and of the same weight or volume. The LCS is spiked with the same analytes at the same concentrations as the matrix spike, when appropriate. When the results of the matrix spike analysis indicate a potential problem due to the sample matrix itself, the LCS results are used to verify that the laboratory can perform the analysis in a clean matrix.

6. 6 Surrogate recoveries. If surrogates are used, the laboratory should evaluate surrogate recovery data from individual samples versus the surrogate control limits developed by the laboratory. Procedures for evaluating the recoveries of multiple surrogates and the associated corrective actions should be defined in an approved project plan.
7. It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Whenever possible, the laboratory should analyze standard reference materials and participate in relevant performance evaluation studies.

4.5.8 Calibration and Standardization

1. Prepare calibration standards using the procedures in Section 4.5.5.6. Refer to Section 4.5.7.2 of this method for proper calibration techniques for both initial calibration and calibration verification. The procedure for either internal or external calibration may be used. In most cases, external standard calibration is used with this method because of the sensitivity of the electron capture detector and the probability of the internal standard being affected by interferences. Because several of the pesticides may coelute on any single column, the analysts should use two calibration mixtures. The specific mixture should be selected to minimize the problem of peak overlap.

Note. *Because of the sensitivity of the electron capture detector, always clean the injection port and column prior to performing the initial calibration*.

The analysis of the multi-component analytes should employ a single-point calibration. A single calibration standard near the mid-point of the expected calibration range of each multi-component analyte is included with the initial calibration of the single component analytes for pattern recognition, so that the analyst is familiar with the patterns and retention times on each column. The calibration standard may be at a lower concentration than the mid-point of the expected range, if appropriate for the project.

For calibration verification (each 12-hr shift), all target analytes specified in the project plan must be injected.

2. Establish the GC operating conditions appropriate for the configuration (single-column or dual column). Optimize the instrumental conditions for resolution of the target analytes and sensitivity. An initial oven temperature of < 140 - 150 °C may be necessary to resolve the four BHC isomers. A final temperature of between 240 °C and 270 °C may be necessary to elute decachlorobiphenyl. The use of injector pressure programming will improve the chromatography of late eluting peaks.

Note. *Once established, the same operating conditions must be used for both calibrations and sample analyses.*

3. A 2-µl injection volume of each calibration standard is recommended. Other injection volumes may be employed, provided that the analyst can demonstrate adequate sensitivity for the compounds of interest.

4. Because of the low concentration of pesticide standards injected on a GC/ECD, column adsorption may be a problem when the GC has not been used for a day or more. Therefore, the GC column should be primed (or deactivated) by injecting a pesticide standard mixture approximately 20 times more concentrated than the midconcentration standard. Inject this standard mixture prior to beginning the initial calibration or calibration verification.

***Note**. Several analytes, including aldrin, may be observed in the injection just following this system priming because of carry-over. Always run an acceptable blank prior to running any standards or samples.*

5. Calibration factors

When external standard calibration is employed, calculate the calibration factor for each analyte at each concentration, the mean calibration factor, and the relative standard deviation (RSD) of the calibration factors, using the formulae below. If internal standard calibration is employed, refer to Method 8000 for the calculation of response factors.

Calculate the calibration factor for each analyte at each concentration as:

$$CF = \frac{\text{Peak Area (or Height) of the compound in the Standard}}{\text{Mass of the Compound Injected (ng)}}$$

Calculate the mean calibration factor for each analyte as:

$$\text{mean CF} = \overline{CF} = \frac{\sum_{i=1}^{n} CF_i}{n}$$

where n is the number of standards analyzed.

Calculate the standard deviation (SD) and the RSD of the calibration factors for each analyte as:

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (CF_i - \overline{CF})^2}{n-1}} \qquad RSD = \frac{SD}{\overline{CF}} \times 100$$

If the RSD for each analyte is < 20%, then the response of the instrument is considered linear and the mean calibration factor may be used to quantitate sample results. If the RSD is greater than 20%, the analyst should consider other calibration options, which may include either a linear calibration not through the origin or a non-linear calibration model (*e.g.*, a polynomial equation).

6. Retention time windows. Absolute retention times are generally used for compound identification. When absolute retention times are used, retention time windows are crucial to the identification of target compounds. Retention time windows are established to compensate for minor shifts in absolute retention times as a result of sample loadings and normal chromatographic variability. The width of the retention time window should be carefully established to minimize the occurrence of both false positive and false negative results. Tight retention time windows may result in false negatives and/or may cause

unnecessary reanalysis of samples when surrogates or spiked compounds are erroneously not identified. Overly wide retention time windows may result in false positive results that cannot be confirmed upon further analysis. Other approaches to compound identification may be employed, provided that the analyst can demonstrate and document that the approaches are appropriate for the intended application.

Before establishing the retention time windows, make sure that the gas chromatographic system is operating within optimum conditions. The widths of the retention time windows should be determined by the experienced analyst.

4.5.9 Analytical Procedure and Analysis

1. The same GC operating conditions used for the initial calibration must be employed for the analysis of samples.
2. Verify calibration at least once each 12-hr shift by injecting calibration verification standards prior to conducting any sample analyses. Analysts should alternate the use of high and low concentration mixtures of single-component analytes and multicomponent analytes for calibration verification. A calibration standard must also be injected at intervals of not less than once every twenty samples (after every 10 samples is recommended to minimize the number of samples requiring re-injection when QC limits are exceeded) and at the end of the analysis sequence. See section 4.5.7.2 for additional guidance on the frequency of the standard injections.

   The calibration factor for each analyte should not exceed a ±20 percent difference from the mean calibration factor calculated for the initial calibration.

   If the calibration does not meet the ±20% limit on the basis of each compound, check the instrument operating conditions, and if necessary, restore them to the original settings, and inject another aliquot of the calibration verification standard. If the response for the analyte is still not within ±20%, then a new initial calibration must be prepared. The effects of a failing calibration verification standard on sample results are discussed in section 4.5.9.
3. Compare the retention time of each analyte in the calibration standard with the absolute retention time windows. Each analyte in each subsequent standard run during the 12-hr period must fall within its respective retention time window. If not, the gas chromatographic system must either be adjusted so that a second analysis of the standard does result in all analytes falling within their retention time windows, or a new initial calibration must be performed and new retention time windows established. As noted, other approaches to compound identification may be employed, provided that the analyst can demonstrate and document that the approaches are appropriate for the intended application.
4. Inject a measured aliquot of the concentrated sample extract. A 2-µl aliquot is suggested, however, the same injection volume should be used for both the calibration standards and the sample extracts, unless the analyst can demonstrate acceptable performance using different volumes or conditions. Record the volume injected and the resulting peak size in area units.
5. Confirmation. Tentative identification of an analyte (either single-component or multi-component) occurs when a peak from a sample extract falls within the daily retention time window. Confirmation is necessary when the sample composition is not well characterized. Confirmatory techniques such as gas chromatography with a dissimilar column or a mass spectrometer should be used. See section 4.5.11 of this method for information on the use of GC/MS as a confirmation technique.

When results are confirmed using a second GC column of dissimilar stationary phase, the analyst should check the agreement between the quantitative result on both columns once the identification has been confirmed.

6. When using the external calibration procedure, determine the quantity of each component peak in the sample chromatogram which corresponds to the compounds used for calibration purposes, as follows. The appropriate selection of a baseline from which the peak area or height can be determined is necessary for proper quantitation.

   For aqueous samples:

   $$\text{Concentration } (\mu g/l) = \frac{(Ax)(Vt)(D)}{(CF)(Vi)(Vs)}$$

   where:

   Ax = Area (or height) of the peak for the analyte in the sample.

   Vt = Total volume of the concentrated extract (µl).

   D = Dilution factor, if the sample or extract was diluted prior to analysis. If no dilution was made, D = 1. The dilution factor is always dimensionless.

   CF = Mean calibration factor from the initial calibration (area/ng).

   Vi = Volume of the extract injected (µl). The injection volume for samples and calibration standards should be the same, unless the analyst can demonstrate acceptable performance using different volumes or conditions.

   Vs = Volume of the aqueous sample extracted in ml. If units of liters are used for this term, multiply the results by 1000.

   Using the units given here for these terms will result in a concentration in units of ng/ml, which is equivalent to µg/l.

   For non-aqueous samples:

   $$\text{Concentration } (\mu g/kg) = \frac{(Ax)(Vt)(D)}{(CF)(Vi)(Ws)}$$

   where :

   Ax, Vt, D, CF, and Vi are the same as for aqueous samples

   Ws = Weight of sample extracted (g). The wet weight or dry weight may be used, depending upon the specific application of the data. If units of kilograms are used for this term, multiply the results by 1000.

   Using the units given here for these terms will result in a concentration in units of ng/g, which is equivalent to µg/kg.

   If the responses exceed the calibration range of the system, dilute the extract and reanalyze. Peak height measurements are recommended over peak area integration when overlapping peaks cause errors in area integration.

   If partially overlapping or coeluting peaks are found, change GC columns or try GC/MS quantitation.

7. Each sample analysis employing external standard calibration must be bracketed with an acceptable initial calibration, calibration verification standards (each 12- hr analytical shift), or calibration standards interspersed within the samples. The results from these bracketing standards must meet the calibration verification criteria.

Although analysis of a single mid-concentration standard (standard mixture or multi-component analyte) will satisfy the minimum requirements, analysts are urged to use different calibration verification standards during organochlorine pesticide analyses. Also, multi-level standards (mixtures or multi-component analytes) are highly recommended to ensure that the detector response remains stable for all analytes over the calibration range.

When a calibration verification standard fails to meet the QC criteria, all samples that were injected after the last standard that last met the QC criteria must be evaluated to prevent misquantitations and possible false negative results, and reinjection of the sample extracts may be necessary. More frequent analyses of standards will minimize the number of sample extracts that would have to be reinjected if the QC limits are violated for standard analysis.

However, if the standard analyzed after a group of samples exhibits a response for an analyte that is above the acceptance limit, *i.e*., >20%, and the analyte was not detected in the specific samples analyzed during the analytical shift, then the extracts for those samples do not need to be reanalyzed, as the verification standard has demonstrated that the analyte would have been detected were it present. In contrast, if an analyte above the QC limits was detected in a sample extract, then reinjection is necessary to ensure accurate quantitation. If an analyte was not detected in the sample and the standard response is more than 20% below the initial calibration response, then reinjection is necessary to ensure that the detector response has not deteriorated to the point that the analyte would not have been detected even though it was present (*i.e*., a false negative result).

8. Sample injections may continue for as long as the calibration verification standards and standards interspersed with the samples meet instrument QC requirements. It is recommended that standards be analyzed after every 10 samples (required after every 20 samples and at the end of a set) to minimize the number of samples that must be re-injected when the standards fail the QC limits. The sequence ends when the set of samples has been injected or when qualitative and/or quantitative QC criteria are exceeded.

9. The use of internal standard calibration techniques does not require that all sample results be bracketed with calibration verification standards. However, when internal standard calibration is used, the retention times of the internal standards and the area responses of the internal standards should be checked for each analysis. Retention time shifts of >30 sec from the retention time of the most recent calibration standard and/or changes in internal standard areas of more than -50 to +100% are cause for concern and must be investigated.

10. If the peak response is less than 2.5 times the baseline noise level, the validity of the quantitative result may be questionable. Consult with the source of the sample to determine whether further concentration of the sample is warranted.

11. Use the calibration standards analyzed during the sequence to evaluate retention time stability. Each subsequent injection of a standard during the 12-hr analytical shift (*i.e*., those standards injected every 20 samples, or more frequently) must be checked against retention time windows. If any of these subsequent standards fall outside their absolute retention time windows, the GC system is out of control. Determine the cause of the problem and correct it. If the problem cannot be corrected, a new initial calibration must be performed.

12. The identification of mixtures (*i.e*., chlordane and toxaphene) is not based on a single peak, but rather on the characteristic peaks that comprise the "fingerprint" of the mixture, using both the retention times and shapes of the indicator peaks. Quantitation is based on the areas of the characteristic peaks as compared to the areas of the corresponding peaks at the same retention times in the calibration standard, using either internal or external

calibration procedures. See section 4.5.11 for information on the use of GC/MS as a confirmation technique.

13. If compound identification or quantitation is precluded due to interference (*e.g.*, broad, rounded peaks or ill-defined baselines), cleanup of the extract or replacement of the capillary column or detector is warranted. Rerun the sample on another instrument to determine if the problem results from analytical hardware or the sample matrix.

4.5.10 Quantitation of Multi-Component Analytes

Multi-component analytes present problems in measurement. Suggestions are offered in the following sections for handling toxaphene, Strobane, chlordane, BHC, and DDT.

1. Toxaphene is manufactured by the chlorination of camphenes, and Strobane results from the chlorination of a mixture of camphenes and pinenes. Quantitation of toxaphene or Strobane is difficult, but reasonable accuracy can be obtained. To calculate toxaphene from GC/ECD results:

   Adjust the sample size so that the major toxaphene peaks are 10 - 70% of full-scale deflection (FSD). Inject a toxaphene standard that is estimated to be within ±10 ng of the sample amount. Quantitate toxaphene using the total area of the toxaphene pattern or using 4 to 6 major peaks.

   While toxaphene contains a large number of compounds that will produce well resolved peaks in a GC/ECD chromatogram, it also contains many other components that are not chromatographically resolved. This unresolved complex mixture results in the "hump" in the chromatogram that is characteristic of this mixture. Although the resolved peaks are important for the identification of the mixture, the area of the unresolved complex mixture contributes a significant portion of the area of the total response.

   To measure total area, construct the baseline of toxaphene in the sample chromatogram between the retention times of the first and last eluting toxaphene components in the standard. In order to use the total area approach, the pattern in the sample chromatogram must be compared to that of the standard to ensure that all of the major components in the standard are present in the sample. Otherwise, the sample concentration may be significantly underestimated.

   Toxaphene may also be quantitated on the basis of 4 to 6 major peaks. A collaborative study of a series of toxaphene residues evaluated several approaches to quantitation of this compound, including the use of the total area of the peaks in the toxaphene chromatogram and the use of a subset of 4 to 6 peaks. That study indicated that the use of 4 to 6 peaks provides results that agree well with the total peak area approach and may avoid difficulties when interferences with toxaphene peaks are present in the early portion of the chromatogram from compounds such as DDT. Whichever approach is employed should be documented and available to the data user, if necessary.

   When toxaphene is determined using the 4 to 6 peaks approach, the analyst must take care to evaluate the relative areas of the peaks chosen in the sample and standard chromatograms. It is highly unlikely that the peaks will match exactly, but the analyst should not employ peaks from the sample chromatogram whose relative sizes or areas appear to be disproportionally larger or smaller in the sample compared to the standard.

   The heights or areas of the 4 to 6 peaks that are selected should be summed together and used to determine the toxaphene concentration. Alternatively, use each peak in the

standard to calculate a calibration factor for that peak, using the total mass of toxaphene in the standard. These calibration factors are then used to calculate the concentration of each corresponding peak in the sample chromatogram and the 4 to 6 resulting concentrations are averaged to provide the final result for the sample.

2. Technical chlordane is a mixture of at least 11 major components and 30 or more minor components that have been used to prepare specific pesticide formulations. The exact percentages of *cis*-chlordane and *trans*-chlordane in technical material are not completely defined, and are not consistent from batch to batch. Moreover, changes may occur when the technical material is used to prepare specific pesticide formulations. The approach used for evaluating and reporting chlordane results will depend on the end use of the results and the analyst's skill in interpreting this multicomponent pesticide residue. The following sections discuss three options: reporting technical chlordane (CAS number 12789-03-6), reporting chlordane (not otherwise specified, or n.o.s., CAS number 57-74-9), and reporting the individual chlordane components that can be identified under individual CAS numbers.

   When the GC pattern of the residue resembles that of technical chlordane, the analyst may quantitate chlordane residues by comparing the total area of the chlordane chromatogram using three to five major peaks or the total area. If the heptachlor epoxide peak is relatively small, include it as part of the total chlordane area for calculation of the residue. If heptachlor and/or heptachlor epoxide are much out of proportion, calculate these separately and subtract their areas from the total area to give a corrected chlordane area.

Note. *Octachloro epoxide, a metabolite of chlordane, can easily be mistaken for heptachlor epoxide on a nonpolar GC column.*

   To measure the total area of the chlordane chromatogram, inject an amount of a technical chlordane standard which will produce a chromatogram in which the major peaks are approximately the same size as those in the sample chromatograms. Construct the baseline of technical chlordane in the standard chromatogram between the retention times of the first and last eluting chlordane components. Use this area and the mass of technical chlordane in the standard to calculate a calibration factor. Construct a similar baseline in the sample chromatogram, measure the area, and use the calibration factor to calculate the concentration in the sample.

   The GC pattern of a chlordane residue in a sample may differ considerably from that of the technical chlordane standard. In such instances, it may not be practical to relate a sample chromatogram back to the pesticide active ingredient technical chlordane. Therefore, depending on the objectives of the analysis, the analyst may choose to report the sum of all the identifiable chlordane components as "chlordane (n.o.s.)" under CAS number 57-74-9.

   The third option is to quantitate the peaks of *cis*-chlordane, *trans*-chlordane, and heptachlor separately against the appropriate reference materials, and report these individual components under their respective CAS numbers.

   To measure the total area of the chlordane chromatogram, inject an amount of a technical chlordane standard which will produce a chromatogram in which the major peaks are approximately the same size as those in the sample chromatograms.

3. Hexachlorocyclohexane is known as BHC, from the former name, benzene hexachloride. Technical grade BHC is a cream colored amorphous solid with a very characteristic musty odor. It consists of a mixture of six chemically distinct isomers and one or more heptachlorocyclohexanes and octachlorocyclohexanes. Commercial BHC preparations may

show a wide variance in the percentage of individual isomers present. Quantitate each isomer (α, β, γ, and δ) separately against a standard of the respective pure isomer.

4. Technical DDT consists primarily of a mixture of 4,4'-DDT (approximately 75%) and 2,4'-DDT. As DDT weathers, 4,4'-DDE, 2,4'-DDE, 4,4'-DDD, and 2,4'-DDD are formed. Since the 4,4'-isomers of DDT, DDE, and DDD predominate in the environment, and these are the isomers normally regulated by EPA, sample extracts should be quantitated against standards of the respective pure isomers of 4,4'-DDT, 4,4'-DDE, and 4,4'-DDD.

4.5.11 GC/MS Confirmation

GC/MS confirmation may be used in conjunction with either single-column or dual-column analysis if the concentration is sufficient for detection by GC/MS.

1. Full-scan GC/MS will normally require a concentration of approximately 10 ng/µL in the final extract for each single-component compound. Ion trap or selected ion monitoring will normally require a concentration of approximately 1 ng/µL.
2. The GC/MS must be calibrated for the specific target pesticides when it is used for quantitative analysis. If GC/MS is used only for confirmation of the identification of the target analytes, then the analyst must demonstrate that those pesticides identified by GC/ECD can be confirmed by GC/MS. This demonstration may be accomplished by analyzing a single-point standard containing the analytes of interest at or below the concentrations reported in the GC/ECD analysis.
3. GC/MS is not recommended for confirmation when concentrations are below 1 ng/µL in the extract, unless a more sensitive mass spectrometer is employed.
4. GC/MS confirmation should be accomplished by analyzing the same extract that is used for GC/ECD analysis and the extract of the associated method blank.
5. If a base/neutral/acid extraction of an aqueous sample was performed for an analysis of semivolatile organics, then that extract and the associated blank may be used for GC/MS confirmation if the surrogates and internal standards do not interfere and if it is demonstrated that the analyte is stable during acid/base partitioning. However, if the compounds are *not* detected in the base/neutral/acid extract, then GC/MS analysis of the pesticide extract should be performed.
6. When system performance does not meet the established QC requirements, Chromatographic system maintenance as corrective action is required, and may include the application of splitter connections as described below.

For dual-columns which are connected using a press-fit Y-shaped glass splitter or a Y-shaped fused-silica connector, clean and deactivate the splitter port insert or replace with a cleaned and deactivated splitter. Break off the first few centimeters (up to 30 cm) of the injection port side of the column. Remove the columns and solvent backflush according to the manufacturer's instructions. If these procedures fail to eliminate the degradation problem, it may be necessary to deactivate the metal injector body and/or replace the columns.

4.6 POLYCHLORINATED BIPHENOLS (PCBs) IN FISH TISSUE AND SEDIMENTS

4.6.1 Scope and Application

1. This method may be used for both saltwater and freshwater fish tissue and sediments
2. This method may be used to determine the concentrations of polychlorinated biphenyls (PCBs) or as individual PCB congeners in extracts using open-tubular, capillary columns with electron capture detectors (ECD) or electrolytic conductivity detectors (ELCD). The 21 PCB congeners listed below (Table 4.9) have been determined by this method, using either a single- or dual column analysis system.

Table 4.9. Indicator List of Polychlorinated Biphenyls (PCBs)

Compound	IUPAC (PCB) No.	Chemical Abstract Service (CAS) Registry No.
2,4'-Dichlorobiphenyl	8	34883-43-7
2,2',5-Trichlorobiphenyl	18	37680-65-2
2,4,4'-Trichlorobiphenyl	28	7012-37-5
2,2',3,5'-Tetrachlorobiphenyl	44	41464-39-5
2,2',5,5'-Tetrachlorobiphenyl	52	35693-99-3
2,3',4,4'-Tetrachlorobiphenyl	66	32598-10-0
3,3',4,4'-Tetrachlorobiphenyl	77	32598-13-3
2,2',4,5,5'-Pentachlorobiphenyl	101	37680-73-2
2,3,3',4,4'-Pentachlorobiphenyl	105	32598-14-4
2,3,3',4',6-Pentachlorobiphenyl	110	38380-03-9
2,3,4,4',5- Pentachlorobipheny	118	31508-00-6
3,3,4,4',5- Pentachlorobiphenyl	126	57465-28-8
2,2',3,3',4,4'-Hexachlorobiphenyl	128	38380-07-3
2,2',3,4,4',5'-Hexachlorobiphenyl	138	35065-28-2
2,2',4,4',5,5'-Hexachlorobiphenyl	153	35065-27-1
2,2',3,3',4,4',5-Heptachlorobiphenyl	170	35065-30-6
2,2',3,4,4',5,5'-Heptachlorobiphenyl	180	35065-29-3
2,2',3,4',5,5',6-Heptachlorobiphenyl	187	52663-68-0
2,2',3,3',4,4',5,6-Octachlorobiphenyl	195	52663-78-2
2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl	206	40486-72-9
2,2',3,3',4,4',5,5',6,6'-Decachlorobiphenyl	209	2051-24-3

4.6.2 Summary of Method

Fish tissue or sediment samples are extracted with hexane-acetone (1:1) or methylene chloride-acetone (1:1) using the ultrasonic extraction technique described in Sec. 4.4. Extracts for PCB analysis are subjected to a sequential sulfuric acid/potassium permanganate cleanup designed specifically for these analytes. This cleanup technique will remove many single component organochlorine or organophosphorus pesticides. Therefore, this method is not applicable to the analysis of those compounds. The extract is analyzed by injecting a measured aliquot into a gas chromatograph equipped with a narrow- or wide-bore fused-silica capillary column and an electron capture detector (GC/ECD) or an electrolytic conductivity detector (GC/ELCD).

4.6.3 Interferences

1. Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis by analyzing method blanks. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be necessary.

2. Interferences co-extracted from the samples will vary considerably from matrix to matrix. While general cleanup techniques are referenced or provided as part of this method, unique samples may require additional cleanup approaches to achieve desired degrees of discrimination and quantitation. Sources of interference in this method can be grouped into four broad categories, as follows:

   Contaminated solvents, reagents, or sample processing hardware.

   Contaminated GC carrier gas, parts, column surfaces, or detector surfaces.

   Compounds extracted from the sample matrix to which the detector will respond, such as single-component chlorinated pesticides, including DDT analogs (DDT, DDE, and DDD).

***Note**. A standard of the DDT analogs should be injected to determine which of the PCB peaks may be subject to interferences on the analytical columns used.*

   Coelution of related analytes -- All PCB congeners cannot be separated using the GC columns and procedures described in this method. If this procedure is expanded to encompass other congeners, then the analyst must either document the resolution of the congeners in question or establish procedures for reporting the results of coeluting congeners that are appropriate for the intended application.

3. Interferences by phthalate esters introduced during sample preparation can pose a major problem in PCB determinations. Interferences from phthalate esters are minimized by avoiding contact with any plastic materials and checking all solvents and reagents for phthalate contamination.

4. Cross-contamination of clean glassware can routinely occur when plastics are handled during extraction steps, especially when solvent-wetted surfaces are handled. Glassware must be scrupulously cleaned. Clean all glassware as soon as possible after use by rinsing with the last solvent used. This should be followed by detergent washing with hot water, and rinses with tap water and organic-free reagent water. Drain the glassware, and dry it in an oven at 130 °C for several hours, or rinse with methanol and drain.

***Note**. Oven-drying of glassware used for PCB analysis can increase contamination because PCBs are readily volatilized in the oven and spread to other glassware. Therefore, exercise caution, and do not dry glassware from samples containing high concentrations of PCBs with glassware that may be used for trace analyses.*

5. Sulfur (S_8) is readily extracted from soil samples and may cause chromatographic interferences in the determination of PCBs. Sulfur contamination should be expected with samples and should be removed with either copper or tetrabutylammonium sulfite. The mixture is shaken and the extract is removed from the sulfur cleanup reagent.

4.6.4 Equipment and Supplies

1. Gas chromatograph. An analytical system complete with GC suitable for on-column and split-splitless injection and necessary accessories including syringes, analytical columns, gases, electron capture detectors, and recorder/integrator or data system. Electrolytic conductivity detectors may also be employed if appropriate for project needs. If the dual-column option is employed, the GC must be equipped with two separate detectors.

2. GC columns. The method describes procedures for single- and dual-column analyses. The single-column approach involves one analysis to determine that a compound is present, and a second analysis to confirm the identity of the compound. The single-column approach may employ either narrow-bore (< 0.32-mm ID) columns or wide-bore (0.53-mm ID) columns. The dual-column approach generally employs a single injection that is split between two columns that are mounted in a single GC. The dual-column approach generally employs wide-bore (0.53-mm ID) columns, but columns of other diameters may be employed if the analyst can demonstrate and document acceptable performance for the intended application. A third alternative is to employ dual columns mounted in a single GC, but with each column connected to a separate injector and a separate detector.

   The listing of these columns is not intended to exclude the use of other columns that are available or that may be developed. Laboratories may use these columns or other columns provided that the laboratories document method performance data (*e.g.*, chromatographic resolution, analyte breakdown, and sensitivity) appropriate for the intended application.

   Narrow-bore columns for single-column analysis (use both columns to confirm compound identifications unless another confirmation technique such as GC/MS is employed). Narrow-bore columns should be installed in split/splitless (Grob-type) injectors.

   30-m x 0.25-mm or 0.32-mm ID fused-silica capillary column chemically bonded with SE-54, 1-µm film thickness.

   30-m x 0.25-mm ID fused-silica capillary column chemically bonded with 35 percent phenyl methylpolysiloxane, 2.5 µm coating thickness, 1-µm film thickness.

   Wide-bore columns for single-column analysis (use two of the three columns listed to confirm compound identifications unless another confirmation technique such as GC/MS is employed). Wide-bore columns should be installed in 1/4-inch injectors, with deactivated liners designed specifically for use with these columns.

   30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 35 percent phenyl methylpolysiloxane, 0.5-µm or 0.83-µm film thickness.

   30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 14% cyanopropylmethylpolysiloxane , 1.0-µm film thickness.

   30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54, 1.5-µm film thickness.

   Wide-bore columns for dual-column analysis.

   Column pair 1:

   30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54, 0.5-µm film thickness.

   30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 14% cyanopropylmethylpolysiloxane, 1.0-µm film thickness.

Column pair 1 is mounted in a press-fit Y-shaped glass 3-way union or a Y-shaped fused-silica connector or equivalent. When connecting columns to a press-fit Y-shaped connector, a better seal may be achieved by first soaking the ends of the capillary columns in alcohol for about 10 sec to soften the polyimide coating.

Column pair 2:

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54, 0.83-µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 14% cyanopropylmethylpolysiloxane1.0-µm film thickness.

Column pair 2 is mounted in an 8-in. deactivated glass injection tee or equivalent.

Column pair 3:

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with SE-54, 1.5-µm film thickness.

30-m x 0.53-mm ID fused-silica capillary column chemically bonded with 35 percent phenyl methylpolysiloxane, 0.5-µm film thickness.

Column pair 3 is mounted in separate injectors and separate detectors.

3. Column rinsing kit -- Bonded-phase column rinse kit
4. Volumetric flasks -- 10-mL and 25-mL, for preparation of standards.
5. Analytical balance, capable of weighing to 0.0001 g.

4.6.5 Reagents and Standards

Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers. Store the standard solutions (stock, composite, calibration, internal, and surrogate) at ≤6 °C in polytetrafluoroethylene (PTFE)-sealed containers in the dark. When a lot of standards is prepared, aliquots of that lot should be stored in individual small vials. All stock standard solutions must be replaced after one year, or sooner if routine QC (see section 4.6.7) indicates a problem. All other standard solutions must be replaced after six months or sooner if routine QC indicates a problem.

1. Organic-free reagent water.
2. Standard stock solutions

   Stock standard solutions (1000 mg/L). May be prepared from pure standard materials or can be purchased as certified solutions. Prepare stock standard solutions by accurately weighing 0.0100 g of pure compound. Dissolve the compound in isooctane or hexane and dilute to volume in a 10-ml volumetric flask. If compound purity is 96 percent or greater, the weight can be used without correction to calculate the concentration of the stock standard solution. Commercially-prepared stock standard solutions may be used at any concentration if they are certified by the manufacturer or by an independent source.

3. Calibration standards for PCB congeners

   If results are to be determined for individual PCB congeners, then standards for the pure congeners must be prepared. This procedure may be appropriate for other congeners as well, but the analyst must either document the resolution of the congeners in question or establish procedures for reporting the results of coeluting congeners that are appropriate for the intended application.

   Stock standards may be prepared or may be purchased as commercially-prepared solutions. Prepare a minimum of five calibration standards containing equal concentrations of two conengers by dilution of the stock standard with isooctane or hexane. The concentrations should correspond to the expected range of concentrations found in real samples and should bracket the linear range of the detector

4. Internal standard

   When PCB congeners are to be determined, the use of an internal standard is highly recommended. Decachlorobiphenyl may be used as an internal standard, added to each sample extract prior to analysis, and included in each of the initial calibration standards.

5. Surrogate standards

   The performance of the method should be monitored using surrogate compounds.

   Surrogate standards are added to all samples, method blanks, matrix spikes, and calibration standards. The choice of surrogate compounds will depend on analysis mode chosen, *e.g*., congeners. The following compounds are recommended as surrogates. Other surrogates may be used, provided that the analyst can demonstrate and document performance appropriate for the data quality needs of the particular application.

   When PCB congeners are to be determined, decachlorobiphenyl is recommended for use as an internal standard, and therefore it cannot also be used as a surrogate. Tetrachloro-*m*-xylene may be used as a surrogate for PCB congener analysis. The recommended spiking solution concentration is 5 mg/L in acetone.

   If decachlorobiphenyl is a target congener for the analysis, 2,2',4,4',5,5'- hexabromo-biphenyl may be used as an internal standard or a surrogate.

6. DDT Analog standard.

   Used to determine if the commonly found DDT analogs (DDT, DDE, and DDD) elute at the same retention times as any of the target analytes (congeners). A single standard containing all three compounds should be sufficient. The concentration of the standard is left to the judgement of the analyst.

4.6.6 GC Specifications

This method allows the analyst to choose between a single-column and a dual-column configuration in the injector port. The columns listed in this section were the columns used to develop the method performance data. Listing these columns in this method is not intended to exclude the use of other columns that are available or that may be developed. Wide-bore or narrow-bore columns may be used with either option. Laboratories may use either the columns listed in this method or other capillary columns or columns of other dimensions, provided that the laboratories document method performance data (*e.g*., chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.

Single-column analysis

This capillary GC/ECD method allows the analyst the option of using 0.25-mm or 0.32-mm ID capillary columns (narrow-bore) or 0.53-mm ID capillary columns (wide-bore). Narrow-bore columns generally provide greater chromatographic resolution than widebore columns, although narrow-bore columns have a lower sample capacity. As a result, narrow-bore columns may be more suitable for relatively clean samples or for extracts that have been prepared with one or more of the clean-up options referenced in the method. Wide-bore columns (0.53-mm ID) may be more suitable for more complex environmental and waste matrices. However, the choice of the appropriate column diameter is left to the professional judgement of the analyst.

Dual-column analysis

The dual-column/dual-detector approach recommends the use of two 30-m x 0.53-mm ID fused-silica open-tubular columns of different polarities, thus, different selectivities towards the target analytes. The columns may be connected to an injection tee and separate electron capture detectors, or to both separate injectors and separate detectors. However, the choice of the appropriate column dimensions is left to the professional judgement of the analyst.

GC temperature programs and flow rates.

Establish the GC temperature program and flow rate necessary to separate the analytes. When determining PCBs as congeners, difficulties may be encountered with coelution of congener 153 and other sample components. Each laboratory must determine retention times and retention time windows for their specific application of the method. Once established, the same operating conditions must be used for the analysis of samples and standards.

4.6.7 Quality Control and Assurance

1. Refer to the QAPP for guidance on quality assurance (QA) and quality control (QC) protocols. When inconsistencies exist between QC guidelines, method-specific QC criteria take precedence over both technique-specific criteria and technique-specific QC criteria take precedence over the criteria in the QAPP. Each laboratory should maintain a formal quality assurance program. Each lab must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.
2. Include a calibration standard after each group of 20 samples, (however it is recommended that a calibration standard be included after every 10 samples to minimize the number of repeat injections) in the analysis sequence as a calibration check. Thus, injections of method blank extracts, matrix spike samples, and other non-standards are counted in the total. Solvent blanks, injected as a check on cross-contamination, need not be counted in the total. The response factors for the calibration verification standard should be within ±20% of the initial calibration. When this calibration verification standard falls out of this acceptance window, the laboratory should stop analyses and take corrective action.

   Whenever quantitation is accomplished using an internal standard, internal standards must be evaluated for acceptance. The measured area of the internal standard must be no more than 50 percent different from the average area calculated during initial calibration. When the internal standard peak area is outside the limit, all samples that fall outside the QC

criteria must be reanalyzed. The retention times of the internal standards must also be evaluated. A retention time shift of >30 sec necessitates reanalysis of the affected sample.

3. The lab must demonstrate initial proficiency with each sample prep and determinative method combination it utilizes, by generating data of acceptable accuracy and precision for target analytes in a clean matrix. If an autosampler is used to perform sample dilutions, before using the autosampler to dilute samples, the lab should satisfy itself that those dilutions are of equivalent or better accuracy than is achieved by an experienced analyst performing manual dilutions. It is suggested that the QC reference sample concentrate contain PCBs as congeners at 10-50 mg/l in the concentrate. A 1-ml spike of this concentrate into 1 L of reagent water will yield a sample concentration of10 µg/l.

4. Calculate the average recovery and the standard deviation of the recoveries of the analytes in each of the four QC reference samples.

5. Before processing samples, the analyst must demonstrate that all parts of the equipment in contact with the sample and reagents are interference-free through the analysis of a method blank. Each time samples are extracted, cleaned up, and analyzed, and when there is a change in reagents, a method blank must be prepared and analyzed for the compounds of interest as a safeguard against chronic contamination. If a peak is observed within the retention time window of any analyte that would prevent the determination of that analyte, determine the source and eliminate it, if possible, before processing the samples. The blanks should be carried through all stages of sample preparation and analysis. When new reagents or chemicals are received, the lab should monitor the preparation and/or analysis blanks associated with samples for any signs of contamination. It is not necessary to test every new batch of reagents or chemicals prior to sample preparation if the source shows no prior problems. However, if reagents are changed during a preparation batch, separate blanks need to be prepared for each set of reagents.

6. Sample quality control for preparation and analysis. The lab must also have procedures for documenting the effect of the matrix on method performance (precision, accuracy. method sensitivity). At a minimum, this should include the analysis of QC samples including a method blank, a matrix spike, a duplicate, and a laboratory control sample (LCS) in each analytical batch and the addition of surrogates to each field sample and QC sample when surrogates are used. Any method blanks, matrix spike samples, and replicate samples should be subjected to the same analytical procedures as those used on actual samples.

   Documenting the effect of the matrix should include the analysis of at least one matrix spike and one duplicate unspiked sample or one matrix spike/matrix spike duplicate pair. The decision on whether to prepare and analyze duplicate samples or a matrix spike/matrix spike duplicate must be based on knowledge of the samples in the sample batch. If samples are expected to contain target analytes, the laboratories should use a matrix spike and matrix spike duplicate pair, spiked with a congener mixture. However, when specific congeners are known to be present or expected in samples, the specific congener should be used for spiking.

   A laboratory control sample (LCS) should be included with each analytical batch. The LCS consists of an aliquot of a clean (control) matrix similar to the sample matrix and of the same weight or volume. The LCS is spiked with the same analytes at the same concentrations as the matrix spike, when appropriate. When the results of the matrix spike analysis indicate a potential problem due to the sample matrix itself, the LCS results are used to verify that the laboratory can perform the analysis in a clean matrix.

7. Surrogate recoveries. If surrogates are used, the laboratory should evaluate surrogate recovery data from individual samples versus the surrogate control limits developed by the laboratory. Procedures for evaluating the recoveries of multiple surrogates and the associated corrective actions should be defined in an approved project plan.

8. It is recommended that the laboratory adopt additional quality assurance practices with this method. The specific practices depend upon the needs of the laboratory and the nature of the samples. Whenever possible, the laboratory should analyze standard reference materials and participate in relevant performance evaluation studies.

4.6.8 Calibration and Standardization

1. Prepare calibration standards using the procedures in section 4.6.5. Refer to section 4.6.7.2 for proper calibration techniques for both initial calibration and calibration verification. When PCBs are to be determined as congeners, the use of internal standard calibration is highly recommended. Therefore, the calibration standards must contain the internal standard (see section 4.6.5.4) at the same concentration as the sample extracts.

Note. *Because of the sensitivity of the electron capture detector, always clean the injection port and column prior to performing the initial calibration.*

2. When PCBs are to be quantitatively determined as congeners, an initial multi-point calibration must be performed that includes standards for all target analytes (congeners).

3. Establish the GC operating conditions appropriate for the configuration (single-column or dual column). Optimize the instrumental conditions for resolution of the target compounds and sensitivity. A final temperature of between 240 °C and 275 °C may be needed to elute decachlorobipheny. The use of injector pressure programming will improve the chromatography of late eluting peaks. Once established, the same operating conditions must be used for both calibrations and sample analyses.

4. A 2-µl injection of each calibration standard is recommended. Other injection volumes may be employed, provided that the analyst can demonstrate adequate sensitivity for the compounds of interest.

5. Record the peak area (or height) for each congener peak to be used for quantitation.

6. When determining PCB congeners by the internal standard procedure, calculate the response factor (RF) for each congener in the calibration standards relative to the internal standard, decachlorobiphenyl, using the equation that follows.

$$RF = \frac{A_s \times C_{is}}{A_{is} \times C_s}$$

where:

A_s = Peak area (or height) of the analyte or surrogate.

A_{is} = Peak area (or height) of the internal standard.

C_s = Concentration of the analyte or surrogate, in µg/l.

C_{is} = Concentration of the internal standard, in µg/l.

7. The response factors or calibration factors from the initial calibration are used to evaluate the linearity of the initial calibration, if a linear calibration model is used. This involves the calculation of the mean response or calibration factor, the standard deviation, and the relative standard deviation (RSD) for each congener peak.

8. Retention time windows. Absolute retention times are generally used for compound identification. When absolute retention times are used, retention time windows are crucial to the identification of target compounds, and must be established. Retention time windows are established to compensate for minor shifts in absolute retention times as a result of sample loadings and normal chromatographic variability. The width of the retention time window must be carefully established to minimize the occurrence of both false positive and false negative results. Tight retention time windows may result in false negatives and/or may cause unnecessary reanalysis of samples when surrogates or spiked compounds are erroneously not identified. Overly wide retention time windows may result in false positive results that cannot be confirmed upon further analysis. Other approaches to compound identification may be employed, provided that the analyst can demonstrate and document that the approaches are appropriate for the intended application. When PCBs are determined as congeners by an internal standard technique, absolute retention times may be used in conjunction with relative retention times (relative to the internal standard).

   When conducting congener analysis, it is important to determine that common single-component pesticides such as DDT, DDD, and DDE do not elute at the same retention times as the target congeners. Therefore, in conjunction with determining the retention time windows of the congeners, the analyst should analyze a standard containing the DDT analogs. This standard need only be analyzed when the retention time windows are determined. It is not considered part of the routine initial calibration or calibration verification steps in the method, nor is there any performance criteria associated with the analysis of this standard. If PCB congener analysis is performed and any of the DDT analogs elute at the same retention time as a PCB congener of interest, then the analyst must adjust the GC conditions to achieve better resolution.

4.6.9 Gas Chromatography Analasis of Sample Extracts

1. The same GC operating conditions used for the initial calibration must be employed for the analysis of samples.
2. Verify calibration at least once each 12-hr shift by injecting calibration verification standards prior to conducting any sample analyses. A calibration standard must also be injected at intervals of not less than once every twenty samples (after every 10 samples is recommended to minimize the number of samples requiring reinjection when QC limits are exceeded) and at the end of the analysis sequence.

   The calibration factor for each analyte calculated from the calibration verification standard (CF_v) should not exceed a difference of more than ±20 percent when compared to the mean calibration factor from the initial calibration curve.

$$\% \text{ Difference} = \frac{\overline{CF} - CF_v}{\overline{CF}} \times 100$$

   When internal standard calibration is used for PCB congeners, the response factor calculated from the calibration verification standard (RF_v) should not exceed a ±20 percent difference when compared to the mean response factor from the initial calibration.

$$\% \text{ Difference} = \frac{\overline{RF} - RF_v}{\overline{RF}} \times 100$$

If the calibration does not meet the ±20% limit on the basis of each compound, check the instrument operating conditions, and if necessary, restore them to the original settings, and inject another aliquot of the calibration verification standard. If the response for the analyte is still not within ±20%, then a new initial calibration must be prepared.

3. Inject a measured aliquot of the concentrated sample extract. A 2-µl aliquot is suggested, however, other injection volumes may be employed, provided that the analyst can demonstrate adequate sensitivity for the compounds of interest. The same injection volume should be used for both the calibration standards and the sample extracts, unless the analyst can demonstrate acceptable performance using different volumes or conditions. Record the volume injected and the resulting peak size in area units.

4. Qualitative identifications of target analytes are made by examination of the sample chromatograms, as described in section 4.6.10.1.

5. Quantitative results are determined for each identified analyte (congener), using the procedures described in sections 4.6.10 and 4.6.11 for either the internal or the external calibration procedure. If the responses in the sample chromatogram exceed the calibration range of the system, dilute the extract and reanalyze. Peak height measurements are recommended over peak area when overlapping peaks cause errors in area integration.

6. Each sample analysis employing external standard calibration must be bracketed with an acceptable initial calibration, calibration verification standard(s) (each 12-hr analytical shift), or calibration standards interspersed within the samples. The results from these bracketing standards must meet the calibration verification criteria discussed in #2 above.

   Multi-level standards (mixtures or multi-component analytes) are highly recommended to ensure that detector response remains stable for all analytes over the calibration range. When a calibration verification standard fails to meet the QC criteria, all samples that were injected after the last standard that met the QC criteria must be evaluated to prevent misquantitations and possible false negative results, and reinjection of the sample extracts may be required. More frequent analyses of standards will minimize the number of sample extracts that would have to be reinjected if the QC limits are violated for the standard analysis. However, if the standard analyzed after a group of samples exhibits a response for an analyte that is above the acceptance limit, *i.e*., >20%, and the analyte was not detected in the specific samples analyzed during the analytical shift, then the extracts for those samples do not need to be reanalyzed, since the verification standard has demonstrated that the analyte would have been detected if it were present. In contrast, if an analyte above the QC limits was detected in a sample extract, then reinjection is necessary to ensure accurate quantitation.

   If an analyte was not detected in the sample and the standard response is more than 20% below the initial calibration response, then reinjection is necessary. The purpose of this reinjection is to ensure that the analyte could be detected, if present, despite the change in the detector response, *e.g*., to protect against a false negative result.

7. Sample injections may continue for as long as the calibration verification standards and standards interspersed with the samples meet instrument QC requirements. It is recommended that standards be analyzed after every 10 samples (required after every 20 samples and at the end of a set) to minimize the number of samples that must be re-injected when the standards fail the QC limits. The sequence ends when the set of samples has been injected or when qualitative or quantitative QC criteria are exceeded.

8. The use of internal standard calibration techniques does not require that all sample results be bracketed with calibration verification standards. However, when internal standard calibration is used, the retention times of the internal standards and the area responses of the internal standards should be checked for each analysis. Retention time shifts of more than 30 sec. from the retention time of the most recent calibration standard and/or changes in internal standard areas of more than -50 to +100% are cause for concern and must be investigated.
9. If the peak response is less than 2.5 times the baseline noise level, the validity of the quantitative result may be questionable. The analyst should consult with the source of the sample to determine whether further concentration of the sample is warranted.
10. Use the calibration standards analyzed during the sequence to evaluate retention time stability. If any of the standards fall outside their daily retention time windows, the system is out of control. Determine the cause of the problem and correct it.
11. If compound identification or quantitation is precluded due to interferences (*e.g*., broad, rounded peaks or ill-defined baselines are present), corrective action is warranted. Cleanup of the extract or replacement of the capillary column or detector may be necessary. The analyst may begin by rerunning the sample on another instrument to determine if the problem results from analytical hardware or the sample matrix.

4.6.10 Qualitative Identification

1. The identification of PCBs as congeners using this method with an electron capture detector is based on agreement between the retention times of peaks in the sample chromatogram with the retention time windows established through the analysis of standards of the target analytes.

   Tentative identification of an analyte occurs when a peak from a sample extract falls within the established retention time window for a specific target analyte. Confirmation is necessary when the sample composition is not well characterized. See section 4.6.12.4 of this method for information on the use of GC/MS as a confirmation technique.

   When results are confirmed using a second GC column of dissimilar stationary phase, the analyst should check the agreement between the quantitative results on both columns once the identification has been confirmed.
2. When simultaneous analyses are performed from a single injection (the dual-column GC configuration described in section 4.6.6), it is not practical to designate one column as the analytical (primary) column and the other as the confirmation column. Since the calibration standards are analyzed on both columns, both columns must meet the calibration acceptance criteria. If the retention times of the peaks on both columns fall within the retention time windows on the respective columns, then the target analyte identification has been confirmed.
3. The results of a single column/single injection analysis may be confirmed on a second, dissimilar, GC column. In order to be used for confirmation, retention time windows must have been established for the second GC column, and the analyst must demonstrate the sensitivity of the second column analysis. This demonstration must include the analysis of a standard of the target analyte at a concentration at least as low as the concentration estimated from the primary analysis. That standard may be individual congeners.

4.6.11 Quantitative Identification

PCBs as Congeners

1. The quantitation of PCB congeners is accomplished by the comparison of the sample chromatogram to those of the PCB congener standards, using the internal standard technique. Calculate the concentration of each congener.
2. Depending on project requirements, the PCB congener results may be reported as congeners, or may be summed and reported as total PCBs.
3. The analytical procedures for these congeners may be appropriate for the analysis of other congeners not specifically included in this method and may be used as a template for the development of such a procedure. However, all PCB congeners cannot be separated using the GC columns and procedures described in this method. If this procedure is expanded to encompass other congeners, then the analyst must either document the resolution of the congeners in question or establish procedures for report.

4.6.12 Confirmation

1. Tentative identification of an analyte occurs when a peak from a sample extract falls within the daily retention time window. Confirmation is necessary when the sample composition is not well characterized. Confirmatory techniques such as gas chromatography with a dissimilar column or a mass spectrometer should be used.
2. When results are confirmed using a second GC column of dissimilar stationary phase, the analyst should check the agreement between the quantitative results on both columns once the identification has been confirmed.
3. When the dual-column approach is employed, the target phenols are identified and confirmed when they meet the identification criteria on both columns.
4. GC/MS confirmation. GC/MS confirmation may be used in conjunction with either single-or dual-column analysis if the concentration is sufficient for detection by GC/MS.

   Full-scan quadrupole GC/MS will normally require a higher concentration of the analyte of interest than full-scan ion trap or selected ion monitoring techniques. The concentrations will be instrument-dependent, but values for full-scan quadrupole GC/MS may be as high as10 ng/µl in the final extract, while ion trap or SIM may only be a concentration of 1 ng/µl.

   The GC/MS must be calibrated for the target analytes when it is used for quantitative analysis. If GC/MS is used only for confirmation of the identification of the target analytes, then the analyst must demonstrate that those PCBs identified by GC/ECD can be confirmed by GC/MS. This demonstration may be accomplished by analyzing a single-point standard containing the analytes of interest at or below the concentrations reported in the GC/ECD analysis.

   GC/MS confirmation should be accomplished by analyzing the same extract used for GC/ECD analysis and the extract of the associated blank.
5. GC/AED confirmation may be used in conjunction with either single-column or dual-column analysis if the concentration is sufficient for detection by GC/AED.

4.7 POLYNUCLEAR AROMATIC HYDROCARBONS (PAHS) IN SEDIMENTS ONLY

4.7.1 Scope and Application

1. This method may be used for both saltwater and freshwater sediments.
2. This method is used to determine the concentration of 25 semivolatile organic compounds (Table 4.10) in extracts prepared from sediment samples.

Table 4.10. Indicator List of Polynuclear Aromatic Hydrocarbons (PAHs)

Compound	Chemical Abstract Service (CAS) Registry No.
Acenaphthene	83-32-9
Acenaphthylene	208-96-8
Anthracene	120-12-7
Benz(a)anthracene	56-55-3
Benzo(b)fluoranthene	205-99-2
Benzo(k)fluoranthene	207-08-9
Benzo(g,h,i)perylene	191-24-2
Benzo(a)pyrene	50-32-8
Benzo(e)pyrene	192-97-2
Biphenyl	92-52-4
Chrysene	218-01-09
Dibenz(a,h)anthracene	53-70-3
Dibenzothiophene	132-65-0
2,6-dimethylnaphthalene	581-42-0
Fluoranthene	206-44-0
Fluorene	86-73-7
Indeno(1,2,3-c,d)pyrene	193-39-5
1-methylnaphthalene	90-12-9
2-methylnaphthalene	91-57-6
1-methylphenanthrene	832-69-9
Naphthalene	91-20-3
Phenanthrene	85-01-8
Perylene	77392-71-3
Pyrene	129-00-0
2,3,5-trimethylnaphthalene	2245-38-7

3. This method can be used to quantitate most neutral, acidic, and basic organic compounds that are soluble in methylene chloride (or other suitable solvents provided that the desired performance data can be generated) and capable of being eluted, without derivatization, as sharp peaks from a gas chromatographic fused-silica capillary column coated with a slightly polar silicone. Such compounds include polynuclear aromatic hydrocarbons.
4. In most cases, this method is not appropriate for the quantitation of multicomponent analytes, *e.g*., Toxaphene, Chlordane, Aroclor*, etc*., because of limited sensitivity for those analytes (see sections 4.5 and 4.6).
5. The lower limit of quantitation for this method when determining an individual compound is approximately 660 µg/kg (wet weight) for sediment samples. Lower limits of quantitation will be proportionately higher for sample extracts that require dilution to avoid saturation of the detector.

4.7.2 Summary of Method

The semivolatile compounds are introduced into the GC/MS by injecting the sample extract into a gas chromatograph (GC) equipped with a narrow-bore fused-silica capillary column. The GC column is temperature-programmed to separate the analytes, which are then detected with a mass spectrometer (MS) connected to the gas chromatograph. Analytes eluted from the capillary column are introduced into the mass spectrometer via a jet separator or a direct connection. Identification of target analytes is accomplished by comparing their mass spectra with the electron impact (or electron impact-like) spectra of authentic standards. Quantitation is accomplished by comparing the response of a major (quantitation) ion relative to an internal standard using an appropriate calibration curve for the intended application.

4.7.3 Interferences

1. Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis by analyzing method blanks. Specific selection of reagents and purification of solvents by distillation in all-glass systems may be necessary.
2. Raw GC/MS data from all blanks, samples, and spikes must be evaluated for interferences. Determine if the source of interference is in the preparation and/or cleanup of the samples and take corrective action to eliminate the problem.
3. Contamination by carryover can occur whenever high-concentration and low-concentration samples are sequentially analyzed. To reduce carryover, the sample syringe must be rinsed with solvent between sample injections. Whenever an unusually concentrated sample is encountered, it should be followed by the analysis of solvent to check for cross contamination.

4.7.4 Equipment and Supplies

1. Gas chromatograph
   An analytical system equipped with a temperature-programmable gas chromatograph suitable for splitless injection and all required accessories, including syringes, analytical columns, and gases. The capillary column should be directly coupled to the source.
2. Column
   30-m x 0.25-mm ID (or 0.32-mm ID) 0.25, 0.5, or 1-µm film thickness silicone-coated fused-silica capillary column. The columns listed in this section were the columns used in developing the method. The listing of these columns in this method is not intended to exclude the use of other columns that may be developed. Laboratories may use these columns or other capillary columns provided that the laboratories document method performance data (*e.g.*, chromatographic resolution, analyte breakdown, and sensitivity) that are appropriate for the intended application.
3. Mass spectrometer
   Capable of scanning from 35 to 500 amu every 1 sec or less, using 70 volts (nominal) electron energy in the electron impact ionization mode. The mass spectrometer must be capable of producing a mass spectrum for decafluorotriphenylphosphine (DFTPP) which meets the criteria as outlined in section 4.7.10.1.

An ion trap mass spectrometer may be used if it is capable of axial modulation to reduce ion-molecule reactions and can produce electron impact-like spectra that match those in the EPA/NIST Library. The mass spectrometer must be capable of producing a mass spectrum for DFTPP which meets the criteria alos outlined in section 4.7.10.1

4. GC/MS interface
   Any GC-to-MS interface may be used that gives acceptable calibration points for each compound of interest and achieves acceptable tuning performance criteria. For a narrow-bore capillary column, the interface is usually capillary-direct into the mass spectrometer source.

5. Data system
   A computer system should be interfaced to the mass spectrometer. The system must allow the continuous acquisition and storage on machine-readable media of all mass spectra obtained throughout the duration of the chromatographic program. The computer should have software that can search any GC/MS data file for ions of a specific mass and that can plot such ion abundances versus time or scan number. This type of plot is defined as an Extracted Ion Current Profile (EICP). Software should also be available that allows integrating the abundances in any EICP between specified time or scan-number limits. The most recent version of the EPA/NIST Mass Spectral Library should also be available.

6. Guard column (optional) -- (deactivated fused-silica, 0.25-mm ID x 6-m) between the injection port and the analytical column joined with column connectors

7. Syringe. 10-µL.

8. Volumetric flasks, Class A -- Appropriate sizes equipped with ground-glass stoppers.

9. Balance. Analytical, capable of weighing 0.0001 g.

10. Bottles -- Glass with polytetrafluoroethylene (PTFE)-lined screw caps or crimp tops.

4.7.5 Reagents and Standards

1. Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Reagents should be stored in glass to prevent the leaching of contaminants from plastic containers.

2. Organic-free reagent water

3. Solvents. Acetone, hexane, methylene chloride, isooctane, carbon disulfide, toluene, and other appropriate solvents. All solvents should be pesticide quality or equivalent. Solvents should be degassed prior to use.

4. Stock standard solutions (1000 mg/L)
   Standard solutions may be prepared from pure standard materials or purchased. Commercially-prepared stock standards may be used at any concentration if they are certified by the manufacturer or by an independent source.

   Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in pesticide quality acetone or other suitable solvent and dilute to volume in a 10-mL volumetric flask. Larger volumes can be used at the convenience of the

analyst. When compound purity is assayed to be 96% or greater, the weight may be used without correction to calculate the concentration of the stock standard.

Transfer the stock standard solutions into bottles equipped with PTFE lined screw-caps. Store, protected from light, at ≤6 °C or as recommended by the standard manufacturer. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them.

Stock standard solutions must be replaced after 1 year or sooner if comparison with quality control check samples indicates a problem.

It is recommended that nitrosamine compounds be placed together in a separate calibration mix and not combined with other calibration mixes. When using a premixed certified standard, consult the manufacturer's instructions for additional guidance.

Mixes with hydrochloride salts may contain hydrochloric acid, which can cause analytical difficulties. When using a premixed certified standard, consult the manufacturer's instructions for additional guidance.

5. Internal standard solutions
The internal standards recommended are 1,4-dichlorobenzene-d_4, naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}. Other compounds may be used as internal standards as long as the criteria in section 4.7.10.1.2 are met.

Dissolve 0.200 g of each compound with a small volume of carbon disulfide. Transfer to a 50-mL volumetric flask and dilute to volume with methylene chloride so that the final solvent is approximately 20% carbon disulfide. Most of the compounds are also soluble in small volumes of methanol, acetone, or toluene, except for perylene-d_{12}. The resulting solution will contain each standard at a concentration of 4,000 ng/µL. Each 1-mL sample extract undergoing analysis should be spiked with 10 µL of the internal standard solution, resulting in a concentration of 40 ng/µL of each internal standard. Store away from any light source at ≤6°C when not in use (-10°C is recommended). When using premixed certified solutions, store according to the manufacturer's documented holding time and storage temperature recommendations.

If a more sensitive mass spectrometer is employed to achieve lower quantitation levels, a more dilute internal standard solution may be required. Area counts of the internal standard peaks should be between 50-200% of the area of the target analytes in the mid-point calibration analysis.

6. GC/MS tuning standard
A methylene chloride solution containing 50 ng/µL of decafluorotriphenylphosphine (DFTPP) should be prepared. The standard should also contain 50 ng/µL each of 4,4'-DDT, pentachlorophenol, and benzidine to verify injection port inertness and GC column performance. Alternate concentrations may be used to compensate for different injection volumes if the total amount injected is 50 ng or less. Store away from any light source at ≤6°C when not in use (-10°C is recommended). If a more sensitive mass spectrometer is employed to achieve lower quantitation levels, a more dilute tuning solution may be necessary. When using premixed certified solutions, store according to the manufacturer's documented holding time and storage temperature recommendations.

7. Calibration standards
A minimum of five calibration standards should be prepared at different concentrations. At least one of the calibration standards should correspond to a sample concentration at or below that necessary to meet the data quality objectives of the project. The remaining

standards should correspond to the range of concentrations found in actual samples but should not exceed the working range of the GC/MS system. Each standard and/or series of calibration standards prepared at a given concentration should contain all the desired project-specific target analytes for which quantitation and quantitative results are to be reported by this method.

It is the intent of EPA that all target analytes for a particular analysis be included in the calibration standard(s). The laboratory shall not report a quantitative result for a target analyte that was not included in the calibration standard(s).

Each 1-mL aliquot of calibration standard should be spiked with 10 µL of the internal standard solution prior to analysis. All standards should be stored away from any light source at ≤6 °C when not in use (-10 °C is recommended), and should be freshly prepared once a year, or sooner if check standards indicate a problem. The calibration verification standard should be prepared, as necessary, and stored at ≤6 °C. When using premixed certified solutions, store according to the manufacturer's documented holding time and storage temperature recommendations.

8. Surrogate standards
The recommended surrogates are phenol-d_6, 2-fluorophenol, 2,4,6-tribromophenol, nitrobenzene-d_5, 2-fluorobiphenyl, and p-terphenyl-d_{14}.

NOTE: In the presence of samples containing residual chlorine, phenol-d_6 has been known to react to form chlorinated phenolic compounds that are not detected as the original spiked surrogate. Sample preservation precautions should be used when residual chlorine is known to be present in order to minimize degradation of deuterated phenols or any other susceptible target analyte.

Surrogate standard check. Determine what the appropriate concentration should be for the blank extracts after all extraction, cleanup, and concentration steps. Inject the concentration into the GC/MS to determine recovery of surrogate standards. It is recommended that this check be done whenever a new surrogate spiking solution is prepared.

If a more sensitive mass spectrometer is employed to achieve lower quantitation levels, a more dilute surrogate solution may be necessary.

9. Matrix spike and laboratory control standards
The same standard may be used as the laboratory control standard (LCS) and the spiking solution should be the same source as used for the initial calibration standards to restrict the influence of standard accuracy on the determination of recovery through preparation and analysis.

Matrix spike check. Determine what concentration should be in the blank extracts after all extraction, cleanup, and concentration steps. Inject this concentration into the GC/MS to determine recovery. It is recommended that this check be done whenever a new matrix spiking solution is prepared.

If a more sensitive mass spectrometer is employed to achieve lower quantitation levels, a more dilute matrix and LCS spiking solution may be necessary.

4.7.6 Quality Control

1. Refer to the QAPP for guidance on quality assurance and quality control protocols. When inconsistencies exist between QC guidelines, method-specific QC criteria take precedence over both technique-specific criteria and those criteria given the QAPP, and technique-

specific QC criteria take precedence over the criteria in the QAPP. Any effort involving the collection of analytical data should include development of a structured and systematic planning document, such as a Sampling and Analysis Plan (SAP), which translates project objectives and specifications into directions for those that will implement the project and assess the results. Each lab should maintain a formal quality assurance program. Each lab must work with the Information Management group (Marlys Cappaert, 541-754-4467 Cappaert.Marlys@epamail.epa.gov) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit. The laboratory should also maintain records to document the quality of the data generated. All data sheets and quality control data should be maintained for reference or inspection.

2. Quality control procedures necessary to evaluate the GC system operation include evaluation of retention time windows, calibration verification and chromatographic analysis of samples. In addition, discussions regarding the instrument QC requirements listed below can be found in the referenced sections of this method,

   The GC/MS must be tuned to meet the recommended DFTPP criteria prior to the initial calibration and for each 12-hr period during which analyses are performed.

   There must be an initial calibration of the GC/MS system as described in section 4.7.10.1. In addition, the initial calibration curve should be verified immediately after performing the standard analyses using a second source standard (prepared using standards different from the calibration standards). The suggested acceptance limits for this initial calibration verification analysis are 70 - 130%. Alternative acceptance limits may be appropriate based on the desired project-specific data quality objectives. Quantitative sample analyses should not proceed for those analytes that fail the second source standard initial calibration verification. However, analyses may continue for those analytes that fail the criteria with an understanding these results could be used for screening purposes and would be considered estimated values.

   The GC/MS system must meet the calibration verification acceptance criteria in section 4.7.10.2 each 12 hrs. The RRT of the sample component must fall within the RRT window of the standard component provided in section 4.7.11.2.

3. Initial demonstration of proficiency. Each laboratory must demonstrate initial proficiency with each sample preparation and determinative method combination it utilizes by generating data of acceptable accuracy and precision for target analytes in a clean matrix. The laboratory must also repeat the following operations whenever new staff members are trained or significant changes in instrumentation are made.

4. Before processing samples, the analyst must demonstrate that all parts of the equipment in contact with the sample and reagents are interference-free. This is accomplished through the analysis of a method blank. Each time samples are extracted, cleaned up, and analyzed, a method blank must be prepared and analyzed for the compounds of interest as a safeguard against chronic laboratory contamination. If a peak is observed within the retention time window of any analyte that would prevent the determination of that analyte, determine the source and eliminate it, if possible, before processing the samples. The blanks should be carried through all stages of sample preparation and analysis. When new reagents or chemicals are received, the lab should monitor the preparation and/or analysis blanks associated with samples for any signs of contamination. It is not necessary to test every new batch of reagents or chemicals prior to sample preparation if the source shows no prior problems. However, if reagents are changed during a preparation batch, separate blanks need to be prepared for each set of reagents.

5. The laboratory must also have procedures for documenting the effect of the matrix on method performance (precision, accuracy, method sensitivity). At a minimum, this should include the analysis of QC samples including a method blank, a matrix spike, a duplicate, and a laboratory control sample (LCS) in each analytical batch and the addition of surrogates to each field sample and QC sample when surrogates are used. Any method blanks, matrix spike samples, and replicate samples should be subjected to the same analytical procedures as those used on actual samples.

   Documenting the effect of the matrix should include the analysis of at least one matrix spike and one duplicate unspiked sample or one matrix spike/matrix spike duplicate pair. The decision on whether to prepare and analyze duplicate samples or a matrix spike/matrix spike duplicate must be based on knowledge of the samples in the sample batch. If samples are expected to contain target analytes, laboratories may use a matrix spike and a duplicate analysis of an unspiked field sample. If samples are not expected to contain target analytes, then laboratories should use a matrix spike and matrix spike duplicate pair. Consult Method 8000 for information on developing acceptance criteria for the MS/MSD.

   A laboratory control sample (LCS) should be included with each analytical batch. The LCS consists of an aliquot of a clean (control) matrix similar to the sample matrix and of the same weight or volume. The LCS is spiked with the same analytes at the same concentrations as the matrix spike, when appropriate. When the results of the matrix spike analysis indicate a potential problem due to the sample matrix itself, the LCS results are used to verify that the laboratory can perform the analysis in a clean matrix.

6. Surrogate recoveries. If surrogates are used, the laboratory should evaluate surrogate recovery data from individual samples versus the surrogate control limits developed by the laboratory. Procedures for evaluating the recoveries of multiple surrogates and the associated corrective actions should be defined in an approved project plan.

4.7.7 Calibration and Standardization

4.7.7.1 Initial Calibration

1. Establish the GC/MS operating conditions as follows:

Mass range:	35-500 amu
Scan time:	≤1 sec/scan
Initial temperature:	40 °C, hold for 4 min
Temperature program:	40-320 °C at 10 °C/min
Final temperature:	320 °C, hold until 2 min after benzo[g,h,i]perylene elutes
Injector temperature:	250-300 °C
Transfer line temperature:	250-300 °C
Source temperature:	According to manufacturer's specifications
Injector:	Grob-type, splitless
Injection volume:	1-2 µL
Carrier gas:	Hydrogen at 50 cm/sec or helium at 30 cm/sec

   Set axial modulation, manifold temperature, and emission current to manufacturer's recommendations

Split injection is allowed if the sensitivity of the mass spectrometer is sufficient.

2. The GC/MS system must be hardware-tuned such that injecting 50 ng or less of DFTPP meets the manufacturer's specified acceptance criteria. The tuning criteria may depend on the type of instrumentation, *e.g.*, Time-of-Flight, Ion Trap, etc. In these cases it would be appropriate to follow the manufacturer's tuning instructions or some other consistent tuning criteria. However, no matter which tuning criteria is selected, the system calibration must not begin until the tuning acceptance criteria are met with the sample analyses performed under the same conditions as the calibration standards.

   In the absence of specific recommendations on how to acquire the mass spectrum of DFTPP from the instrument manufacturer, the following approach should be used: Three scans (the peak apex scan and the scans immediately preceding and following the apex) are acquired and averaged. Background subtraction is required, and must be accomplished using a single scan acquired within 20 scans of the elution of DFTPP. The background subtraction should be designed only to eliminate column bleed or instrument background ions. Do not subtract part of the DFTPP peak or any other discrete peak that does not coelute with DFTPP.

   Use the DFTPP mass intensity criteria in the manufacturer's instructions as primary tuning acceptance criteria. Alternatively, other documented tuning criteria may be used provided that method performance is not adversely affected. The analyst is always free to choose criteria that are tighter than those included in this method or to use other documented criteria provided they are used consistently throughout the initial calibration, calibration verification, and sample analyses.

Note. *All subsequent standards, samples, MS/MSDs, and blanks associated with a DFTPP analysis must use the identical mass spectrometer instrument conditions.*

   The GC/MS tuning standard solution should also be used to assess GC column performance and injection port inertness. Degradation of DDT to DDE and DDD should not exceed 20%.

   If degradation is excessive and/or poor chromatography is noted, the injection port may require cleaning. It may also be necessary to break off the first 6 to12 in. of the capillary column. The use of a guard column (section 4.7.4.6) between the injection port and the analytical column may help prolong analytical column performance life.

3. The internal standards selected should permit most of the components of interest in a chromatogram to have retention times of 0.80-1.20 relative to one of the internal standards. Use the base peak ion from the specific internal standard as the primary ion for quantitation. If interferences are noted, use the next most intense ion as the quantitation ion (*e.g.*, for 1,4-dichlorobenzene-d_4, use m/z 150 for quantitation).

4. Analyze 1-2 µL of each calibration standard (containing the compounds for quantitation and the appropriate surrogates and internal standards) and tabulate the area of the primary ion against concentration for each target analyte. A set of at least five calibration standards is necessary (see section 4.7.5.7). Alternate injection volumes may be used if the applicable quality control requirements for using this method are met. The injection volume must be the same for all standards and sample extracts.

5. Initial calibration calculations

6. Calculate response factors (RFs) for each target analyte relative to one of the internal standards as follows:

$$RF = \frac{A_s \times C_{is}}{A_{is} \times C_s}$$

where,

A_s = Peak area (or height) of the analyte or surrogate.

A_{is} = Peak area (or height) of the internal standard.

C_s = Concentration of the analyte or surrogate, in µg/L.

C_{is} = Concentration of the internal standard, in µg/L.

Calculate the mean response factor and the relative standard deviation (RSD) of the response factors for each target analyte using the following equations. The RSD must be ≤20% for each target analyte. It is recommended that a minimum response factor for the most common target analytes be demonstrated for each individual calibration level to ensure that these compounds are behaving as expected. In addition, meeting minimum response factor criteria for the lowest calibration standard is critical in establishing and demonstrating the desired sensitivity. Due to the large number of compounds that may be analyzed by this method, some compounds will fail to meet these criteria. For these occasions, it is acknowledged that the failing compounds may not be critical to the specific project and therefore they may be used as qualified data or estimated values for screening purposes.

$$\text{mean RF} = \overline{RF} = \frac{\sum_{i=1}^{n} RFi}{n}$$

$$SD = \sqrt{\frac{\sum_{i=1}^{n}\left(RFi - \overline{RF}\right)^2}{n-1}} \qquad RSD = \frac{SD}{\overline{RF}} \times 100$$

Where:

RFi = RF for each of the calibration standards

$\overline{RF}$ = mean RF for each compound from the initial calibration

n = number of calibration standards, *e.g.,* 5

If more than 10% of the compounds included with the initial calibration exceed the 20% RSD limit and do not meet the minimum correlation coefficient (0.99) for alternate curve fits, then the chromatographic system is considered too reactive for analysis to begin. Clean or replace the injector liner and/or capillary column, then repeat the calibration procedure.

7. Evaluation of retention times. The relative retention time (RRT) of each target analyte in each calibration standard should agree within 0.06 RRT units. Late-eluting target analytes usually have much better agreement.

$$RRT = \frac{\text{Retention time of the anlaysis}}{\text{Retention time of the internal standard}}$$

8. Linearity of target analytes. If the RSD of any target analyte is 20% or less, then the relative response factor is assumed to be constant over the calibration range, and the average relative response factor may be used for quantitation (section 4.7.12.2).

If the RSD of any target analyte is greater than 20%. One of the options must be applied to GC/MS calibration in this situation, or a new initial calibration must be performed. The average RF should not be used for compounds that have an RSD greater than 20% unless the concentration is reported as estimated.

When the RSD exceeds 20%, the plotting and visual inspection of a calibration curve can be a useful diagnostic tool. The inspection may indicate analytical problems, including errors in standard preparation, the presence of active sites in the chromatographic system, analytes that exhibit poor chromatographic behavior, *etc*.

Due to the large number of compounds that may be analyzed by this method, some compounds may fail to meet either the 20% RSD, minimum correlation coefficient criteria (0.99). Any calibration method but it should be used consistently. It is considered inappropriate once the calibration analyses are completed to select an alternative calibration procedure in order to pass the recommended criteria on a case-by-case basis. If compounds fail to meet these criteria, the associated concentrations may still be determined but they must be reported as estimated. In order to report nondetects, it must be demonstrated that there is adequate sensitivity to detect the failed compounds at the applicable lower quantitation limit.

4.7.7.2 GC/MS Calibration Verification

1. Prior to the analysis of samples or calibration standards, inject 50 ng or less of the DFTPP standard into the GC/MS system. The resultant mass spectrum for DFTPP must meet the criteria as outlined in section 4.7.10.1 before sample analysis begins. These criteria must be demonstrated each 12-hr shift during which samples are analyzed.
2. The initial calibration function for each target analyte should be checked immediately after the first occurrence in the region of the middle of the calibration range with a standard from a source different from that used for the initial calibration. The value determined from the second source check should be within 30% of the expected concentration. An alternative recovery limit may be appropriate based on the desired project-specific data quality objectives. Quantitative sample analyses should not proceed for those analytes that fail the second source standard initial calibration verification. However, analyses may continue for those analytes that fail the criteria with an understanding these results could be used for screening purposes and would be considered estimated values.
3. The initial calibration for each compound of interest should be verified once every 12 hrs prior to sample analysis, using the introduction technique and conditions used for samples. This is accomplished by analyzing a calibration standard (containing all the compounds for quantitation) at a concentration either near the midpoint concentration for the calibrating range of the GC/MS or near the action level for the project. The results must be compared against the most recent initial calibration curve and should meet the verification acceptance criteria below.

***Note**. The DFTPP and calibration verification standard may be combined into a single standard as long as both tuning and calibration verification acceptance criteria for the project can be met without interferences.*

4. A method blank is analyzed prior to sample analyses in order to ensure that the total system (introduction device, transfer lines and GC/MS system) is contaminant-free. If the method blank indicates contamination, then it may be appropriate to analyze a solvent blank to demonstrate that the contamination is not a result of carryover from standards or samples.

5. Calibration verification standard criteria. Each of the most common target analytes in the calibration verification standard should meet the minimum response factors. This criterion is particularly important when the common target analytes are also critical project-required compounds. This is the same check that is applied during the initial calibration.

   If the minimum response factors are not met, the system should be evaluated, and corrective action should be taken before sample analysis begins. Possible problems include standard mixture degradation, injection port inlet contamination, contamination at the front end of the analytical column, and active sites in the column or chromatographic system.

   All target compounds of interest must be evaluated using a 20% criterion. Use percent difference when performing the average response factor model calibration. Use percent drift when calibrating using a regression fit model. If the percent difference or percent drift for a compound is less than or equal to 20%, then the initial calibration for that compound is assumed to be valid. Due to the large numbers of compounds that may be analyzed by this method, it is expected that some compounds will fail to meet the criterion. If the criterion is not met (*i.e*., greater than 20% difference or drift) for more than 20% of the compounds included in the initial calibration, then corrective action must be taken prior to the analysis of samples. In cases where compounds fail, they may still be reported as non-detects if it can be demonstrated that there was adequate sensitivity to detect the compound at the applicable quantitation limit. For situations when the failed compound is present, the concentrations must be reported as estimated values.

   Problems similar to those listed under initial calibration could affect the ability to pass the calibration verification standard analysis. If the problem cannot be corrected by other measures, a new initial calibration must be generated. The calibration verification criteria must be met before sample analysis begins.

   The method of linear regression analysis has the potential for a significant bias to the lower portion of a calibration curve, while the relative percent difference and quadratic methods of calibration do not have this potential bias. When calculating the calibration curves using the linear regression model, a minimum quantitation check on the viability of the lowest calibration point should be performed by re-fitting the response from the low concentration calibration standard back into the curve. It is not necessary to re-analyze a low concentration standard, rather the data system can recalculate the concentrations as if it were an unknown sample. The recalculated concentration of the low calibration point should be within ± 30% of the standard's true concentration. Other recovery criteria may be applicable depending on the project's data quality objectives and for those situations the minimum quantitation check criteria should be outlined in a laboratory standard operating procedure, or a project-specific Quality Assurance Project Plan. Analytes which do not meet the minimum quantitation calibration re-fitting criteria should not be considered and corrective action such as redefining the lower limit of quantitation and/or reporting those specific target analytes as estimated when the concentration is at or near the lowest calibration point may be appropriate.

6. Internal standard retention time. The retention times of the internal standards in the calibration verification standard must be evaluated immediately after or during data acquisition. If the retention time for any internal standard changes by more than 30 sec from that in the mid-point standard level of the most recent initial calibration sequence, then the chromatographic system must be inspected for malfunctions and corrections must be made, as required. When corrections are made, reanalysis of samples analyzed while the system was malfunctioning is required.

7. Internal standard response. If the EICP area for any of the internal standards in the calibration verification standard changes by a factor of two (-50% to +100%) from that in the mid-point standard level of the most recent initial calibration sequence, the mass spectrometer must be inspected for malfunctions and corrections must be made, as appropriate. When corrections are made, reanalysis of samples analyzed while the system was malfunctioning is required.

4.7.8 Procedures

1. Samples are prepared using ultrasonic extraction method described in section 4.4.
2. Cleanup procedures may not be necessary for a relatively clean sample matrix, but most extracts from environmental and waste samples will require additional preparation before analysis. The specific cleanup procedure used will depend on the nature of the sample to be analyzed and the data quality objectives for the measurements.

4.7.8.1 GC/MS Analysis of Samples

1. It is highly recommended that sample extracts be screened on a GC/FID or GC/PID using the same type of capillary column used in the GC/MS system. This will minimize contamination of the GC/MS system from unexpectedly high concentrations of organic compounds.
2. Allow the sample extract to warm to room temperature. Just prior to analysis, add 10 µL of the internal standard solution to the 1 mL of concentrated sample extract obtained from sample preparation.
3. Inject an aliquot of the sample extract into the GC/MS system, using the same operating conditions that were used for the calibration. The volume to be injected should include an appropriate concentration that is within the calibration range of base/neutral and acid surrogates using the surrogate solution as noted in section 4.7.5.8. The injection volume must be the same volume that was used for the calibration standards.
4. If the response for any quantitation ion exceeds the initial calibration range of the GC/MS system, the sample extract must be diluted and reanalyzed. Additional internal standard solution must be added to the diluted extract to maintain the same concentration as in the calibration standards (usually 40 ng/µL, or other concentrations as appropriate, if a more sensitive GC/MS system is being used). Secondary ion quantitation should be used only when there are sample interferences with the primary ion.

   Monitor internal standard retention times in all samples, spikes, blanks, and standards to effectively check drifting, method performance, poor injection execution, and anticipate the need for system inspection and/or maintenance. Internal standard responses (area counts) must be monitored in all samples, spikes, blanks for similar reasons. If the EICP area for any of the internal standards in samples, spikes and blanks changes by a factor of two (-50% to +100%) from the areas determined in the continuing calibration analyzed that day, corrective action must be taken. The samples, spikes or blanks should be reanalyzed or the data should be qualified.

   When ions from a compound in the sample saturate the detector, this analysis should be followed by the analysis of an instrument blank consisting of clean solvent. If the blank analysis is not free of interferences, then the system must be decontaminated. Sample analysis may not resume until the blank analysis is demonstrated to be free of interferences.

Contamination from one sample to the next on the instrument usually takes place in the syringe. If adequate syringe washes are employed, then carryover from high concentration samples can usually be avoided.

All dilutions should keep the response of the major constituents (previously saturated peaks) in the upper half of the linear range of the curve.

5. The use of selected ion monitoring (SIM) is acceptable for applications requiring quantitation limits below the normal range of electron impact mass spectrometry. However, SIM may provide a lesser degree of confidence in the compound identification, since less mass spectral information is available. Using the primary ion for quantitation and the secondary ions for confirmation set up the collection groups based on their retention times. The selected ions are nominal ions and most compounds have small mass defect, usually less than 0.2 amu, in their spectra. These mass defects should be used in the acquisition table. The dwell time may be automatically calculated by the laboratory's GC/MS software or manually calculated using the following formula. The total scan time should be less than 1,000 msec and produce at least 5 to 10 scans per chromatographic peak. The start and stop times for the SIM groups are determined from the full scan analysis using the formula below:

$$\text{Dwell Time for the Group} = \frac{\text{Scan Time (msec.)}}{\text{Total Ions in the Group}}$$

4.7.8.2 Analyte identification

The qualitative identification of compounds determined by this method is based on retention time and on comparison of the sample mass spectrum, after background correction, with characteristic ions in a reference mass spectrum. The reference mass spectrum must be generated by the laboratory using the conditions of this method. The characteristic ions from the reference mass spectrum are defined as the three ions of greatest relative intensity, or any ions over 30% relative intensity, if less than three such ions occur in the reference spectrum. Compounds are identified when the following six criteria are met.

1. The intensities of the characteristic ions of a compound must maximize in the same scan or within one scan of each other. Selection of a peak by a data system target compound search routine where the search is based on the presence of a target chromatographic peak containing ions specific for the target compound at a compound-specific retention time will be accepted as meeting this criterion.
2. The RRT of the sample component is within ± 0.06 RRT units of the RRT of the standard component.
3. The relative intensities of the characteristic ions agree within 30% of the relative intensities of these ions in the reference spectrum. (Example: For an ion with an abundance of 50% in the reference spectrum, the corresponding abundance in a sample spectrum can range between 20 and 80%.) Use professional judgement in interpretation where interferences are observed.
4. Structural isomers that produce very similar mass spectra should be identified as individual isomers if they have sufficiently different GC retention times. Sufficient GC resolution is achieved if the height of the valley between two isomer peaks is <50% of the average of the two peak heights. Otherwise, structural isomers are identified as isomeric pairs. The resolution should be verified on the mid-point concentration of the initial calibration as well

as the laboratory designated continuing calibration verification level if closely eluting isomers are to be reported (e.g., benzo(b)fluoranthene and benzo(k)fluoranthene).

5. Identification is hampered when sample components are not resolved chromatographically and produce mass spectra containing ions contributed by more than one analyte. When gas chromatographic peaks obviously represent more than one sample component *(i.e*., a broadened peak with shoulder(s) or a valley between two or more maxima), appropriate selection of analyte spectra and background spectra is important.
6. Examination of extracted ion current profiles of appropriate ions can aid in the selection of spectra and in qualitative identification of compounds. When analytes coelute (*i.e*., only one chromatographic peak is apparent), the identification criteria may be met, but each analyte spectrum will contain extraneous ions contributed by the coeluting compound.

4.7.9 Quantitation

1. Once a target compound has been identified, the quantitation of that compound will be based on the integrated abundance of the primary characteristic ion from the EICP. It is highly recommended to use the integration produced by the software if the integration is correct because the software should produce more consistent integrations. However, manual integrations may be necessary when the software does not produce proper integrations because baseline selection is improper; the correct peak is missed; a coelution is integrated; the peak is partially integrated; etc. The analyst is responsible for ensuring that the integration is correct whether performed by the software or done manually.

   Manual integrations should not be substituted for proper maintenance of the instrument or setup of the method (*e.g*. retention time updates, integration parameter files, *etc*). The analyst should seek to minimize manual integration by properly maintaining the instrument, updating retention times, and configuring peak integration parameters.
2. If the RSD of a compound's response factor is 20% or less, the concentration in the extract may be determined using the average response factor ($\overline{RF}$) from initial calibration data.
3. Where applicable, the concentration of any non-target analytes identified in the sample should be estimated. The same formula as in section 4.8.10.1.5 should be used with the following modifications: The areas Ax and Ais should be from the total ion chromatograms, and the RF for the compound should be assumed to be 1.
4. The resulting concentration should be reported indicating that the value is an estimate. Use the nearest internal standard free of interferences.
5. Structural isomers that produce very similar mass spectra must be quantitated as individual isomers if they have sufficiently different GC retention times. Sufficient GC resolution is achieved if the height of the valley between two isomer peaks is <50% of the average of the two peak heights. Otherwise, structural isomers are identified as isomeric pairs. The resolution should be verified on the mid-point concentration of the initial calibration as well as the laboratory designated continuing calibration verification level if closely eluting isomers are to be reported (*e.g*., benzo(b)fluoranthene and benzo(k)fluoranthene).

5.0 SEDIMENTS

5.1 SEDIMENT GRAIN SIZE AND CHARACTERIZATION

Suggested analytical methods for sediment grain size are described in section 5.1. However, some laboratories participating in the survey may choose to employ other analytical methods. Labs engaged by EPA or the State may use a different analytical method as long as the lab is able to achieve the same performance requirements as the standard methods. Performance data must be submitted to EPA prior to initiating any analyses. Methods performance requirements for this program identify detection limit, precision and accuracy objectives for each indicator. Method performance requirements for sediment grain size are shown in Table 5.1

Table 5.1. Laboratory method performance requirements for sediment grain size.

Indicator/Data Type	Maximum Allowable Accuracy (Bias) Goal	Maximum Allowable Precision Goal	Completeness Goal
Particle Size	NA	10%	95%

5.1.1 Scope of Application

Silts and Clays are those particles that pass through a 63 µm mesh sieve. Materials retained on the sieve are primarily sands (63 µm – 2 mm) and occasionally small amounts of gravel (2 mm - 64 mm). This method is suitable for both saltwater and freshwater sediments.

5.1.2 Sample Storage and Equipment

Sediment samples must be chilled at 4-5°C prior to processing. Samples must not be allowed to dry before grain size analyses are conducted. Sieves used to determine sediment grain size will not be used for other purposes (*e.g*., benthic sorting). All wet sieving procedures are to be carried out using stainless steel screens. Fine screens (63 µm mesh) will be cleaned with copious amounts of water to prevent clogging of mesh openings. Screens must not be cleaned with brushes, which may distort openings. Sediments will not be forced through screens.

An analytical balance accurate to 0.1 mg will be used for all weighing. Prior to each use, the balance will be zeroed, and its calibration will be checked using a standard weight. The same standard weight (each standard is numbered) will be used for all weight measurements for a particular batch of samples.

5.1.3 Procedures for Silt-Clay Content Determination

5.1.3.1 Sediment Preparation

1. Sediment samples will be retrieved from cold storage and brought to room temperature. Sample numbers will be recorded on a silt-clay analysis data sheet.
2. Sediments will be removed from storage bags, placed in a clean 250 ml glass beaker and homogenized by stirring the sediment with a small spatula with a small amount of deionized water added for lubrication (if necessary) for at least three minutes. After stirring, rinse sediment from the spatula back into beaker using deionized water.
3. The amount of sediment to be processed depends upon sediment type. With more sample, the grains interfere with each other too much during settling and may flocculate; with too little sample, the experimental error in weighing becomes large with respect to sample size.

4. For sandy sediments, ~45-50 g wet weight are removed from the 250 ml glass beaker and placed in a clean 100 ml glass beaker for wet sieving. For muddy sediments, ~20-25 g wet weight are removed from the 250 ml glass beaker to a 100 ml glass beaker for wet sieving.
5. The remaining unused sediment will be returned to the original storage bag and held in cold storage until all QA/QC checks for this sample have been passed.

5.1.3.2 Dispersion of Sediment Clay Fraction and Wet Sieving the Sample

1. Make-up a 5 g/L stock solution of dispersant. Add 5 grams of sodium exametaphosphate "Calgon"to 1 liter of deionized water.
2. Add 20 ml of dispersant solution (100 mg hexametaphosphate) and 30 ml distilled water to the sample. Stir, using a magnetic stirrer for 1 to 5 mins to break-up sediment aggregrates.
3. After stirring, the sample will be wet sieved through a 63 µm mesh sieve into a large evaporation dish and wash all fines into the sieve using as little distilled water as possible.
4. The volume of sediment + water in the evaporation dish must be < 900 ml to allow for rinsing the sample into a 1000 ml graduated cylinder.

5.1.3.3 Treatment of the Silt and Clay Fraction (particles < 63 µm)

1. Carefully transfer mud in evaporation dish to a 1000 ml graduated cylinder. Rinse the mud (generally medium-coarse silt-size (16-63 µm particles) found at the bottom of the dish into the graduated cylinder using deionized water, being careful not to exceed 1000 ml mark.
2. Set the sediment fraction remaining in the sieve aside.
3. Fill the cylinder with deionized water up to the 1000 ml mark. Using a metal stirring rod, vigorously stir the water column from bottom to top, using short strokes, starting at the base of the column and working upwards. Keep stirring until the material is distributed uniformly throughout the column. End up stirring with long, smooth strokes the full length of the column. Be careful not to break the water surface as material could be lost. Place a beaker with tap water next to the cylinder and insert a thermometer to record water temperature.
4. Immediately (<20 sec) after stirring, withdraw 40 ml of sample using a 40 ml volumetric pipette. Expel sample into a recorded 50 ml glass beaker. Rinse pipette with a small volume of deionized water, and add the rinse to the 50 ml beaker. If the sample is taken in two parts (*i.e.*, two 20-ml samples), the cylinder will be stirred between extractions and samples withdrawn after each stirring and added to the beaker.
5. The 50 ml glass beaker will be placed in an oven at 100°C until dry (typically 24 hours). A randomly selected subsample of each batch will be reweighed after an additional 24 hour drying period, as a check for the stability of the dry weight measurement.

5.1.3.4 Treatment of Sand (>63 mm) Fraction Retained on Sieve

If necessary, remove shell and shells fragments, pieces of wood and algae. Air dry and record weight on the biomass data sheet.

The sand fraction (>63 µm) is transferred to a recorded 50 ml glass beaker and placed in an oven at 100°C until dry (typically for 24 hrs). A randomly selected subsample of each batch is reweighed after an additional 24-hour drying period to check for stability of the dry weight measurement.

5.1.3.5 Analysis of Samples

1. After drying, remove beaker from oven and let equilibrate with the atmosphere for 1.5 hr before weighing. Weigh each fraction sample to nearest 0.001 grams and record weight.
2. Calculate weight of silt-clay fraction using correction factor as follows:

$$\text{Silt-Clay wt.} = \text{Net wt.} \times \frac{\text{(total volume in cylinder)}}{\text{(sample volume from cylinder)}} - \text{dispersant wt.}$$

***Note**: The total volume in cylinder is 1000 ml. The sample volume from cylinder is 40 ml. Using the prescribed methods, this results in a dispersant weight of 4 mg.*

3. Calculate percent silt-clay fraction

$$\%\ \text{silt-clay} = \frac{\text{silt-clay wt.}}{\text{(sand wt. + silt-clay wt.)}} \times 100$$

4. Calculate percent sand

   % sand = 100 - % silt-clay

   ***Note**: (100 - % mud) is not, in all cases, equal to the percent sand, since gravel sized particles (>2mm but < 64 mm) may be present in some samples*

5.1.4 Procedures for Percent Water Content

The percent water content of the sample is needed to correct sediment dry weights for salt content, since salts are left behind during the drying process.

5.1.4.1 Sample Preparation

1. Retrieve sediment samples from cold storage and bring to room temperature. Record sample numbers on data sheet. Samples that have dried cannot be used.
2. Remove sediment from storage bag, place in a 250 ml beaker and homogenize. Homogenization will be accomplished by stirring the sediment with a small spatula for at least three minutes. Do not rinse the spatula and do not add water to the beaker during the homogenization process.
3. Place approximately 5-10 grams wet weight of sediment into a clean recorded 50 ml glass beaker and weight immediately. The sample must not be allowed to stand at room temperature for more than a few minutes since evaporation will affect the water content measurement. Store any unused sediment from each sample at 4°-5°C for QA/QC analysis and other sediment analyses.
4. Place the sample in a drying oven at 100°C until dry (typically 24 hours). Store dry samples in a dessicator containing hydrous silica gel until cooled (1 hour).

   ***Note**: dry samples may absorb moisture from wet sample, thus dry samples should be removed before placing moist samples in the oven.*

5. All weight is recorded on data sheets to the nearest 0.001 gram. Ten percent of randomly selected subsamples of each batch are reweighed after an additional 24 hour drying period as a check for stability for the dry weight measurement (a change of <0.1% is expected).

5.1.4.2 Calculation of Sediment Water Content

Water loss (ml) = Gross wet wt. – Gross dry wt.

$$\% \text{ water} = \frac{(\text{Gross wet wt.} - \text{Gross dry wt.})}{\text{Net wet wt .}} \times 100$$

For saline sediments a correction factor must be applied because dry salts are included in the dry sediment weight. For this value use the bottom salinity recorded during field measurements:

Salt wt. (g) = Water loss (ml) x Salinity (mg/ml or ppt)

Corrected dry weight = Gross dry wt. – Salt wt.

$$\text{Corrected percent water} = \frac{(\text{Gross wet wt.} - \text{Corrected dry wt.})}{\text{Net wet wt.}} \times 100$$

Note: *Assume a water density of 1g/ml for the (fresh) water that evaporates*

5.1.5 Procedures for Sediment Grain Size Distribution

This procedure is used to determine the percent by weight of soil and clays in sediment samples. It provides a method for determination of weight percent quantiles for sediments as well as the quantile deviation of skewness.

5.1.5.1 Sample Preparation

1. Retrieve sediment samples from cold storage and bring to room temperature. Record sample numbers on data sheet. Return unused portion to storage.
2. For sandy sediments, remove ~45-50 g wet weight. For muddy sediment remove ~20-25 g wet weight. Place sediment into a 250 ml beaker for homogenization.
3. If the sample is ≤20% silt-clay, skip the next step and proceed to step 5 of this section.
4. If the sample is greater than 20% silt-clay, the organics in the sample must be removed as follows: Initially, add enough deionized water to cover the sample. Add small quantities of 30% H_2O_2 to the sample, stirring until any effervescence ceases. Cover beaker with large watch glass cover if frothing is excessive. If the solution heats excessively, cool the beaker in a water bath. Continue adding H_2O_2 until frothing ceases, then slowly heat to 60°-70°C (H_2O_2 decomposes above 70°C). Observe for 10 minutes to ensure that the possibility of a strong reaction has passed. Add H_2O_2 until no further reaction occurs. An accepted alternative method, such as the combustion method, may also be used so long as it has been documented and does not impact the QA.
5. Homogenize the sample by stirring with a spatula and a small amount of deionized water for at least three minutes.

5.1.5.2 Dispersion of Clay Fraction of Sediments and Wet Sieving the Sample

1. Make-up a 5g/L stock solution of dispersant. Add 5 grams of sodium exametaphosphate "Calgon"to 1 liter of deionized water.
2. Add 20 ml of the dispersant solution (100 mg of hexametaphosphate) and 30 ml of distilled water to the sample. Stir, using a magnetic stirrer for one to five minutes to break-up sediment aggregrates.

3. After stirring, the sample will be wet sieved through a 63 µm mesh sieve into a large evaporation dish and wash all fines into the sieve using as little distilled water as possible.
4. The volume of sediment + water in the evaporation dish must be < 900 ml to allow for rinsing the sample into a 1000 ml graduated cylinder.

5.1.5.3 Treatment of the Silt and Clay Fraction (particles < 63 µm)

1. Carefully transfer mud in evaporation dish to a 1000 ml graduated cylinder. Rinse the mud (generally medium-coarse silt-size (16-63 µm particles) found at the bottom of the dish into the graduated cylinder using deionized water, being careful not to exceed 1000 ml mark.
2. Set the sediment fraction remaining in the sieve aside.
3. Fill the cylinder with deionized water up to the 1000 ml mark. Using a metal stirring rod, vigorously stir the water column from bottom to top, using short strokes, starting at the base of the column and working upwards. Keep stirring until the material is distributed uniformly throughout the column. End up stirring with long, smooth strokes the full length of the column. Be careful not to break the water surface as material could be lost. Place a beaker with tap water next to the cylinder and insert a thermometer to record water temperature.

5.1.5.4 Analysis of the Silt and Clay (<63µm) Fraction

1. Stir the cylinder to suspend the sample. As soon as the stir rod emerges for the last time, start the timer. At the end of 20 seconds, insert the pipette to a depth of 20 cm and withdraw exactly 20 ml. This is the most important single step in this exercise as subsequent analyses are based on the calculation of the total mud weight.
2. Transfer the pipette sample fractions to separate recorded 50 ml glass beakers. Each pipette withdrawal should be rinsed with a small volume of deionized water which is then added to the 50 ml sample beaker.
3. Continue to withdraw 20-ml samples with the 20 ml volumetric pipette at the depths and times indicated in Table 5.2 for the recorded water temperature.

Table 5.2. Sampling Time Intervals

		PIPETTE	Elapsed time for withdrawal of sample in hours, minutes, and seconds (h:mm:ss).								
PHI DIAMETER	DIAMETER (microns)	SAMPLING DEPTH (cm)	18 degrees C	19 degrees C	20 degrees C	21 degrees C	22 degrees C	23 degrees C	24 degrees C	25 degrees C	26 degrees C
		Start Time:	0:00:00	0:00:00	0:00:00	0:00:00	0:00:00	0:00:00	0:00:00	0:00:00	0:00:00
4.0	62.5	20	0:00:20	0:00:20	0:00:20	0:00:20	0:00:20	0:00:20	0:00:20	0:00:20	0:00:20
4.5	44	20	0:02:02	0:01:59	0:01:56	0:01:53	0:01:50	0:01:48	0:01:45	0:01:43	0:01:40
		Restir until:	0:03:00	0:03:00	0:03:00	0:03:00	0:03:00	0:03:00	0:03:00	0:03:00	0:03:00
5.0	31	10	0:05:02	0:04:59	0:04:57	0:04:54	0:04:51	0:04:48	0:04:46	0:04:44	0:04:41
5.5	22	10	0:07:03	0:06:57	0:06:51	0:06:46	0:06:40	0:06:35	0:06:30	0:06:26	0:06:21
6.0	16	10	0:10:40	0:10:28	0:10:18	0:10:07	0:09:57	0:09:47	0:09:38	0:09:29	0:09:19
6.5	11	10	0:19:12	0:18:49	0:18:26	0:18:03	0:17:42	0:17:21	0:17:01	0:16:42	0:16:22
7.0	7.8	10	0:35:14	0:34:27	0:33:42	0:32:56	0:32:13	0:31:32	0:30:53	0:30:15	0:29:36
7.5	5.5	10	1:07:49	1:06:14	1:04:44	1:03:13	1:01:46	1:00:24	0:59:05	0:57:49	0:56:30
8.0	3.9	10	2:11:55	2:08:46	2:05:47	2:02:45	1:59:53	1:57:09	1:54:32	1:52:02	1:49:23
8.5	2.8	5	2:08:03	2:05:00	2:02:06	1:59:10	1:56:23	1:53:43	1:51:11	1:48:46	1:46.12
9.0	2.0	5	4:08:06	4:02:07	3:56:26	3:50.41	3.45:13	3:40.01	3:35:03	3.30:18	3.25 16

4. Each beaker should be placed in an oven at 100°C for 24 hrs. All weights are to be recorded on the data sheet.

5.1.5.5 Calculations for Determining Sample Withdrawal Times for Pipette Analysis

Sample withdrawal times for pipette analysis are based upon Stoke's law written as:

$$T = \frac{Depth}{1500*A*(d2)}$$

where:
T is time in minutes,
Depth is in centimeters,
A is a constant, and
d is the particle diameter in millimeters.

The A value is a function of temperature, gravity, and density of particles. Assume a density of 2.65 (associated with quartz or clay minerals). The following table relates various temperatures to the constant A.

Temp. (°C)	20	21	22	23	24	25	26
A	3.57	3.66	3.75	3.84	3.93	4.02	4.12

5.1.5.6 Removal Of Carbonates (if warranted)

1. If a sediment contains >50%, by weight, of calcareous material, the sample is described as a carbonate sediment. Process carbonate sediments as follows.
2. Record the weight of the sand fraction to 0.001 grams. A 10% (by volume) HCl solution will be added to the dried and weighed sediment. Cover the sediment completely with HCl and let sit for four hours. Additional acid will be added and if foaming is apparent, the sample will be left to stand for several more hours. This process will be repeated until no further reaction occurs with subsequent additions of HCl.
3. The sample will be transferred to a 63 µm sieve and washed using copious amounts of deionized water. This will remove any salts formed during the acidification step.
4. Transfer the sample to a 100 ml glass beaker, dry, and weigh to 0.001 grams.
5. Calculate the sediment carbonate content

$$\% \text{ carbonate} = \frac{(\text{wt. of sand (before acid)} - \text{wt. of sand (after acid)})}{\text{wt. of sand (before acid)}}$$

5.1.5.7 Treatment Of The Sand (>63µm) Fraction

1. 1.Transfer the >63 µm sand fraction to a 250 ml glass beaker and place in a drying oven at 100°C until dry (typically 24 hrs).
2. Transfer the dried sediment into the top of a stack of clean, stainless steel sieves composed of 500 µm (1.0 Ø), 355 µm (1.5 Ø), 250 µm (2.0 Ø), 180 µm (2.5 Ø), and 125 µm (3.0 Ø), 90 µm (~3.5 Ø) and 63 µm (4.0 Ø) sieve with a closed pan on the bottom. Shake on a rotary tapper (Ro-tap) for 15 minutes.
3. Weigh each sieved fraction as follows: Tare a 100 ml beaker to zero; add the 500 µm (1.0 Ø) sediment fraction and weigh to 0.001 grams. Next add the 355 µm (1.5 Ø) fraction to the

beaker. Proceed to add subsequent fractions until all weighed. Record the individual and cumulative weights of the sand fraction to 0.001 grams.

5.1.6 Calculations for Sediment Grain Size Distributions

1. Calculate total weight of mud (silt-clay) in sample (obtained at 20 sec withdrawal intervals).

   Total mud weight (g) = (sample wt. x 50) - dispersant weight

   Dispersant weight = 0.1 gram

   Note: the amount of mud in each 20 ml withdrawal is equal to 1/50 of the total amount of mud remaining in the 1000 ml cylinder at the withdrawal time and at the withdrawal depth.

2. Calculate the total sample weight

   Total sample weight (g) = total mud weight + total sand weight

3. Determine cumulative percentages for each mud (<63 µm size fraction

   Each pipette sample represents material in the column finer than a certain grain size. First multiply each size fraction by 50, subtract the weight of dispersant.

   Fraction wt. (g) for each Ø size = (Wt. of sand for each Ø size X 50) - Dispersant wt. (g)

   Then divide each fraction by the total sample weight, subtract from 1, and multiply the product by 100 to determine cumulative percentages for each sand (<63 µm) fraction.

   $$\text{Cumulative \% (for each Ø)} = \frac{1 - \text{Fraction wt. (g) for each Ø size}}{\text{Total sample weight (g)}} \times 100$$

4. Determine cumulative percentages for each sand (>63 µm) size fraction

   $$\text{\% Wt. of each sand fraction} = \frac{\text{Wt. of sand for each Ø size}}{\text{Total sample weight}} \times 100$$

   Add the percentages incrementally to obtain cumulative weight percentages.

5.1.7 Determination of Statistical Parameters Of Grain Size

1. Plot the cumulative curve of the sample and read the Ø values which correspond to the 24th ($Ø_{25}$), 50th (*i.e.*, median ($Md_{Ø}$)) and 75th ($Ø_{75}$) percentiles by linear interpolation.

2. Calculate the Phi Quartile Deviation (QDØ and Phi Quartile Skewness (SkqØ). Record the $Md_{Ø}$, QDØ, and SkqØ for each sample.

   Phi Quartile Deviation = $(Ø_{75} - Ø_{25}) / 2$

   $$\text{Phi Quartile Skewness} = \frac{(Ø_{25} + Ø_{75}) - (2 \times Md_{Ø})}{2}$$

5.2 ASSESSING SEDIMENT TOXICITY USING ESTUARINE AND MARINE AMPHIPODS

Suggested analytical methods for sediment toxicity are described in sections 5.2 and 5.3. Some laboratories participating in the survey may choose to employ other analytical methods. Labs engaged by EPA or the State may use a different analytical method as long as the lab is able to achieve the same performance requirements as the standard methods. Performance data must be submitted to EPA prior to initiating any analyses. Methods performance requirements for this program identify detection limit, precision and accuracy objectives for each indicator. Method performance requirements for sediment toxicity are shown in Table 5.3.

Table 5.3. Laboratory method performance requirements for sediment toxicity.

Indicator/Data Type	Maximum Allowable Accuracy (Bias) Goal	Maximum Allowable Precision Goal	Completeness Goal
Sediment toxicity	NA	NA	95%

5.2.1 Scope of Application

This method is for use with sediments from oligohaline to fully marine environments. Procedures are described for testing estuarine and marine amphipod crustaceans in the laboratory to evaluate the toxicity of contaminants associated with whole sediments. This method may only be used for salt water sediments.

5.2.2 Summary of Method

Sediments are collected from nearshore coastal sites using a modified Van Veen or ponar grab sampler. A toxicity method is outlined for *Leptocheirus plumulosus,* an Atlantic coast estuarine sediment-burrowing amphipod species. The toxicity test is conducted for 10 d in 1 L glass chambers containing 175 mL of sediment and 800 mL of overlying seawater. Exposure is static (*i.e*., water is not renewed), and the animals are not fed over the 10 d exposure period. Sediment tests include control sediment (sometimes called a negative control). The endpoint in the toxicity test is survival. Procedures are described for use with sediments with pore water salinity ranging from > 0‰ to fully marine.

5.2.3 Interferences

1. Interferences are characteristics of a sediment or sediment test system that can potentially confound interpretation of test results. There are three categories of interfering factors: those characteristics of sediments affecting survival independent of chemical concentration (*i.e*., non-contaminant factors); changes in chemical bioavailability as a function of sediment manipulation or storage; and the presence of indigenous organisms.
2. There are a number of non-contaminant factors that may influence amphipod survival in these tests. The most important and variable factors include sediment particle size, pore water salinity, and pore water ammonia. The physico-chemical properties of each test sediment must be within the tolerance limits of the test organism.
3. Sediment collection, handling, and storage may alter contaminant bioavailablity and concentration by changing the physical, chemical, or biological characteristics of the sediment. These manipulation processes are generally thought to increase availability of organic compounds because of disruption of the equilibrium with organic carbon in the pore water per particle system. Similarly, oxidation of anaerobic sediments increases the availability of certain metals. Because the availability of contaminants may be a function of

the degree of manipulation, it is recommended that handling, storage, and preparation of the sediment for actual testing be as consistent as possible.

Sediment samples should not be sieved to remove indigenous organisms unless there is a good reason to believe indigenous organisms may influence the response of the test organism. However, large indigenous organisms and large debris can be removed using forceps. Reynoldson et al. (1994) observed reduced growth of amphipods, midges, and mayflies in sediments with elevated numbers of oligochaetes and recommended sieving sediments suspected to have high numbers of indigenous oligochaetes. If sediments must be sieved, it may be desirable to analyze samples before and after sieving (e.g., pore-water metals, DOC, and AVS) to document the influence of sieving on sediment chemistry.

4. Testing sediments at temperatures different from that in the field might affect contaminant solubility, partitioning coefficients, or other physical and chemical characteristics. Interaction between sediment and overlying water and the ratio of sediment to overlying water may influence bioavailability.
5. Depletion of aqueous and sediment-sorbed contaminants resulting from uptake by an organism or test chamber may also influence availability. In most cases, the organism is a minor sink for contaminants relative to the sediment. However, within the burrow of an organism, sediment desorption kinetics may limit uptake rates. Desorption of a particular compound from sediment may range from easily reversible (labile; within minutes) to irreversible (non-labile: within days or months).
6. Salinity of the overlying water is an additional factor that can affect the bioavailability of metals. Some metals (*e.g.*, cadmium) are more bioavailable at lower salinities. Therefore, if a sediment sample from a low salinity location is tested with overlying waters of high salinity, there is the potential that metal toxicity may be reduced.

5.2.4 Equipment and Supplies

1. The facility should include separate areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding, acclimation, and culture chambers should not be in a room where sediment tests are conducted, stock solutions or sediments are prepared, or where equipment is cleaned.
2. Equipment and supplies that contact stock solutions, sediments or overlying water should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and supplies that contact sediment or water should be chosen to minimize sorption of test materials from water. Glass, type 316 stainless steel, nylon, high-density polyethylene, polycarbonate and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. High-density plastic containers are recommended for holding, acclimation, and culture chambers.
3. Environmental chamber (or equivalent) with photoperiod and temperature control of 5-25°C.
4. Water purification system capable of producing at least 1 mega-ohm water.
5. Analytical balance capable of accurately weighing to 0.01 mg.
6. One liter glass containers (beakers or wide-mouthed jars) with an internal diameter of 10 cm serve as test chambers. Each test chamber should have a cover. Acceptable test chamber covers include watch glasses, plastic lids, and 9 cm diameter glass culture dishes. It may be necessary to drill a hole in each cover to allow for the insertion of a pipette for aeration. A full list of equipment and supplies is in Table 5.4 below.

Table 5.4. Equipment and Supplies for Culturing and Testing Estuarine and Marine Amphipods.

A. Biological Supplies	Brood stock of test organisms TetraMin® dried wheat leaves	dried alfalfa leaves Neo-Novum® Algae (*e.g*., Pseudoisochrysis paradnxa and Phaeoductylum tricornutum [optional])
B. Glassware	Culture chambers (30 cm x 45 cm x 15 cm plastic wash bin) Test chambers (1 L glass jar or beaker) Glass bowls	Wide-bore pipets (4 to 6 mm ID) Glass disposable pipets Graduated cylinders (assorted sizes, 10 mL to 4 L)
C. Instruments and Equipment	Dissecting microscope Stainless-steel sieves (*e.g*., U.S. Standard No. 25, 30, 35, 10, 50 mesh) Photoperiod timers Light meter Temperature controllers Thermometer Continuous recording thermometer Photoperiod timer Dissolved oxygen meter pH meter Selective ion meter	Ammonia electrode (or ammonia kit) Salinity meter/temperature compensating salinity refractometer Drying oven Desiccator Balance (0.01 mg sensitivity) Refrigerator Freezer Light box Hemacytometer Mortar and pestle or blender
D. Miscellaneous	Air supply and air stones (oil free and regulated) Glass hole-cutting bits Glass glue	Aluminum weighing pans Deionized water Sieve cups (mesh size 10.5 mm) Dissecting probes
E. Chemicals	Acetone (reagent grade) Hexane (reagent grade) Hydrochloric acid (reagent grade) Reagents for preparing synthetic seawater (reagent grade): $CaCl_2$•2 H_2O, KBr, KCl, $MgCl_2$•6 H_2O, $Na_2B_4O_7$•l0 H_2O, NaCl, $NaHCO_3$, Na_2SO_4, $SrCl_2$•6 H_2O	Formalin Ethanol Rose bengal Cadmium chloride Sodium dodecyl sulfate Copper sulfate Detergent (non-phosphate)

5.2.5 Reagents and Water

5.2.5.1 Reagents

See Table 5.2. Section E above for a list of chemicals and reagents. All reagents should be at least reagent grade, unless a test on formulation commercial product, technical-grade, or use-grade material is specified.

5.2.5.2 Sea water

Sea water used to test and culture organisms should be uniform in quality. Acceptable sea water should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (*e.g*., discoloration, unusual behavior). If problems are observed in the culturing or testing of organisms, it is desirable to evaluate the characteristics of the water.

Reconstituted sea water is prepared by adding specified amounts of reagent grade chemicals to high-purity distilled or deionized water. Suitable salt reagents can be reagent grade chemicals, commercial sea salts, such as Forty Fathoms®, Instant Ocean®, or HW Marinemix®. Pre-formulated brine (*e.g*., 60 to 90%) prepared with dry ocean salts or heat-concentrated natural sea water can also be used. Acceptable high-purity water can be prepared using deionization,

distillation, or reverse-osmosis units. Test water can also be prepared by diluting natural water with deionized water.

5.2.6 Sample Manipulation

1. Homogenization

Sediment samples tend to settle during shipment. Water above the sediment is not discarded, but is mixed back into the sediment during homogenization. Sediment samples should not be sieved to remove indigenous organisms unless there is a good reason to believe indigenous organisms may influence the response of the test organism (See Section 5.3.3, #3).

2. Analytical Methodology

The precision, accuracy, and bias of each analytical method used should be determined in the appropriate matrix: that is, sediment or water. Reagent blanks and analytical standards should be analyzed and recoveries should be calculated.

Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from about 1 to 2 cm above the sediment surface in the test chamber. Overlying water samples should not contain any surface debris, any material from the sides of the test chamber, or any sediment. Measurement of test material(s) concentration in sediment at the end of a test can be taken by siphoning most of the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the sediment for chemical analysis.

5.2.7 Quality Control

1. Before a sediment test is conducted in any test facility, the analyst should conduct a "non-toxicant" test with the potential test species in which all test chambers contain a control sediment (sometimes called the negative control), and clean overlying water for the amphipod species to be tested. Survival of the test organism will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to achieve acceptable species-specific control survival. Evaluations may also be conducted of the magnitude of the within- and between-chamber variance in a test. For the test to be acceptable, survival at 10 d must equal or exceed 90% in the control sediments.
2. If the supplier has not conducted five reference toxicity tests with the test organism, the testing laboratory must do so before starting a sediment test. Intralaboratory precision, expressed as a coefficient of variation of the range in response for each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (*e.g*., the same test duration, type of water, age of test organisms) and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical (section 5.3.10).

   Reference toxicants such as cadmium (available as cadmium chloride ($CdCl_2$)), copper (available as copper sulfate ($CuSO_4$)), and sodium dodecyl sulfide (SDS) are available for use. No one reference toxicant can be used to measure the condition of test organisms in respect to another toxicant with a different mode of action. Test conditions for conducting reference-toxicity tests with *L. plumulosus* are outlined in Table 5.5.

Table 5.5. Recommended Test Conditions for Conducting Reference-Toxicity Tests

Parameter	Conditions
1. Test type:	Water-only test
2. Dilution series:	Control and at least 5 test concentrations ((0.5 dilution factor)
3. Toxicant:	Cd, Cu, Sodium dodecyl sulfate (SDS)
4. Temperature:	25°C for *L. plumulosus*
5. Salinity:	20‰
6. Light quality:	Chambers should be kept in dark or colored with opaque material
7. Photoperiod:	24 h D
8. Renewal of water:	None
9. Age and size of test organisms:	*L. plumulosus* 2 - 4 mm (no mature males or females)
10. Test chamber:	1 L glass beaker or jar
11. Volume of water:	800 mL (minimum)
12. Number of organisms per chamber:	n = 20 if 1 per replicate; n = 10 (minimum) if >1 per replicate
13. Number of replicate chamber per /treatment:	1 minimum; 2 recommended
14. Aeration:	Recommended: but not necessary if >90% dissolved oxygen saturation can be achieved without aeration
15. Dilution water:	Culture water, surface water, site water, or reconstituted water
16. Test duration:	96 h
17. Endpoint:	Survival (LC_{50});
18. Test acceptability:	90% control survival

3. Before conducting tests with contaminated sediment, the laboratory should conduct five exposures in control sediment. It is recommended that these five exposures with control sediment be conducted concurrently with the five reference toxicity tests described above.

4. Each lab must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit.

5.2.8 Culturing and Maintaining Test Organisms

1. The quality of test organisms obtained from an outside source, regardless of whether they are from culture or collected from the field, must be verified by conducting a reference-toxicity test concurrently with the sediment test. For cultured organisms, the supplier should provide data with the shipment describing the history of the sensitivity of organisms from the same source culture. For field-collected organisms, the supplier should provide data with the shipment describing the collection location, the time and date of collection, the water salinity and temperature at the time of collection, and collection site sediment for holding and acclimation purposes.

2. *L. plumulosus* used in tests should be pre-reproductive animals that are 2 to 4 mm in length. To obtain animals in this size range, sediment from culture chambers containing mixed-size

amphipods should be poured over a sieve series that consists of the following sequence of mesh sizes: 0.71 mm, 0.50 mm, and 0.25 mm. Animals retained on the 0.50 mm mesh screen should be washed into a shallow glass pan. The smaller animals from this group should be selected for toxicity testing. Gravid females should be avoided.

Alternatively, test animals within a narrow size range are obtained by isolating the smallest amphipods which are allowed to grow until they reach a testable size. To obtain the smallest amphipods, transfer sediment from culture chambers containing mixed-size amphipods over a sieve series that consists of the following sequence of mesh sizes: 1.0 mm, 0.5 mm, and 0.25 mm. Animals retained on the 0.25 mm mesh screen should be small juveniles that are 1.1 to 2.0 mm in length. They will take ~2 weeks to reach testable size after isolation. The amphipods retained on the 0.25 mm screen are washed into a culture chamber that is set up as a normal culture chamber, *i.e.,* containing a thin (-1 cm) sediment layer and maintained under culture conditions for the 2-week interim period. By the end of the 2-week grow-out period, the animals should be of testable size and be within a narrow size and age range.

3. Holding and Acclimation

   Density. Amphipods should be held and acclimated (if necessary) in containers (4 to 8 L volume) that contain a 2 to 4 cm layer of collection site sediment that has been sieved through a 0.5 mm mesh screen. Approximately 350 amphipods should be added to each 8 L container. Amphipod density should not exceed 1 amphipod/cm^2.

   Duration. Depending on temperature and salinity at the collection site, amphipods may have to be acclimated to standard test conditions. If necessary, changes in temperature or salinity to bring amphipods from the collection site conditions to the test conditions should be made gradually. Once test conditions are achieved, amphipods should be maintained at these conditions for at least two days before testing to allow for acclimation. Amphipods held for more than ten days should not be used for testing.

   Temperature. Overlying water temperature must not be changed by more than 3 °C per day during acclimation to the test temperature. Once the test temperature is reached, amphipods must be maintained at that temperature for a minimum of 2 days.

   Salinity. The target test salinity for *L. plumulosus* is 20‰. It is likely that the collection site salinity will be considerably lower than this. Upon arrival in the laboratory, the water used to hold the organisms should be adjusted to 20‰ by adjusting the salinity in the holding container at a rate that must not exceed 5‰ per 24 h. The amphipods should be maintained at 20‰ for 2 days before testing.

5.2.9 Procedure

1. The 10-d sediment toxicity test with *L. plumufosus* must be conducted at the species-specific temperature and salinity with a 24 h light photoperiod at an illuminance of about 500 to 1000 lux. Test chambers are 1 L glass chambers containing 175 mL of sediment and 800 mL of overlying reconstituted seawater. Five replicates are recommended for routine testing. Exposure is static (*i.e*., water is not renewed), and the animals are not fed over the 10 d exposure period. Conditions for conducting a 10-d sediment toxicity test are summarized in Table 5.6.

Table 5.6. Test Conditions for Conducting a10-d Sediment Toxicity Test

Parameter	Conditions
1. Test type:	Whole sediment toxicity test, static
2. Temperature:	25°C for L. plumulosus
3. Salinity	20‰
4. Light quality:	Wide-spectrum fluorescent lights
5. Illuminance:	500 – 1000 lux
6. Photoperiod:	24L:0D
7. Test chamber:	1 L glass beaker or jar with ~10 cm I.D.
8. Sediment volume:	175 mL (2 cm)
9. Overlying water volume:	800 mL
10. Renewal of overlying water:	None
11. Size and life stage of amphipods:	L. plumulosus: 2-4 mm (no mature males or females)
12. Number of organisms per chamber:	20 per test chamber
13. Number of replicate chambers/treatment:	4 (minimum); 5 (recommended)
14. Feeding:	None
15. Aeration:	Water in each test chamber should be aerated overnight before start of test and throughout the test aeration at rate that maintains ≥90% saturation of dissolved oxygen concentration
16. Overlying water:	Clean sea water, natural or reconstituted water
17. Overlying water quality:	Temperature daily; pH, ammonia, salinity, and DO at test start and end.
18. Test duration:	10 d
19. Endpoints:	Survival
20. Test acceptability:	Minimum mean control survival of 90%

2. Introduction of Sediment (Day -1). One day prior to the addition of amphipods, field collected test sediment is homogenized by stirring in the sediment storage container or by using a rolling mill, feed mixer, or other suitable apparatus. Control and reference sediments are included. Excess water on the surface of the sediment can indicate separation of solid and liquid components.

   A 175mL aliquot of thoroughly homogenized sediment is added to each test chamber. It is important that an identical volume be added to each replicate test chamber: at a minimum the volume added should equate to a depth of 2 cm in the test chamber.

3. Addition of Overlying Water (Day -1). To minimize disruption of sediment as test seawater is added, a turbulence reducer should be used. The turbulence reducer may be either a disk cut from polyethylene, nylon, or Teflon® sheeting (4 to 6 mil), or a glass petri dish attached (open face up) to a glass pipette. If a disk is used as the turbulence reducer, it should fit the inside diameter of the test chamber and have attached a length of nylon monofilament (or nontoxic equivalent) line. The turbulence reducer is positioned just above the sediment surface and raised as sea water is added to the 750-mL mark on the side of the test chamber. The turbulence reducer is removed and rinsed with test sea water between replicates of a treatment. A separate turbulence reducer is used for each treatment. The

test chambers should be covered, placed in a temperature controlled water bath and gently aerated.

4. Addition of Amphipods (Day 0). On the day of the test, add amphipods to the test chambers. Approximately one-third more amphipods than are needed for the test should be sieved from the culture or control sediment in the holding container and transferred to a sorting tray. *L. plumulosus* should be isolated using a 0.5 mm sieve. Sieving should be conducted with sea water of the same temperature and salinity as the holding and test water.

   Once isolated, active amphipods should be randomly selected using a transfer pipette or other suitable tool (not forceps), and distributed among dishes or cups containing approximately 150 mL of test sea water until each container has twenty amphipods. The distribution of amphipods to the test chambers must be executed in a randomized fashion.

   Amphipods should be added to test chambers without disruption of the sediment by placing a 6-mil polyethylene, nylon, or Teflon® disk on the water surface and gently pouring the water and amphipods from the sorting container over the disk into the test chamber. The disk should be removed once the amphipods have been introduced. Alternatively, amphipods from the sorting container can be poured into a sieve cup (mesh size ≤0.5 mm) and gently washed into the test chamber with test sea water. Any amphipods remaining in the sorting container should be gently washed into the test chamber using test sea water. The water level should be brought up to the 950 mL mark, the test chamber covered, and aeration continued.

   After the addition of the animals, the test chambers should be examined for animals that may have been injured or stressed during the processes. Injured or stressed animals should be removed. Allow 5 to 10 min for animals to bury into the test sediment. Amphipods that have not burrowed within this time should be replaced with animals from the same sieved population, unless they are repeatedly burrowing into the sediment and immediately emerging in an apparent avoidance response. In that case, the amphipod is not replaced. Record the number of amphipods that are removed.

5. Ending the Test (Day 10). The contents of the test chambers must be sieved (≥ 0.5 mm mesh) to isolate the test animals. Test water should be used for sieving. Material retained on the sieve should be washed into a sorting tray with clean test sea water. The sieve should be slapped forcefully against the surface of the water to ensure that all amphipods are dislodged from the screen

   Material that has been washed from the sieve into the sorting tray should be carefully examined for the presence of amphipods. Numbers of live, missing, and dead amphipods should be recorded for each test chamber. Missing animals are assumed to have died should be considered dead in calculations of the percent survival for each replicate treatment. Amphipods that are inactive but not obviously dead must be observed using a low-power dissecting microscope or a hand-held magnifying glass. Any animal that fails to exhibit movement (*i.e*. neuromuscular twitch of pleopods or antennae) upon gentle prodding with a probe should be considered dead.

6. A summary of the procedure is contained in Table 5.7 below.

Table 5.7. General Activity Schedule for Conducting 10-d Sediment Toxicity Test

Days	Activity
Days -10 to -3	Collect or receive amphipods from supplier and place into collection site sediment. Alternatively, separate 2-4 mm *L. plumulosus* from culture.
Days -9 to -2	Acclimate and observe amphipods to species-specific test conditions.
Day -1	Observe amphipods, monitor water conditions. Add sediment to each test chamber. Place chambers into exposure system, and start aeration.
Day 0	Measure temperature of overlying water in test chambers. Transfer 20 amphipods into each test chamber. Archive 20 test organisms for length determination.
Day 1	Measure temperature. Observe behavior of test organisms and ensure that each test chamber is receiving air. Measure dissolved oxygen in test chambers to which aeration has been cut-off.
Day 2	Measure total water quality (pH, temperature, dissolved oxygen, salinity, total ammonia) of overlying water. Observe behavior of test organisms and ensure that each test chamber is receiving air.
Days 3 to 7	Same as Day 1.
Day 8	Same as Day 2.
Day 9	Same as Day 1
Day 10	Measure temperature. End the test by collecting the amphipods with a sieve.

5.3 SEDIMENT TOXICITY USING FRESHWATER AMPHIPODS

5.3.1 Scope of Application

1. This method is for use with sediments from freshwater aquatic environments. Procedures are described for testing amphipod crustaceans in the laboratory to evaluate the toxicity in terms of survival rate from contaminants associated with whole sediments.
2. This method is used for freshwater sediments. See Section 5.2 for method for assessing salt water sediments.

5.3.2 Summary of Method

This section describes procedures for testing freshwater organisms in the laboratory to evaluate the potential short-term toxicity of sediments. Sediments are collected from the field and the toxicity method will be applied to the amphipod *Hyalella azteca.* The method described here is for conducting a 10-d acute toxicity test. The test is conducted for 10 days in 300-mL chambers containing 100 mL of sediment and 175 mL of overlying water. Overlying water is added daily and test organisms are fed during the toxicity tests. The endpoint in the 10-d toxicity test with *H. azteca* is survival.

5.3.3 Interferences

1. Interferences are characteristics of a sediment or sediment test system that can potentially confound interpretation of test results. There are three categories of interfering factors: those characteristics of sediments affecting survival independent of chemical concentration (*i.e.*, non-contaminant factors); changes in chemical bioavailability as a function of sediment manipulation or storage; and the presence of indigenous organisms.
2. There are a number of non-contaminant factors that may influence amphipod survival in these tests. The most important and variable factors include sediment particle size, pore water salinity, and pore water ammonia. The physico-chemical properties of each test sediment must be within the tolerance limits of the test organism.
3. Sediment collection, handling, and storage may alter contaminant bioavailablity and concentration by changing the physical, chemical, or biological characteristics of the sediment. These manipulation processes are generally thought to increase availability of organic compounds because of disruption of the equilibrium with organic carbon in the pore water per particle system. Similarly, oxidation of anaerobic sediments increases the availability of certain metals. Because the availability of contaminants may be a function of the degree of manipulation, it is recommended that handling, storage, and preparation of the sediment for actual testings be as consistent as possible.
4. Testing sediments at temperatures different from that in the field might affect contaminant solubility, partitioning coefficients, or other physical and chemical characteristics. Interaction between sediment and overlying water and the ratio of sediment to overlying water may influence bioavailability.
5. Depletion of aqueous and sediment-sorbed contaminants resulting from uptake by an organism or test chamber may also influence availability. In most cases, the organism is a minor sink for contaminants relative to the sediment. However, within the burrow of an organism, sediment desorption kinetics may limit uptake rates. Desorption of a particular compound from sediment may range from easily reversible (labile; within minutes) to irreversible (non-labile: within days or months).

6. Sediment samples should not be sieved to remove indigenous organisms unless there is a good reason to believe indigenous organisms may influence the response of the test organism. However, large indigenous organisms and large debris can be removed using forceps. Reynoldson et al. (1994) observed reduced growth of amphipods, midges, and mayflies in sediments with elevated numbers of oligochaetes and recommended sieving sediments suspected to have high numbers of indigenous oligochaetes. If sediments must be sieved, it may be desirable to analyze samples before and after sieving (e.g., pore-water metals, DOC, and AVS) to document the influence of sieving on sediment chemistry.

5.3.4 Equipment and Supplies

1. The facility should include separate areas for culturing and testing to reduce the possibility of contamination by test materials and other substances, especially volatile compounds. Holding, acclimation, and culture chambers should not be in a room where sediment tests are conducted, stock solutions or sediments are prepared, or where equipment is cleaned.
2. Equipment and supplies that contact stock solutions, sediments or overlying water should not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. In addition, equipment and supplies that contact sediment or water should be chosen to minimize sorption of test materials from water. Glass, type 316 stainless steel, nylon. high-density polyethylene, polycarbonate and fluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. High-density plastic containers are recommended for holding, acclimation, and culture chambers.
3. Environmental chamber or equivalent with photoperiod and temperature control of 20-25°C.
4. Water purification system capable of producing at least 1 mega-ohm water.
5. The water-delivery system used in water-renewal testing can be one of several designs. The system should be capable of delivering water to each replicate test chamber. Diluter systems have been successfully modified for sediment testing. The water-delivery system should be calibrated before the test by determining the flow rate of the overlying water. At any particular time during the test, flow rates through any two test chambers should not differ by more than 10%.
6. Test chambers may be constructed in several ways depending on the contaminants of interest. Clear silicone adhesives, suitable for aquaria, sorb some organic compounds that might be difficult to remove. Therefore, as little adhesive as possible should be in contact with the test material. Extra beads of adhesive should be on the outside of the test chambers rather than on the inside. To leach potentially toxic compounds from the adhesive, all new test chambers constructed using silicone adhesives should be held at least 48 h in overlying water before use in a test. A full list of equipment and supplies is in Table 5.8 below.

Table 5.8. Equipment and Supplies for Culturing and Testing the Freshwater Amphipod *H. azteca*

A. Biological Supplies	Brood stock of test organisms Active dry yeast Cerophyl® (dried cereal leaves)	Trout food pellets Diatoms (e.g., *Navicula* sp.)
B. Glassware	Culture chambers Test chambers (300-mL high-form lipless beaker) Juvenile holding beakers (1 L) Wide-bore pipets (4- to 6-mm ID)	Glass disposable pipets Burettes (for hardness and alkalinity determinations) Graduated cylinders (assorted sizes, 10 mL to 2 L)
C. Instruments and Equipment	Dissecting microscope Stainless-steel sieves (*e.g.*, U.S. Standard No. 25, 30, 35, 10, 50 mesh) Photoperiod timers Light meter Temperature controllers Thermometer Continuous recording thermometer Photoperiod timer Dissolved oxygen meter pH meter Selective ion meter	Ammonia electrode (or ammonia kit) Salinity meter/temperature compensating salinity refractometer Drying oven Desiccator Balance (0.01 mg sensitivity) Blender Refrigerator Freezer Light box Hemacytometer
D. Miscellaneous	Ventilation system for test chambers Air supply and airstones (oil free and regulated) Cotton surgical gauze or cheese cloth Stainless-steel screen (no. 60 mesh, for test chambers) Glass hole-cutting bits Plastic mesh (110-µm mesh opening; Nytex® 110)	Aluminum weighing pans Fluorescent light bulbs Nalgene bottles (500 mL and 1000 mL) Air line tubing White plastic dish pan Coiled-web material Shallow pans (plastic (light-colored), glass, stainless steel) Silicon adhesive caulking
E. Chemicals	Detergent (non-phosphate) Acetone (reagent grade) Hexane (reagent grade) Hydrochloric acid (reagent grade)	Copper Sulfate, Potassium Chloride Reagents for reconstituting water Formalin (or Notox®) Sucrose

5.3.5 Reagents and Water

5.3.5.1 Reagents

All reagents should be at least reagent grade, unless a test on formulation commercial product, technical-grade, or use-grade material is specified.

5.3.5.2 Water

1. Water used to test and culture organisms should be uniform in quality. Acceptable freshwater should allow satisfactory survival, growth, or reproduction of the test organisms. Test organisms should not show signs of disease or apparent stress (*e.g.*, discoloration. unusual behavior). If problems are observed in the culturing or testing of organisms, it is desirable to evaluate the characteristics of the water.

2. Reconstituted water should be prepared by adding specified amounts of reagent-grade chemicals to high-purity distilled or deionized water. Deionized water should be obtained from a system capable of producing at least 1mega-ohm water. Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured on each batch of reconstituted water. Reconstituted water should be aerated before use to adjust pH and dissolved oxygen to the

acceptable ranges. It is recommended replenishing reconstituted water every 2 weeks. Below is a summary of salts to be added to 100 liters of deionized water to make up reconstituted water for use in Hyalella azteca sediment toxicity testing (Borgman 1996).

Salt	MW	g/100L
KCl	74.54	0.373
$NaHCO_3$	84	8.4
$MgSO_4$	120.3	3.0075
$CaCl_2$	110.9	11.09
NaBr	102.8	0.103

5.3.6 Sample Manipulation

1. Sediment samples tend to settle during shipment. Water above the sediment should not be discarded, but should be mixed back into the sediment during homogenization.
2. Analytical Methodology: The precision, accuracy, and bias of each analytical method used should be determined in the appropriate matrix: that is, sediment or water. Reagent blanks and analytical standards should be analyzed and recoveries should be calculated.

   Measurement of test material(s) concentration in water can be accomplished by pipeting water samples from about 1 to 2 cm above the sediment surface in the test chamber. Overlying water samples should not contain any surface debris, any material from the sides of the test chamber, or any sediment. Measurement of test material(s) concentration in sediment at the end of a test can be taken by siphoning most of the overlying water without disturbing the surface of the sediment, then removing appropriate aliquots of the sediment for chemical analysis.

5.3.7 Quality Control

1. Before a sediment test is conducted, the analyst must conduct a “non-toxicant” test with each potential test species in which all test chambers contain a control sediment (sometimes called the negative control), and clean overlying water for each amphipod species to be tested. Survival of the test organism will demonstrate whether facilities, water, control sediment, and handling techniques are adequate to achieve acceptable species-specific control survival. Evaluations may also be conducted of the magnitude of the within- and between-chamber variance in a test. For the test to be acceptable, survival at 10 d must equal or exceed 90% for amphipod species in the control sediments.
2. If the supplier has not conducted five reference toxicity tests with the test organism, the testing laboratory must do so before starting a sediment test. Intralaboratory precision, expressed as a coefficient of variation of the range in response for each type of test to be used in a laboratory should be determined by performing five or more tests with different batches of test organisms, using the same reference toxicant, at the same concentrations, with the same test conditions (*e.g.*, the same test duration, type of water, age of test organisms) and same data analysis methods. A reference toxicant concentration series (0.5 or higher) should be selected that will consistently provide partial mortalities at two or more concentrations of the test chemical. Reference toxicants such as sodium (in the form of sodium chloride (NaCl)), potassium (as potassium chloride (KCl)), cadmium (available as cadmium chloride ($CdCl_2$)), and copper (available as copper sulfate ($CuSO_4$)) are available

for use. No one reference toxicant can be used to measure the condition of test organisms in respect to another toxicant with a different mode of action. Test conditions for conducting reference-toxicity tests are outlined in Table 5.9.

Table 5.9. Recommended Test Conditions for Conducting Reference-Toxicity Tests

Parameter	Conditions
1. Test type:	Water-only test
2. Dilution series:	Control and at least 5 test concentrations (0.5 dilution factor)
3. Toxicant:	Na, K, Cd, or Cu,
4. Temperature:	23°± 1°C
5. Light quality:	Wide-spectrum fluorescent lights
6. Illuminance:	100 to 1000 lux
7. Photoperiod:	16L:8D
8. Renewal of water:	None
9. Age of test organisms:	7-14 d old (1-2 d range in age)
10. Test chamber:	30 mL plastic cups (covered with glass or plastic)
11. Volume of water:	20 mL
12. Number of organisms per chamber:	10
13. Replicate chambers/treatment	4 minimum
14. Feeding:	0.1 mL YCT (1800 mg/L stock) on Day 0 and Day 2
15. Substrate:	Nitex® screen (110 mesh)
16. Aeration:	None
17. Dilution water:	Cultured, well water, surface water, site water, or reconstituted
18. Test duration:	96 h
19. Endpoint:	Survival (LC_{50});
20. Test acceptability:	90% control survival

3. Before conducting tests with contaminated sediment, the laboratory should demonstrate its ability to conduct tests by conducting five exposures in control sediment. It is recommended that these five exposures with control sediment be conducted concurrently with the five reference toxicity tests described above.

4. The lab must work with the Information Management group (Marlys Cappaert,541-754-4467, Cappaert.Marlys@epamail.epa.gov,) to ensure their bench sheets and/or data recording spreadsheets are compatible with the electronic deliverables the lab will need to submit.

5.3.8 Culturing and Maintaining Test Organisms

5.3.8.1 General

All organisms in a test must be from the same source. Organisms may be obtained from laboratory cultures or from commercial or government sources. The test organism used should be identified using an appropriate taxonomic key, and verification should be documented. Obtaining organisms from wild populations should be avoided.

A group of organisms should not be used for a test if they appear to be unhealthy, discolored, or otherwise stressed (*e.g.*, >20% mortality for 48 h before the start of a test). If the organisms fail

to meet these criteria, the entire batch should be discarded and a new batch should be obtained. All organisms should be as uniform as possible in age and life stage. Test organisms should be handled as little as possible.

Organisms can be cultured using either static or renewal procedures. Renewal is recommended to limit loss of the culture organisms from a drop in dissolved oxygen or a buildup of waste products. In renewal systems, there should be at least one volume addition/d of culture water to each chamber. In static systems, the overlying water volume should be changed at least weekly by siphoning down to a level just above the substrate and slowly adding fresh water. Extra care should be taken to ensure that proper water quality is maintained in static systems. For example, aeration is needed in static systems to maintain dissolved oxygen at >2.5 mg/L.

It may be desirable for laboratories to periodically perform 96-h water-only reference-toxicity tests to assess the sensitivity of culture organisms. Data from these reference-toxicity tests could be used to assess genetic strain or life-stage sensitivity to select chemicals.

5.3.8.2 Culturing Procedures for Hyalella azteca

1. The culturing procedure must produce 7- to 14-d-old amphipods to start a 10-d sediment test. The 10-d test with should start with a narrow range in size or age (1- to 2-d range in age) to reduce potential variability in growth at the end of the 10-d test. This narrower range would be easiest to obtain using known age organisms instead of sieving the cultures to obtain similar sized amphipods (*i.e*., amphipods within a range of 1- to 2-d old will be more uniform in size than organisms within the range of 7 d).

2. The following procedure can be used to obtain known age amphipods to start a test. Mature amphipods (50 organisms >30-d old at 23°C) are held in 2-L glass beakers containing 1 L of aerated culture water and cotton gauze as a substrate. Amphipods are fed 10 mL of a yeast-Cerophyl®-trout chow (YCT) mixture and 10 mL of the green algae *Selenastrum capricornutum* (about 3.5×10^7 cells/mL). Five mL of each food is added to each culture daily, except for renewal days, when 10 mL of each food is added.

   Water in the culture chambers is changed weekly. Survival of adults and juveniles and production of young amphipods should be measured at this time. The contents of the culture chambers are poured into a translucent white plastic or white enamel pan. After the adults are removed, the remaining amphipods will range in age from <1- to 7-d old. Young amphipods are transferred with a pipet into a 1-L beaker containing culture water and are held for one week before starting a toxicity test. Organisms are fed 10 mL of YCT and 10 mL of green algae on start-up day and 5 mL of each food each following day. Survival of young amphipods should be >80% during this one-week holding period. Some of the adult amphipods can be expected to die in the culture chambers, but mortality greater than about 50% should be cause for concern. Reproductive rates in culture chambers containing 60 adults can be as high as 500 young per week. A decrease in reproductive rate may be caused by a change in water quality, temperature, food quality, or brood stock health. Adult females will continue to reproduce for several months.

3. Laboratories that use mixed-age amphipods for testing must demonstrate that the procedure used to isolate amphipods will produce test organisms that are 7- to 14-d old. For example, amphipods passing through a #35 sieve (500 µm), but stopped by a #45 sieve (355 µm) will average 1.54 mm (SD 0.09) in length. The mean length of these sieved organisms corresponds to that of 6-d-old amphipods. After holding for 3 d before testing to eliminate organisms injured during sieving, these amphipods would be about 9 d old (length 1.84 mm, SD 0.11) at the start of a toxicity test.

In a different but similar method, smaller amphipods are isolated from larger amphipods using a stack of sieves: #30 (600 µm), #40 (425 µm), and #60 (250 µm). Sieves should be held under water to isolate the amphipods. Amphipods may float on the surface of the water if they are exposed to air. Artificial substrate or leaves are placed in the #30 sieve. Culture water is rinsed through the sieves and small amphipods stopped by the #60 sieve are washed into a collecting pan. Larger amphipods in the #30 and #40 sieves are returned to the culture chamber. The smaller amphipods are then placed in 1-L beakers containing culture water and food (about 200 amphipods per beaker) with gentle aeration.

Amphipods should be held and fed at a rate similar to the mass cultures for at least 2 d before the start of a test to eliminate animals injured during handling.

5.3.9 Procedure

1. The recommended 10-d sediment toxicity test with *H. azteca* must be conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 100 to 1000 lux . Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten 7- to 14-d-old amphipods per replicate are used to start a test. The 10-d test should start with a narrow range in size or age of *H. azteca* (*i.e*., 1- to 2-d range in age) to reduce potential variability in growth at the end of a 10-d test. Four replicates are recommended for routine testing. Amphipods in each test chamber are fed 1.0 mL of YCT food daily. Each chamber receives 2 volume additions/d of overlying water. Water renewals may be manual or automated. Conditions for conducting a 10-d sediment toxicity test are summarized in Table 5.10 below.

Table 5.10. Recommended Test Conditions for Conducting 10-d Sediment Toxicity Tests

Parameter	Conditions
1. Test type:	Whole-sediment toxicity test with renewal of overlying water
2. Temperature:	23°± 1°C
3. Light quality:	Wide-spectrum fluorescent lights
4. Illuminance:	100 to 1000 lux
5. Photoperiod:	16L:8D
6. Test chamber:	300 mL high-form lipless beaker
7. Sediment volume	100 mL
8. Overlying water volume:	175 mL
9. Renewal of overlying water:	2 volume additions/d; continuous or intermittent (*e.g*., 1 volume addition every 12 h)
10. Age of organisms:	7- to 14-d old at the start of the test (1- to 2-d range in age)
11. Number of organisms/ chamber:	10
12. Replicate chambers/treatment:	4 recommended
13. Feeding:	YCT food, fed 1.0 mL daily (1800 mg/L stock) to each test chamber.
14. Aeration:	None unless DO in overlying water drops below 2.5 mg/L
15. Test duration:	10 d
16. Endpoint:	Survival
17. Test acceptability:	Min. mean control survival of 80%.

2. Introduction of Sediment (Day -1). The day before the sediment test is started each sediment should be thoroughly homogenized and added to the test chambers. Each test chamber should contain the same amount of sediment, determined either by volume or by weight. Overlying water is added to the chambers on Day -1 in a manner that minimizes suspension of sediment. This can be accomplished by gently pouring water along the sides of the chambers or by pouring water onto a baffle (*e.g.*, a circular piece of Teflon® with a handle attached) placed above the sediment to dissipate the force of the water. A test begins when the organisms are added to the test chambers (Day 0).
3. Addition of Amphipods (Day 0). Amphipods should be introduced into the overlying water below the air-water interface. Test organisms can be pipetted directly into overlying water. The size of the test organisms at the start of the test should be measured using the same measure (length or weight) that will be used to assess their size at the end of the test. For length, a minimum of 20 organisms should be measured. For weight measurement, a larger sample size (*e.g.*, 80) may be desirable because of the relative small mass of the organisms. Test organisms should be handled as little as possible.
4. Feeding (Day 0 to Day 9). For each beaker, 1.0 mL of YCT is added from Day 0 to Day 9. Suspensions of food should be thoroughly mixed before aliquots are taken. In some instances, the addition of the food may alter the availability of the contaminants in the sediment. Furthermore, if too much food is added to the test chamber or if the mortality of test organisms is high, fungal or bacterial growth may develop on the sediment surface. Therefore, the amount of food added to the test chambers is kept to a minimum.
5. Ending a Test (Day 10).Any of the surviving amphipods in the water column or on the surface of the sediment can be pipetted from the beaker before sieving the sediment. Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. A #40 sieve (425-µm mesh) can be used to remove amphipods from sediment. Alternatively, sieving of sediment can be accomplished as follows: (1) pour about half of the overlying water through a #50- (300-µm) mesh sieve, (2) swirl the remaining water to suspend the upper 1 cm of sediment, (3) pour this slurry through the #50-mesh sieve and wash the contents of the sieve into an examination pan, (4) rinse the coarser sediment remaining in the test chamber through a #40- (425-µm) mesh sieve and wash the contents of this second sieve into a second examination pan. Surviving test organisms are removed from the two pans and counted. A summary of the procedure is in Table 5.11.

Table 5.11. General Activity Schedule for Conducting 10-d Sediment Toxicity Test

Day	Activity
Day -7	Separate known-age amphipods from the cultures and place in holding chambers. Begin preparing food for the test. There should be a 1- to 2-d range in age of amphipods used to start the test.
Days -6 to -2	Feed and observe isolated amphipods, monitor water quality (*e.g.*, temperature and dissolved oxygen).
Day -1	Feed and observe isolated amphipods, monitor water quality. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
Day 0	Measure total water quality (pH, temperature, dissolved oxygen, hardness, alkalinity, conductivity, ammonia). Transfer 10 7- to 14-day-old amphipods into each test chamber. Release organisms under the surface of the water. Add 1.0 mL of YCT into each test chamber. Archive 20 test organisms for length determination or archive 80 test organisms for dry weight determination. Observe behavior of test organisms.
Days1 to 8	Add 1.0 mL of YCT food to each test chamber. Measure temperature and dissolved oxygen. Observe behavior of test organisms.
Day 9	Measure total water quality.
Day 10	Measure temperature and dissolved oxygen. End the test by collecting the amphipods with a sieve. Count survivors and prepare organisms for weight or length measurements.

6.0 INFAUNAL BENTHIC MACROINVERTEBRATE COMMUNITIES

6.1 SCOPE AND APPLICATION

This method describes the laboratory procedures used to measure the species composition and abundance of macroinvertebrate fauna found in estuarine or freshwater sediments. The procedure is designed to produce data of consistent quality meeting the measurement quality objectives (MQOs) of 10% total error for the extraction of organisms and 10% total error for the identification and enumeration of extracted fauna (Section 6.6). Upon request to the EPA, and subsequent approval of alternate methods, performance-based evaluations may also be utilized to qualify participating labs.

6.2 SUMMARY OF METHOD

Sediment grab samples will be sieved (0.5 mm mesh; 1.0 mm mesh for CA, OR, and WA) in the field and preserved in 10% buffered formalin prior to shipping to the laboratory. Preserved samples are sorted, identified, and enumerated to the lowest practical taxonomic level (genus or species) using standardized keys and references.

6.3 SAMPLE STORAGE AND TREAMENT

Samples will be preserved in 10% buffered formalin.

Samples should be stored in a cool, dry area away from direct sunlight.

6.4 SORTING

1. Sort all samples under a minimum of 6X dissecting microscope.
2. Remove the macroinvertebrates with forceps and place in internally labeled vials containing 70-80% denatured ethanol. Remove taxa such as polychaetes, oligochaetes, bivalves, gastropods, crustaceans, *etc*. Do not remove empty snail or bivalve sheels; surface-dwelling or strictly pelagic arthropod taxa such as Collembola, Veliidae, Gerridae, Notonectidae, Corixidae, Cladocera, or Copepoda; or terrestrial taxa. Also, do not remove fragments such as legs, antennae, gills, or wings.
3. Once the QC check of the material in the pan has been completed, remove the material from the pan and place it in a separate container with preservative (70-80% ethanol). The container must be appropriately labeled both inside and outside. The lid must be tightly closed and the container archived until all necessary QC checks are completed. Do not discard any sample portion until instructed by the QC Officer and the Project Manager.

6.5 PROCEDURE

6.5.1 Identification and Enumeration – General

1. Empty one sample vial at a time into a Petri dish. Add preservative to keep organisms covered. View the sample under the stereo dissecting microscope.

2. Identify organisms to the lowest practical taxonomic level. Specimens that are difficult to identify will be set aside in vials and preserved for later. These specimens may require further processing (see Section 6.5.2) or a taxonomist with different area of expertise.
3. Count the number of taxa in each sample. Specimens will be identified, counted and removed from Petri dish one at a time. Remove similar organisms to other dishes to be placed in vials.
4. Specimens that can be identified only to genus, family or order will also be included in the total number of taxa in each sample (*e.g*., organisms identified to be within the family *Spionidae* will be counted as one taxon). If a specimen identified to genus, family or order can be identified as one of several taxa already identified in the sample, that organism will not be counted as an additional taxa. For example, if an organism is identified as far as belonging to *Tellinidae*, and the taxonomist believes it could be either *Macoma balthica* or *Macoma mitchelli*, both of which are present in the sample, the specimen would be recorded as *Tellinidae* and would not be included in the taxa count for that sample.
5. If organisms can be identified, they are counted ONLY if:

   The fragment included the head,

   The mollusk shell (bivalve or gastropod) is occupied by a specimen, or

   The specimen is the sole representative of a taxon in the sample.
6. If *early instar or juvenile* specimens can be identified, they are counted:

   As the same taxon if, with confidence, they can be associated with one or more mature specimens that have a more developed morphology.

   As a separate taxon if the specimen is the sole representative of a taxon in the sample.

6.5.2 Subsampling

If the laboratory believes that subsampling for a particular organism (such as Oligochaetes and Chironomids) may be appropriate for a given sample, they should contact Treda Grayson at U.S. EPA Office of Water, 202-566-0916, Grayson.Treda@epamail.epa.gov.

If the number of specimens is greater than 400, proceed as follows:

Distribute the sample on a gridded tray as evenly as possible. Select grids randomly until at least 200 specimens are identified to be mounted. Any remaining specimens in the last grid will also be identified so the total number of specimens in these samples will be slightly >200.

1. Specimens in the remaining grids will be enumerated.
2. Prepare slide mounts using appropriate media and view organisms under compound microscope.

6.6 QUALITY ASSURANCE AND QUALITY CONTROL

6.6.1 Sorting QC

1. Experienced QC Officers will use 6-10x microscopes to check **all** sorted material from the first five samples processed by a sorter to ensure that each meets the 90% sorting efficiency (SE). This will not only apply to inexperienced sorters, but also to those initially

deemed as "experienced." Qualification will only occur when sorters achieve ≥ 90% sorting efficiency for five samples consecutively.

2. The QC Officer will calculate percent sorting efficiency (PSE) for each sample as follows:

$$PSE = \frac{A}{A + B} \times 100$$

where A = number of organisms found by the primary sorter, and B = number of recoveries (organisms missed by the primary sort and found by the QC check).

If the SE for each of these five consecutive samples is ≥ 90% for an individual, this individual is considered "experienced" and can serve as a QC Officer. In the event that an individual fails to achieve ≥ 90% SE, they will be required to sort an additional five samples to continue to monitor their SE. However, if they show marked improvement in their SE prior to completion of the next five samples, whereby they acquire the ≥ 90% SE, the QA Officer may, at his/her discretion, consider this individual "experienced." SE should not be calculated for samples processed by more than one individual.

3. After individuals qualify, 10% (1 of 10, randomly selected) of their samples will be checked.
4. If an "experienced" individual fails to maintain a ≥ 90% sorting efficiency as determined by QC checks, an internal lab QC Officer will perform QC checks on every grid of five consecutive samples until a ≥ 90% sorting efficiency is achieved on all five. During this time, that individual will not be able to perform QC checks.
5. Randomly select 10% of the sample pickates (sort residue) for an internal lab QC check for missed specimens. If samples contain more than 10% of the original number of organisms found in the sample, a project facilitator will make a determination as to whether more of the samples need to be resorted (upon closer examination of the data).

6.6.2 Taxonomic QC

1. On receipt of the samples, the project facilitator will randomly select 10% of the samples to be sent to the QC taxonomist, another experienced taxonomist who did not participate in the original identifications. A chain-of-custody form will be completed and sent with the samples.
2. The QC taxonomist will perform whole-sample re-identifications, with care taken to ensure inclusion of all slide-mounted specimens, completing a separate copy of the taxonomic bench sheet for each sample. Each lab must work with the Information Management group (Marlys Cappaert, Cappaert.Marlys@epamail.epa.gov, 541-754-4467,) to ensure their bench sheets are compatible with the electronic deliverables the lab will need to submit. Each bench sheet must be labeled with the term "QC Re-ID." As each bench sheet is completed, it must be faxed to the project facilitator.
3. The project facilitator will compare the taxonomic results (counts AND identifications) generated by the primary and QC taxonomists for each sample and calculate percent disagreement in enumeration (PDE) and percent taxonomic disagreement (PTD) as measures of taxonomic precision (Stribling et al. 2003) as follows:

$$PDE = \frac{|n1 - n2|}{n1 + n2} \times 100$$

where $n1$ is the number of specimens counted in a sample by the first taxonomist and $n2$ is the number of specimens counted by the QC taxonomist.

$$PTD = \left[1 - \left(\frac{comp_{pos}}{N}\right)\right] \times 100$$

where $comp_{pos}$ is the number of agreements (positive comparisons) and N is the total number of specimens in the larger of the two counts.

4. Unless otherwise specified by project goals and objectives, the measurement quality objective for enumerations will be a mean PDE less than or equal to 5 and a mean PTD less than or equal to 15, calculated from all the samples in the 10% set sent to the QC taxonomist. Results greater than these values will be investigated and logged for indication of error patterns or trends, but all values will generally be considered acceptable for further analysis, unless the investigation reveals significant problems.
5. Corrective action will include determining problem areas (taxa) and consistent disagreements, addressing problems through taxonomist interactions. Disagreements resulting from identification to a specific taxonomic level, creating the possibility to double-count "unique" or "distinct" taxa will also be rectified through corrective actions.
6. The project facilitator will prepare a report or technical memorandum. This document will quantify both aspects of taxonomic precision, assess data acceptability, highlight taxonomic problem areas, and provide recommendations for improving precision. This report will be submitted to the project manager, with copies sent to the primary and QC taxonomists and another copy maintained in the project file.

6.8 REFERENCES

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APPENDIX A

[The following appendix, "APPENDIX J: RECOMMENDED PROCEDURES FOR PREPARING WHOLE FISH COMPOSITE HOMOGENATE SAMPLES", has been excerpted from "Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories. Volume 1: Fish Sampling and Analysis." Third Edition. EPA 823-B-00-007. USEPA, Office of Water. November 2000.]

Note: For NCA purposes, the sections referring to fish sex and age determinations and assessment of morphological abnormalities, as well as additional references to these in the text, are not relevant and may be ignored.

APPENDIX J

RECOMMENDED PROCEDURES FOR PREPARING WHOLE FISH COMPOSITE HOMOGENATE SAMPLES

J.1 GENERAL GUIDELINES

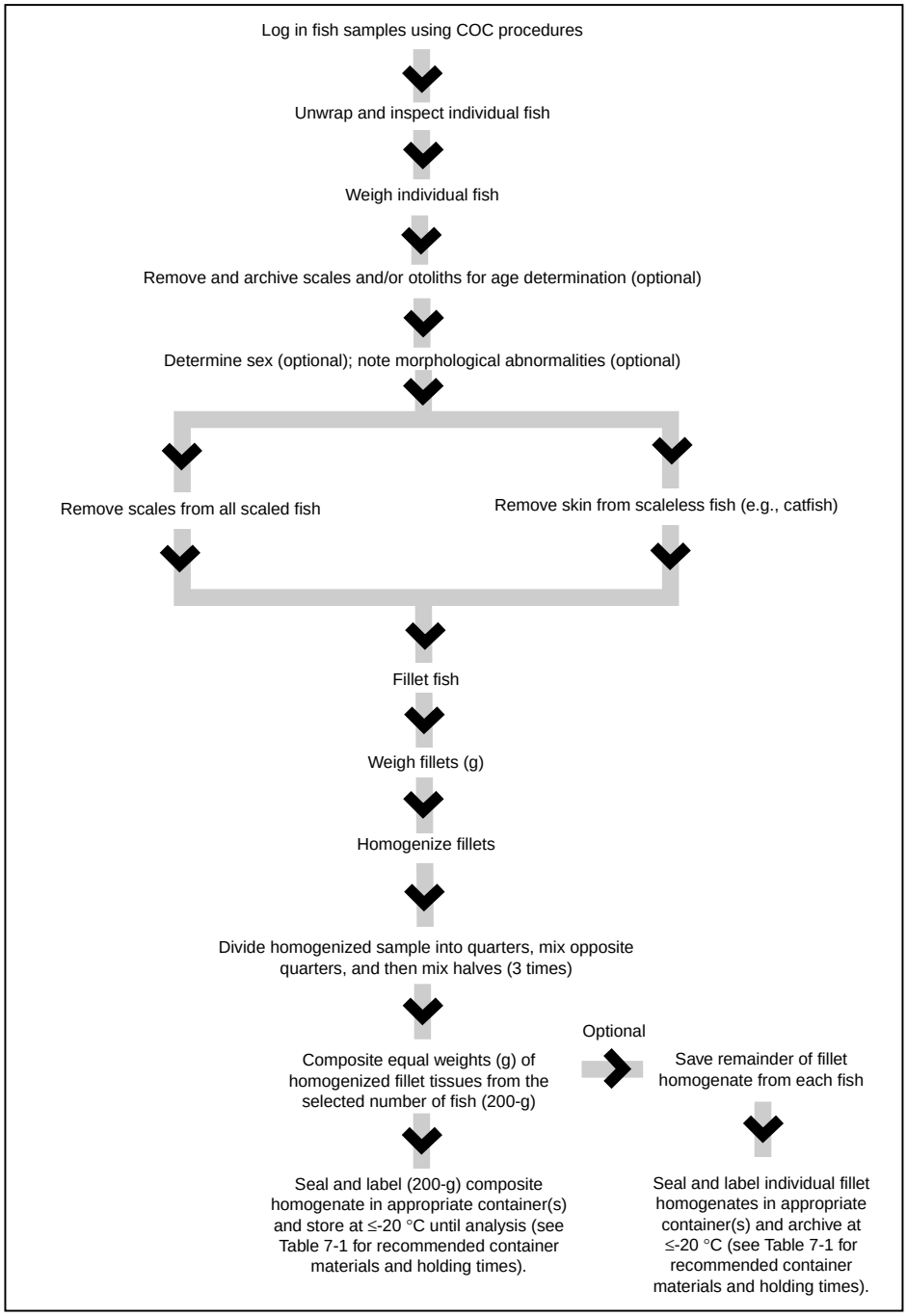
Laboratory processing to prepare whole fish composite samples (diagrammed in Figure J-1) involves

- Inspecting individual fish for foreign material on the surface and rinsing if necessary
- Weighing individual fish
- Examining each fish for morphological abnormalities (optional)
- Removing scales or otoliths for age determination (optional)
- Determining the sex of each fish (optional)
- Preparing individual whole fish homogenates
- Preparing a composite whole fish homogenate.

Whole fish should be shipped on wet or blue ice from the field to the sample processing laboratory if next-day delivery is assured. Fish samples arriving in this manner (chilled but not frozen) should be weighed, scales and/or otoliths removed, and the sex of each fish determined within 48 hours of sample collection. The grinding/homogenization procedure may be carried out more easily and efficiently if the sample has been frozen previously (Stober, 1991). Therefore, the samples should then be frozen ($\leq$-20 °C) in the laboratory prior to being homogenized.

If the fish samples arrive frozen (i.e., on dry ice) at the sample processing laboratory, precautions should be taken during weighing, removal of scales and/or otoliths, and sex determination to ensure that any liquid formed in thawing remains with the sample. **Note:** The liquid will contain target analyte contaminants and lipid material that should be included in the sample for analysis.

The thawed or partially thawed whole fish should then be homogenized individually, and equal weights of each homogenate should be combined to form the composite sample. Individual homogenates and/or composite homogenates may be frozen; however, frozen individual homogenates must be rehomogenized before compositing, and frozen composite homogenates must be rehomogenized before aliquotting for analysis. The maximum holding time from sample collection to analysis for mercury is 28 days at $\leq$-20 °C; for all other analytes, the holding time is 1 year at $\leq$-20 °C (Stober, 1991). Recommended container materials,



COC = Chain of custody.

Figure J-1. Laboratory sample preparation and handling for whole fish composite homogenate samples.

preservation temperatures, and holding times are given in Table J-1. **Note:** Holding times in Table J-1 are maximum times recommended for holding samples from the time they are received at the laboratory until they are analyzed. These holding times are based on guidance that is sometimes administrative rather than technical in nature; there are no promulgated holding time criteria for tissues (U.S. EPA, 1995b). If states choose to use longer holding times, they must demonstrate and document the stability of the target analyte residues over the extended holding times.

J.2 SAMPLE PROCESSING PROCEDURES

Fish sample processing procedures are discussed in more detail in the sections below. Each time custody of a sample or set of samples is transferred from one person to another during processing, the Personal Custody Record of the chain-of-custody (COC) form that originated in the field (Figure 6-8) must be completed and signed by both parties so that possession and location of the samples can be traced at all times (see Section 7.1). As each sample processing procedure is performed, it should be documented directly in a bound laboratory notebook or on standard forms that can be taped or pasted into the notebook. The use of a standard form is recommended to ensure consistency and completeness of the record. Several existing programs have developed forms similar to the sample processing record for whole fish composite samples shown in Figure J-2.

J.2.1 Sample Inspection

Individual fish received for filleting should be unwrapped and inspected carefully to ensure that they have not been compromised in any way (i.e., not properly preserved during shipment). Any specimen deemed unsuitable for further processing and analysis should be discarded and identified on the sample processing record.

J.2.2 Sample Weighing

A wet weight should be determined for each fish. All samples should be weighed on balances that are properly calibrated and of adequate accuracy and precision to meet program data quality objectives. Balance calibration should be checked at the beginning and end of each weighing session and after every 20 weighings in a weighing session.

Fish shipped on wet or blue ice should be weighed directly on a foil-lined balance tray. To prevent cross contamination between individual fish, the foil lining should be replaced after each weighing. Frozen fish (i.e., those shipped on dry ice) should be weighed in clean, tared, noncontaminating containers if they will thaw before the weighing can be completed. Liquid from the thawed sample must be

Table J-1. Recommendations for Container Materials, Preservation, and Holding Times for Fish, Shellfish, and Turtle Tissues from Receipt at Sample Processing Laboratory to Analysis

Analyte	Matrix	Sample container	Storage	
			Preservation	Holding time[a]
Mercury	Tissue (whole specimens, homogenates)	Plastic, borosilicate glass, quartz, and PTFE	Freeze at ≤-20 °C	28 days[b]
Other metals	Tissue (whole specimens, homogenates)	Plastic, borosilicate glass, quartz, and PTFE	Freeze at ≤-20 °C	6 months[c]
Organics	Tissue (whole specimens, homogenates)	Borosilicate glass, quartz, PTFE, and aluminum foil	Freeze at ≤-20 °C	1 year[d]
Metals and organics	Tissue (whole specimens, homogenates)	Borosilicate glass, quartz, and PTFE	Freeze at ≤-20 °C	28 days (mercury); 6 months (for other metals); and 1 year (for organics)
Lipids	Tissue (whole specimens, homogenates)	Plastic, borosilicate glass, quartz, PTFE	Freeze at ≤-20 °C	1 year

PTFE = Polytetrafluoroethylene for Teflon.

[a] Maximum holding times recommended by U.S. EPA (1995b).

[b] This maximum holding time is also recommended by the Puget Sound Estuary Program (1990). The California Department of Fish and Game (1990) and the USGS National Water Quality Assessment Program (Crawford and Luoma, 1993) recommend a maximum holding time of 6 months for all metals, including mercury.

[c] This maximum holding time is also recommended by the California Department of Fish and Game (1990), the 301(h) monitoring program (U.S. EPA, 1986), and the USGS National Water Quality Assessment Program (Crawford and Luoma, 1993). The Puget Sound Estuary Program (1990) recommends a maximum holding time of 2 years.

[d] This maximum holding time is also recommended by the Puget Sound Estuary Program (1990). The California Department of Fish and Game (1990) and the USGS National Water Quality Assessment Program (Crawford and Luoma, 1993) recommend a more conservative maximum holding time of 6 months. EPA (1995a) recommends a maximum holding time of 1 year at ≤-10 °C for dioxins and dibenzofurans.

Sample Processing Record for Fish Contaminant Monitoring Program — Whole Fish Composites

Project No. _____ Sampling Date and Time: _____

STUDY PHASE: Screening ☐; Intensive: Phase I ☐ Phase II ☐

SITE LOCATION

Site Name/Number: _____

County/Parish: _____ Lat./Long.: _____

State Waterbody Segment Number: _____ Waterbody Type: _____

Bottom Feeder – Species Name: _____

Composite Sample #: _____ Number of Individuals: _____

Fish #	Weight (g)	Scales/Otoliths Removed (✓)	Sex (M, F)	Homogenate Prepared (✓)	Weight of homogenate taken for composite (g)
001					
002					
003					
004					
005					
006					
007					
008					
009					
010					
Analyst Initials/Date	/	/	/	/	/

Total Composite Homogenate Weight _____

Predator – Species Name: _____

Composite Sample #: _____ Number of Individuals: _____

Fish #	Weight (g)	Scales/Otoliths Removed (✓)	Sex (M, F)	Homogenate Prepared (✓)	Weight of homogenate taken for composite (g)
001					
002					
003					
004					
005					
006					
007					
008					
009					
010					
Analyst Initials/Date	/	/	/	/	/

Total Composite Homogenate Weight _____

Notes: _____

Figure J-2. Example of a sample processing record for fish contaminant monitoring program—whole fish composites.

kept in the container as part of the sample because it will contain lipid material that has separated from the tissue (Stober, 1991).

All weights should be recorded to the nearest gram on the sample processing record and/or in the laboratory notebook.

J.2.3 Age Determination

Age provides a good indication of the duration of exposure to pollutants (Versar, 1982). A few scales or otoliths (Jearld, 1983) should be removed from each fish and delivered to a fisheries biologist for age determination. For most warm water inland gamefish, 5 to 10 scales should be removed from below the lateral line and behind the pectoral fin. On softrayed fish such as trout and salmon, the scales should be taken just above the lateral line (WDNR, 1988). For catfish and other scaleless fish, the pectoral fin spines should be clipped and saved (Versar, 1982). The scales, spines, or otoliths may be stored by sealing them in small envelopes (such as coin envelopes) or plastic bags labeled with, and cross-referenced by, the identification number assigned to the tissue specimen (Versar, 1982). Removal of scales, spines, or otoliths from each fish should be noted (by a check mark) on the sample processing record.

J.2.4 Sex Determination (Optional)

To determine the sex of a fish, an incision should be made on the ventral surface of the body from a point immediately anterior to the anus toward the head to a point immediately posterior to the pelvic fins. If necessary, a second incision should be made on the left side of the fish from the initial point of the first incision toward the dorsal fin. The resulting flap should be folded back to observe the gonads. Ovaries appear whitish to greenish to golden brown and have a granular texture. Testes appear creamy white and have a smooth texture (Texas Water Commission, 1990). The sex of each fish should be recorded on the sample processing record.

J.2.5 Assessment of Morphological Abnormalities (Optional)

Assessment of gross morphological abnormalities in finfish is optional. This assessment may be conducted in the field (see Section 6.3.1.5) or during initial inspection at the central processing laboratory prior to filleting. States interested in documenting morphological abnormalities should consult Sinderman (1983) and review recommended protocols for fish pathology studies used in the Puget Sound Estuary Program (1990).

J.2.6 Preparation of Individual Homogenates

To ensure even distribution of contaminants throughout tissue samples, whole fish must be ground and homogenized prior to analyses.

Smaller whole fish may be ground in a hand crank meat grinder (fish < 300 g) or a food processor (fish 300-1,000 g). Larger (>1,000 g) fish may be cut into 2.5-cm cubes with a food service band saw and then ground in either a small or large homogenizer. To avoid contamination by metals, grinders and homogenizers used to grind and blend tissue should have tantalum or titanium blades and/or probes. Stainless steel blades and probes have been found to be a potential source of nickel and chromium contamination (due to abrasion at high speeds) and should be avoided.

Grinding and homogenization of biological tissue, especially skin from whole fish samples, is easier when the tissue is partially frozen (Stober, 1991). Chilling the grinder/homogenizer briefly with a few chips of dry ice will reduce the tendency of the tissue to stick to the grinder.

The ground sample should be divided into quarters, opposite quarters mixed together by hand, and the two halves mixed back together. The grinding, quartering, and hand mixing should be repeated two more times. If chunks of tissue are present at this point, the grinding/homogenizing should be repeated. No chunks of tissue should remain because these may not be extracted or digested efficiently. If the sample is to be analyzed for metals only, the ground tissue may be mixed by hand in a polyethylene bag (Stober, 1991). Homogenization of each individual fish should be noted on the sample processing record. At this time, individual whole fish homogenates may be either composited or frozen and stored at $\leq$-20 °C in cleaned containers that are noncontaminating for the analyses to be performed (see Table J-1).

J.2.7 Preparation of Composite Homogenates

Composite homogenates should be prepared from equal weights of individual homogenates. If individual whole fish homogenates have been frozen, they should be thawed partially and rehomogenized prior to compositing. Any associated liquid should be maintained as a part of the sample. The weight of each individual homogenate that is used in the composite homogenate should be recorded, to the nearest gram, on the sample processing record.

Each composite homogenate should be blended by dividing it into quarters, mixing opposite quarters together by hand, and mixing the two halves together. The quartering and mixing should be repeated at least two more times. If the sample is to be analyzed only for metals, the composite homogenate may be mixed by hand in a polyethylene bag (Stober, 1991). At this time, the composite homogenate may be processed for analysis or frozen and stored at $\leq$-20 °C (see Table J-1).

The remainder of each individual homogenate should be archived at $\leq$-20 °C with the designation "Archive" and the expiration date recorded on the sample label. The location of the archived samples should be indicated on the sample processing record under "Notes."

It is essential that the weights of individual homogenates yield a composite homogenate of adequate size to perform all necessary analyses. Weights of individual homogenates required for a composite homogenate, based on the number of fish per composite and the weight of composite homogenate recommended for analyses of all screening study target analytes (see Table 4-1), are given in Table J-2. The total composite weight required for intensive studies may be less than in screening studies if the number of target analytes is reduced significantly.

The recommended sample size of 200 g for screening studies is intended to provide sufficient sample material to (1) analyze for all recommended target analytes (see Table 4-1) at appropriate detection limits, (2) meet minimum QA and QC requirements for the analyses of replicate, matrix spike, and duplicate matrix spike samples (see Section 8.3.3.4), and (3) allow for reanalysis if the QA and QC control limits are not met or if the sample is lost. However, sample size requirements may vary among laboratories and the analytical methods used. Therefore, it is the responsibility of each program manager to consult with the analytical laboratory supervisor to determine the actual weights of composite homogenates required to analyze for all selected target analytes at appropriate detection limits.

J.3 REFERENCES

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APPENDIX B

Statement of Work

Determination of Parent and Alkyl Polycyclic Aromatic Hydrocarbons In Environmental Samples Related to BP Oil Spill

The Environmental Protection Agency (EPA) is currently responding to multiple environmental issues as a result of the oil spill and dispersant usage in the Gulf of Mexico. Of particular concern are the environmental impacts of crude oil to the ecological systems along the Gulf coast from Texas to Florida, and possibly to upper continental coastline along the eastern United States. Potentially thousands of environmental samples will be collected and analyzed for volatile organic compounds (VOCs), metals, and semi-volatile organic compounds (SVOCs), including Polycyclic Aromatic Hydrocarbons (PAH). The SVOC analysis, to date, have only included the parent PAH compounds typically analyzed and reported by such analytical protocols as found in EPA's Solid Waste Guidance document SW-846, METHOD 8270D SEMIVOLATILE ORGANIC COMPOUNDS BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS). The EPA has now determined a need to evaluate the toxic effects of the spilled crude oil on the aquatic ecosystems in the greater Gulf area, and must expand the list of SVOCs to include alkyl substituted PAH compounds.

1.0 PURPOSE

There are limited analytical methods currently published that address the analysis of alkyl PAH. SW-846 Method 8272, which is a new method to be released in the next SW-846 update, and EPA's Contract Laboratory Program's (CLP) "Modified Analysis" protocol based upon the existing CLP SVOC method, both address analysis of alkyl PAHs. Also ASTM Method D7363-07 presents a method for determination of parent and alkyl PAH in sediment pore waters. This method, however, uses solid-phase microextraction GC/MS techniques that may not be appropriate for sea water and oily waters.

The purpose of this Statement of Work (SOW) is to provide an analytical laboratory an analytical protocol for the sample preparation and analysis of parent PAH and alkyl PAH compounds in water and sediment. The SOW is a combined protocol derived from SW-846, CLP and ASTM. Web-link references to these published methods are included in this SOW. Reporting protocols will also be incorporated into this SOW to allow the laboratory to deliver chromatograms to EPA for the purpose of "fingerprinting" in case correlation analysis needs to be made between environmental samples and the raw crude oil spilling from the BP oil platform in the Gulf.

2.0 SPECIAL REQUIREMENTS

The laboratory performing the analyses presented in this SOW must have demonstrated experience in the analysis of SVOCs, including the parent PAH and alkyl PAH compounds listed in Table 1. The laboratory should also have demonstrated experience in oil correlation analysis via GC/MS fingerprinting. The laboratory must also demonstrate the existence of a formal laboratory Quality System. This may be demonstrated by showing proof of an existing accreditation certificate issued by NELAC or proof of an existing contract issued through the EPA CLP. The laboratory must certify in writing that they have current GC/MS capability and capacity to provide for analytical results within 72 hours of sample receipt at a frequency of approximately 20 samples per day.

3.0 QUALITY ASSURANCE (QA) REQUIREMENTS

Laboratory must perform all analysis under a formal Laboratory Quality Assurance Program. At a minimum, the laboratory must have a Quality Program that address items in Sections 1.0 through 7.0 of Exhibit E taken from the EPA CLP SOM1.1 found at the following: http://www.epa.gov/superfund/programs/clp/som1.htm. The laboratory must have a QA/QC

program in place to assess the quality of the data. EPA requires that recipients of funds for work involving environmental data comply with the American National Standard ANSI/ASQC E4-1994, "Specifications and Guidelines for Quality Systems for Environmental Data Collection and Environmental Technology Programs." Information concerning quality specifications for non-EPA organizations is available at http://www.epa.gov/quality/exmural.html. EPA reserves the right to inspect all laboratory Quality Assurance documents, including method specific Standard Operating Procedures (SOPs). EPA also reserves the right to inspect all raw data and to perform on-site inspections of the laboratory during the period of performance of any contract agreed upon for this project.

4.0 DETAILED TASK DESCRIPTION

Table 1 lists the target analytes required for this SOW. Analyses are to be performed using the Selected Ion Monitoring (SIM) technique. Table 1 lists the specific ions that are to be monitored and quantified.

Table 1. Primary Ions Monitored for Each Target Analyte during GC/MS Analysis

Compound	Ion	Compound	Ion
n-alkanes (C10-C35)*	57	Pyrene P	202
Naphthalene-d8 (IS)	136	C1-Fluoranthenes/Pyrenes	216
Naphthalene P	128	C2-Fluoranthenes/Pyrenes	230
2-Methylnaphthalene P	142	C3-Fluoranthenes/Pyrenes	244
1-Methylnaphthalene P	142	1-Methylpyrene R	216
C2-naphthalenes	156	2-methylfluoranthene R	216
C3-naphthalenes	170	Pyrene-d10 (S)	212
C4-naphthalenes	184	Chrysene-d12 (IS)	240
2-Methylnaphthalene-d10(MS)	152	Chrysene P	228
Acenaphthene-d10 (IS)	162	C1-chrysenes	242
Acenaphthylene P	152	C2-chrysenes	256
Acenaphthene P	153	C3-chrysenes	270
Acenaphthylene-d8 (S)	160	C4-chrysenes	284
Fluorene-d10 (IS)	176	6-methylchrysene R	242
Fluorene P	166	Benzo (a) anthracene P	228
C1-fluorenes	180	Benzo (a) pyrene-d12 (S)	264
C2-fluorenes	194	Perylene-d12 (IS)	264
C3-fluorenes	208	Benzo(b)fluoranthene P	252
Phenanthrene-d10	188	Benzo(k)fluoranthene P	252
Phenanthrene P	178	Benzo(e)pyrene P	252
C1-phenanthrenes/anthracenes	192	Benzo(a)pyrene P	252
C2-phenanthrenes/anthracenes	206	Perylene P	252
C3-phenanthrenes/anthracenes	220	Indeno(1,2,3-cd)pyrene P	276
C4-phenanthrenes/anthracenes	234	Dibenzo(a,h)anthracene P	278
2,3-Dimethylanthracene R	206	Benzo(g,h,i)perylene P	276
1-Methylphenanthrene R	192	Hopanes (191 family)*	191
Anthracene P	178	Steranes (217 family)*	217
Anthracene-d10 (S)	188	Steranes (218 family)*	218
Fluoranthene-d10 (IS)	212		
Fluoranthene P	202		

* = Not to be quantified. For fingerprinting analysis only; IS = Internal Standard; S = Surrogate Compound; P = Parent PAH; MS = Matrix Spike Compound; R = Reference Compound

4.1 Sample Preparation

It is to be expected that water and sediment samples will be analyzed, and there is the potential that oil from the spill may be analyzed as well. Sample preparation procedures to follow for samples received under this SOW are as follows:

Water

Water samples and water samples containing slight oil contamination (visible sheen, but very little water - oil interface) should be extracted using a continuous liquid-liquid extraction technique detailed in EPA's CLP protocols for SVOCs. This can be found in Exhibit D of SOM1.1 at http://www.epa.gov/superfund/programs/clp/som1.htm. with the following exceptions: Do not use the internal standard and deuterated monitoring compounds (DMC) listed in the SOM protocol. Instead use the internal standards and surrogate compounds listed in Table 1. All references to SMO (sample management office) shall be ignored. Instead for any consultation of protocol or issues, discuss with the EPA personnel designated in the SOW. Also, there is concern that the ASTM protocol for using solid phase microextraction may not be applicable for the oil spill, and therefore the micorextraction technique is not to be used.

For samples having a definite water – oil interface, the laboratory should contact the EPA Regional sample requestor for instructions, e.g. shake sample and extract as a whole sample, or separate and analyze water and oil as separate phases.

Sediment

The laboratory should follow the Soil/Sediment protocols detailed in the EPA CLP SOM1.1. Any of the three listed techniques for sample extraction many be used. For extracts that appear "clean" after concentration, the GPC procedure given in SOM1.1 may be skipped. For sample extracts with obvious high levels of oil matrix, the alumina cleanup procedure detailed in 40 CFR Part 300, Appendix C, Section 4.6.3.1 may be used. The surrogates listed above shall still be used if cleanup is performed via the CFR protocol.

Oil

Samples received that are obvious oil samples may be analyzed using a direct dilution process. A measured aliquot of the oil sample is placed in a 10 mL volumetric flask and brought to volume with methylene chloride. The amount of oil diluted shall be at the laboratory's discretion, but the dilution shall be such that the best detection levels may be achieved while preventing undue contamination of the analytical GC column and GC/MS. The sample and methylene chloride shall be shaken and then allowed to settle until all obvious non-soluble material has settled to the bottom. An aliquot of the oil/methylene chloride extract shall be then analyzed by GC/MS. Surrogates are to be added to the oil sample aliquot before methylene chloride is added.

4.2 Instrumental Analysis

All analysis are to be made on GC/MS instrumentation and are to be made in the selected Ion Mode (SIM). The laboratory should follow the protocols given in the EPA SOM1.1 method for SVOC SIM analyses with the exception that the analytes, internal standard compounds and surrogates listed in Table 1 are to be analyzed instead of those listed in SOM1.1. All parent

PAH, alkyl PAH, and matrix spike compounds must be calculated using the internal standard method in which response factors are calculated. The laboratory must tune each GC/MS instrument to meet the criteria listed in SOM1.1. The laboratory must also calibrate target analytes and surrogates using a minimum of a 5-point calibration curve, as detailed in SOM 1.1. The laboratory must calibrate target analytes and surrogates at the concentrations listed in Table 2. The 5-point calibration analysis is to be performed before any samples are analyzed under this SOW. Each parent PAH and surrogate listed in Table 1 is to be calibrated against the internal standard listed directly above it in Table 1. For example, anthracene is to be calibrated using phenanthrene as the internal standard. The % RSD should be calculated for each PAH using the instructions given in SOM1.1. Each parent PAH should have a %RSD no greater than 20%. After 15 samples, including QC samples, have been analyzed, or in situations in which the GC/MS has been inoperative for 6 hours or longer, a calibration verification analysis must be made to ensure the applicability of the 5-point calibration curve. The lab must analyze the single point calibration verification at a concentration level consistent with either Level 3 or Level 4 in Table 2. After the calibration verification analysis, the laboratory should calculate the %Difference according to instructions in SOM1.1. The % Difference must be no greater than **+/- 30%.** If the % Difference is greater than 30%, the laboratory may reanalyze the calibration verification again. If the instrument can still not meet the %Difference criteria, the laboratory must perform instrument maintenance and analyze another 5-point calibration analysis.

There are no 5-point calibration or continuing calibration criteria for the alkyl PAH compounds. Alkyl PAH compounds are calculated against the internal standard listed directly above the alkyl PAH in Table 1. The same response factor used for the parent PAH is to be used for the alkyl PAH associated with the parent PAH. Specifically: C2, C3, and C4 naphthalenes will use the same response factor as naphthalene; C1, C2, and C3 fluorenes will use the same response factor as fluorene; C1, C2, C3 and C4 phenanthrenes/anthracenes shall use the same response factor as phenanthrene; C1, C2, and C3 fluoranthenes/pyrenes shall use the same response factor as fluoranthene; C1, C2, C3 and C4 chrysenes shall use the same response factor as chrysene.

** Reference compounds have been added to the list of analytes that are to be included in calibration analysis. A response factor for each of the reference compounds is to be calculated at each calibration level. The response factor will be used for informational purposes only. The reference compound is not to be specifically analyzed for in the environmental samples. For example, 1-methylpyrene should be included in the calibration analysis and a response factor is to be determined for the compounds using Fluoranthene-d10 as the internal standard. When the environmental samples have are analyzed only the total C1-Fluoranthenes/Pyrenes will be calculated using the response factor that was calculated for pyrene.

Table 2. Calibration Concentrations for Target PAH and Surrogate Compounds: Initial 5-Point Calibration

Compound Class	Calibration Level 1 (ng/µL)	Calibration Level 2 (ng/µL)	Calibration Level 3 (ng/µL)	Calibration Level 4 (ng/µL)	Calibration Level 5 (ng/µL)
PAH	0.10	0.20	0.40	0.80	1.0
Surrogates	0.10	0.20	0.40	0.80	1.0
Internal Standards	0.40	0.40	0.40	0.40	0.40
Reference Compounds	0.10	0.20	0.40	0.80	1.0

SOW Required Detection Limits:

For this SOW, the instrument and sample preparation scheme must be such that a Reporting limit of 0.1 µg/L can be achieved for water samples. Sediment samples must meet a reporting limit of 4 µg/kg. For oily samples requiring only dilution with methylene chloride for sample preparation, the laboratory should strive to reach as low of a reporting limit as feasible.

4.3 Special Quality Control Analyses

The laboratory must ensure their analytical systems are in control. For each batch of 15 samples, the laboratory must analyze reagent blanks for each matrix analyzed. The laboratory shall ensure that no PAH constituents are present in the laboratory blank analysis. If the blank samples are contaminated with PAH, the laboratory must re-analyze any samples associated with the blanks, at no additional cost to the government.

For every batch of 15 samples, a matrix spike (MS) and matrix spike duplicate (MSD) must be analyzed. The MS/MSD pair shall consist of 2-methylnaphthalene-d10. The concentration of the compound is left to the discretion of the laboratory, but should be at a concentration that falls within the mid-range level of the 5-point calibration curve. Percent recovery is calculated for each MS and MSD. The MS and MSD percent recoveries must lie between 50 – 150% recovery. If this criterion is not met, all samples associated with the MS/MSD shall be reanalyzed at no additional cost to the government.

5.0 DELIVERABLES

Copies of all raw and calculated analytical data associated with this SOW shall remain in possession of the laboratory at least until 365 days from the time results have been submitted to EPA.

A copy of calculated results for the parent PAH and alkyl PAH compounds shall be submitted via electronic spreadsheet to each EPA recipient listed in the Task Order directive from EPA. These results must contain at a minimum the results of the PAH analysis, as well as results for surrogate analysis. A copy of the 5-point calibration analysis must be included with the tabulated results of the sample analysis. This must include the calculated response factors for each parent PAH, each surrogate, and each reference compound. Also, electronic pdf copies of various chromatograms shall also be submitted with the calculated results. The chromatograms required are the following:

(a) Total ion chromatogram in which all compounds present in the sample, including internal standards and surrogates are shown.
(b) Extracted ion chromatogram detailing the fingerprint of the m/z 57 mass ion, which is characteristic of normal and branched alkanes. The chromatogram retention time window must be such that normal alkanes from C10 through 35 are displayed in a single chromatogram.
(c) Extracted ion chromatogram detailing the fingerprint of the m/z 57 mass ion characteristic of the normal alkanes C17 and C18, and the branched alkanes, Pristane and Phytane. The chromatogram retention time window must be set to start 0.5 minutes before the elution of C17 and end 0.5 minutes after the elution of Phytane.
(d) Extracted ion chromatogram detailing the fingerprint of the m/z 156 mass ion, which is characteristic of the C2- naphthalenes. These compounds should lie retention time wise

between C14 and C15 normal alkanes. For the pattern recognition of these compounds see page 16 of the ASTM D7363-07 method.

(e) Extracted ion chromatogram detailing the fingerprint of the m/z 170 mass ion, which is characteristic of the C3- naphthalenes. These compounds should lie retention time wise between C15 and C16 normal alkanes. For the pattern recognition of these compounds see page 16 of the ASTM D7363-07 method.

(f) Extracted ion chromatogram detailing the fingerprint of the m/z 184 mass ion, which is characteristic of the C4- naphthalenes. These compounds should lie retention time wise between C16 and C17 normal alkanes. For the pattern recognition of these compounds see page 16 of the ASTM D7363-07 method. If an internal standard or surrogate compound containing mass 184 also elutes in this time frame, normalize the height of the chromatogram on the C4 naphthalenes.

(g) Extracted ion chromatogram detailing the fingerprint of the m/z 180 mass ion, which is characteristic of the C1-fluorenes. For the pattern recognition of these compounds see page 19 of the ASTM D7363-07 method.

(h) Extracted ion chromatogram detailing the fingerprint of the m/z 194 mass ion, which is characteristic of the C2-fluorenes. For the pattern recognition of these compounds see page 19 of the ASTM D7363-07 method.

(i) Extracted ion chromatogram detailing the fingerprint of the m/z 208 mass ion, which is characteristic of the C3-fluorenes. For the pattern recognition of these compounds see page 19 of the ASTM D7363-07 method. If an internal standard or surrogate compound containing mass 208 also elutes in this time frame, normalize the height of the chromatogram on the C3-fluorenes.

(j) Extracted ion chromatogram detailing the fingerprint of the m/z 192 mass ion, which is characteristic of the C1-phenanthrenes. These compounds should lie retention time wise between C19 and C20 normal alkanes. For the pattern recognition of these compounds see page 20 of the ASTM D7363-07 method.

(k) Extracted ion chromatogram detailing the fingerprint of the m/z 206 mass ion, which is characteristic of the C2-phenanthrenes. These compounds should lie retention time wise between C20 and C21 normal alkanes. For the pattern recognition of these compounds see page 20 of the ASTM D7363-07 method.

(l) Extracted ion chromatogram detailing the fingerprint of the m/z 220 mass ion, which is characteristic of the C3-phenanthrenes. These compounds should lie retention time wise between C21 and C22 normal alkanes. For the pattern recognition of these compounds see page 20 of the ASTM D7363-07 method.

(m) Extracted ion chromatogram detailing the fingerprint of the m/z 234 mass ion, which is characteristic of the C4-phenanthrenes. These compounds should lie retention time wise between C22 and C23 normal alkanes. For the pattern recognition of these compounds see page 20 of the ASTM D7363-07 method.

(n) Extracted ion chromatogram detailing the fingerprint of the m/z 216 mass ion, which is characteristic of the C1-Fluoranthenes.

(o) Extracted ion chromatogram detailing the fingerprint of the m/z 230 mass ion, which is characteristic of the C2-Fluoranthenes.

(p) Extracted ion chromatogram detailing the fingerprint of the m/z 244 mass ion, which is characteristic of the C3-Fluoranthenes.

(q) Extracted ion chromatogram detailing the fingerprint of the m/z 242 mass ion, which is characteristic of the C1-chrysenes.

(r) Extracted ion chromatogram detailing the fingerprint of the m/z 256 mass ion, which is characteristic of the C2-chrysenes.

(s) Extracted ion chromatogram detailing the fingerprint of the m/z 270 mass ion, which is characteristic of the C3-chrysenes.

(t) Extracted ion chromatogram detailing the fingerprint of the m/z 284 mass ion, which is characteristic of the C4-chrysenes.

(u) Extracted ion chromatogram detailing the fingerprint of the m/z 191 mass ion, which is characteristic of the 27 through 34 carbon hopane family.

(v) Extracted ion chromatogram detailing the fingerprint of the m/z 217 mass ion, which is characteristic of the 27 through 29 carbon alpha, beta sterane family.

(w) Extracted ion chromatogram detailing the fingerprint of the m/z 218 mass ion, which is characteristic of the 27 through 29 carbon alpha, beta sterane family.

One should reference a journal citation or petroleum geochemistry for the retention time location of the sterane and hopane families. As an example: Daling and Faksness: *Environmental Forensics* (2002) 3, pp. 263-278; Improved and Standardized Methodology for Oil Spill Fingerprinting.

A reference grade crude oil sample shall be diluted in methylene chloride and analyzed using the exact GC and GC/MS conditions that are used to detect, quantify, and fingerprint the analytes given above. The laboratory must use the South Louisiana Sweet Crude oil sample provided by RT Corporation, Laramie, WY. If the laboratory does not possess this crude oil, they must procure a sample from RTC. This reference sample shall be used to determine exact retention times and patterns for the PAH homologues and hopane and sterane families.

Every effort should be made to ensure that the chromatograms are presented in such a manner as to be able to easily distinguish each individual homologue of the alkylated PAH series and the hopane and sterane families.

5.1 Laboratory Deliverable Turnaround times

Quantified spreadsheet results must be delivered to EPA within 72 hours of receipt of sample. The required fingerprint chromatograms must be delivered to EPA within 5 days of receipt of samples.

APPENDIX C

Summary of EPA Analytical Methods for Dispersant Analysis

Summary of EPA Analytical Methods for Dispersant Analysis

Compound	CAS	EPA Method ID	Technology	Reporting Limits	EPA Benchmark
Propylene Glycol	57-55-6	EPA SW 846 Modified 8270	Direct Inject GC/MS	500 ug/L	500,000 ug/L
2-Butoxyethanol	111-76-2	EPA R5/6 LC	Direct Inject LC/MS/MS	125 ug/L	165 ug/L
Di(Propylene Glycol) Butyl Ether	29911-28-2	EPA R5/6 LC	Direct Inject LC/MS/MS	1 ug/L	ND
2-Ethylhexanol	104-76-7	EPA SW 846 Method 8260	Heated purge GC/MS	10 ug/L	ND
Dioctylsulfosuccinate, sodium salt	577-11-7	EPA RAM-DOSS	LC/MS/MS	20 ug/L	40 ug/L

CAS: Chemical Abstract Service number
ND: Not determined at this time
SW 846: "Test Methods for Evaluating Solid Waste, Physical/Chemical Methods"
See (http://www.epa.gov/epawaste/hazard/testmethods/sw846/index.htm)
LC/MS/MS: Liquid Chromatograph with Tandem MassSpectrometry