Surface Water Ambient Monitoring Program (SWAMP)

Quality Assurance Project Plan

for

Screening of cyanotoxins in lakes/reservoirs and coastal wetlands in the San Diego Region

San Diego Regional Water Quality Control Board
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GROUP A ELEMENTS: PROJECT MANAGEMENT

Quality Assurance Project Plan

For

Screening of cyanotoxins in lakes/reservoirs and coastal wetlands in the San Diego Region

San Diego Regional Water Quality Control Board

Version 3.0

June 11, 2013

APPROVAL SIGNATURES

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Title:	Name:	Signature:	Date*:
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Field Sampling Coordinator	Carey Nagoda	On file	June 13, 2013

STATE BOARD (SWRCB):

Title:	Name:	Signature:	Date*:
SWAMP Database Manager (SWRCB/SWAMP)	Stacey Swenson	On file	June 26, 2013
SWAMP QA Officer (SWRCB/SWAMP)	Beverly van Buuren	On file	June 12, 2013

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Title:	Name:	Signature:	Date*:
WPCL QC Officer	Gail Cho	On file	June 26, 2013

2. Table of Contents

Group A Elements: Project Management…………………………………………………2 of 231

1. Title and Approval Sheets………………………………………………….............3
2. Table of Contents……………………………………………………………………...4
3. Distribution List………………………………………………………….............7
4. Project/Task Organization………………………………………………………………7
5. Problem Definition/Background…………………………………………………………9
6. Project/Task Description…………………………………………………………..12
7. Quality Objectives and Criteria for Data Measurement…………………….............21
8. Special Training Needs/Certification…………………………………...............25
9. Documents and Records……………………………………………….............25

Group B Elements: Data Generation and Acquisition……………………………………...27

10. Sampling Process Design………………………………………………………….27
11. Sampling Methods…………………………………………………………………...28
12. Sample Handling and Custody………………………………………………….31
13. Analytical Methods and Field Measurements……………………………………..33
14. Quality Control……………………………………………………………………35
15. Instrument/Equipment Testing, Inspection, and Maintenance………………………37
16. Instrument/Equipment Calibration and Frequency………………………………..38
17. Inspection/Acceptance of Supplies and Consumables……………………………….38
18. Non-Direct Measurements (Existing Data)…………………………………….38
19. Data Management………………………………………………………………………39

Group C Elements: Assessment and Oversight………………………………………………40

20. Assessments & Response Actions……………………………………………...40
21. Reports to Management…………………………………………………………...40

Group D Elements: Data Validation and Usability…………………………………………41

22. Data Review, Verification, and Validation Requirements…………………………...41
23. Verification and Validation of Methods……………………………………………..41
24. Reconciliation with User Requirements……………………………………………..42

References………………………………………………………………………………..43

List of Tables

4-1. Personnel responsibilities…………………………………………………………….7
5-1. WHO guidelines for algae and cyanobacteria in fresh water……………………….11
5-2. Action levels for cyanotoxins under selected scenarios…………………………….12
6-1. List of potential lakes/reservoirs and coastal wetlands for cyanotoxin sampling……………………………………………………………………………….14
6-2. Alternate lakes/reservoirs and coastal wetlands for cyanotoxin sampling………….15
6-3. Hydrologic units and subareas of the water bodies chosen for cyanotoxin sampling……………………………………………………………………………….18
7-1. Measurement of analyses type and applicable data quality indicators……………...21
7-2. Data quality objectives for field measurements…………………………….…………24
7-3. Data quality objectives for laboratory measurements…………………….………...24
11-1. Sample preparation requirements per site……………………………….………...30
12-1. Sample preservation and storage requirements…………………………….……...32
13-1. Specifications for water quality parameters measured with Quanta Hydrolab……………………………………………………………………………33
13-2. Specifications for alkalinity and salinity field measurements…………….............34
13-3. Laboratory analytical methods……………………………………………………35
14-1. Quality control checks and information provided…………………………………36
19-1. Parties responsible for entering data into SWAMP………………………………..39

List of Figures

4-1. Organizational chart…………………………………………………………….9
6-1. Project schedule……………………………………………………….............17
6-2. San Diego Region location map…………………………………………………18
6-3. Proposed cyanotoxin sampling locations of lakes/reservoirs in the San Diego Region……………………………………………………………………………19
6-4. Proposed cyanotoxin sampling locations of coastal wetlands in the San Diego Region……………………………………………………………………………20

Appendices

APPENDIX A: Chain-of-Custody (COC) form………………………………..............45
APPENDIX B: Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1 (Fetscher et al., 2012)…………………………..…………47
APPENDIX C: Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis (EPA Method 440.0)……………..84
APPENDIX D: Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis (EPA Method 365.5)………………………………...96
APPENDIX E: Methods of Analysis by the U.S. Geological Survey National Water Quality Laboratory. Evaluation of Alkaline Persulfate Digestion as an Alternative to Kjeldahl Digestion for Determination of Total and Dissolved Nitrogen and Phosphorous in Water (USGS Method I-2650-03)……………………………………………...106

APPENDIX F: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Single Reagent) (EPA Method 365.2)……………………………………………………………………107
APPENDIX G: Phosphorous, Total (Colorimetric, Automated, Block Digester AA II) (EPA Method 365.4)……………………………………………………………………115
APPENDIX H: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent) (EPA Method 365.3)……………………………………………………………...….121
APPENDIX I: Determination of Ammonia Nitrogen by Semi-Automated Colorimetry (EPA Method 350.1)…………………………………………………………….....126
APPENDIX J: Nitrogen, Nitrate-Nitrite (Colorimetric, Automated, Cadmium Reduction) (EPA Method 353.2)…………………………………………………………….....142
APPENDIX K: In Vitro Determination of Chlorophyll a and Pheophytin a in Marine and Freshwater Algae by Fluorescence (EPA Method 445.0)…………………………...…151
APPENDIX L: Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments (Van Heukelem and Thomas, 2001)…………………………………………………....175
APPENDIX M: Method validation of microcystins in water and tissue by enhanced liquid chromatography tandem mass spectrometry (Mekebri et al., 2009)………...…..195
APPENDIX N: Characterization and deployment of Solid Phase Adsorption Toxin Tracking (SPATT) resin for monitoring of microcystins in fresh and saltwater (Kudela, 2011)……………………………………………………………………………....205
APPENDIX O: HACH Method 8203, Alkalinity…………………………................215
APPENDIX P: Field sheets and checklists……………………………………..............222
APPENDIX Q: Approval Signatures…………………………………………………...225

3. Distribution List

The key personnel to oversee the implementation of the QAPP are listed below.

Title:	Name (Affiliation):	Tel. No.:
Project Director and Contract Manager	Lilian Busse (SD RWQCB)	(858) 736-7332
Project Manager	Meredith Howard (SCCWRP)	(714) 755-3263
SWAMP Database Manager	Stacey Swenson (Moss Landing)	(831) 771-4114
SWAMP QA Officer	Beverly van Buuren (Moss Landing)	(206) 297-1378
SWAMP QA Specialist	Eric von der Geest (Moss Landing)	(831) 771-4400
WPCL QC Officer	Gail Cho (DFW-WPCL)	(916) 358-2858
Field Sampling Coordinator	Carey Nagoda (SD RWQCB)	(858) 627-3933

4. Project/Task Organization

4.1 Involved parties and roles

This section of the QAPP identifies the management elements of the cyanotoxin study. It includes a description of the staff organization, tasks involved in implementing this study, and the roles and responsibilities of the contributing parties. The Project QA managers will advise on the project but will not participate in the day to day execution of the study. Table 4-1 lists the personnel and corresponding responsibilities for completing this study.

Table 4-1. Personnel responsibilities

Name	***Organizational Affiliation***	***Role***	***Contact Information (Telephone & e-mail address)***
Lilian Busse	SD RWQCB	Project Director Contract Manager	(858) 736-7332 Lilian.Busse@waterboards.ca.gov
Meredith Howard	SCCWRP	Project Manager	(714) 755-3263 meredithh@sccwrp.org
Stacey Swenson	SWRCB/Moss Landing	SWAMP Database Manager	(831) 771-4114 sswenson@mlml.calstate.edu
Beverly van Buuren	SWRCB/Moss Landing	SWAMP QA Officer	(206) 297-1378 bvanbuuren@mlml.calstate.edu
Eric von der Geest	SWRCB/Moss Landing	SWAMP QA Specialist	(831) 771-4400 evondergeest@mlml.calstate.edu
Gail Cho	DFW-WPCL	WPCL QC Officer	(916) 358-2858 Gail.Cho@wildlife.ca.gov
Carey Nagoda	SD RWQCB	Field Sampling Coordinator	(858) 627-3933 Carey.Nagoda@waterboards.ca.gov

Lilian Busse (SD RWQCB) will serve as the project director and contract manager. The project director will review, evaluate and approve the study design and sample site locations, coordinate with other monitoring efforts in the study areas, develop reporting deadlines, and verify

completion of all tasks. As contract manager, Lilian will monitor laboratory contract progress, authorize payments, and maintain records.

Meredith Howard (SCCWRP) is the project manager and is responsible for providing technical assistance for the preparation of field sampling and coordination of laboratory activities. The duties include overseeing the collection and storage of samples, assisting in the implementation of field components, and managing all laboratory activities for the analysis of nutrients, chlorophyll-a, pigments, and continuous cyanotoxin samplers.

Stacey Swenson, Beverly van Buuren and **Eric von der Geest** (Moss Landing) will manage the Surface Water Ambient Program (SWAMP) database activities for this project. They will be responsible for providing the project team with necessary templates for data input and verify that the quality assurance and quality control procedures found in this QAPP meet the standards developed for SWAMP as set forth in the Electronic Template for EPA QAPP guidelines and the SWAMP Measurement Quality Objectives (MQOs).

Gail Cho (DFW-WPCL) will serve as the QC Officer for all laboratory analyses conducted at the WPCL. Duties include overseeing cyanotoxin filter analyses (LC-MS methods) and data management. The QC Officer will ensure that the QAPP guidelines are being met and request corrective actions when necessary.

Carey Nagoda (SD RWQCB) will coordinate all field sampling efforts for this project. Duties include developing the schedule for the field team, maintaining adequate supplies and equipment, conducting the sampling, and ensuring proper sample preservation and shipment to appropriate laboratories. The Field Sampling Coordinator is responsible for keeping all field records and entering the field-generated data into the SWAMP database.

4.2 Quality Assurance Officer role

The Quality Assurance Officer will be responsible for maintaining the QAPP and for ensuring that personnel have the most current approved version of the QAPP. Prior to conducting any sampling activities, the Quality Assurance Officer shall coordinate with the project team to ensure all mandatory QA protocols are understood, SWAMP templates are prepared, and all necessary chain-of-custody (COC) and analysis authorization (AA) forms are generated.

4.3 Persons responsible for QAPP update and maintenance

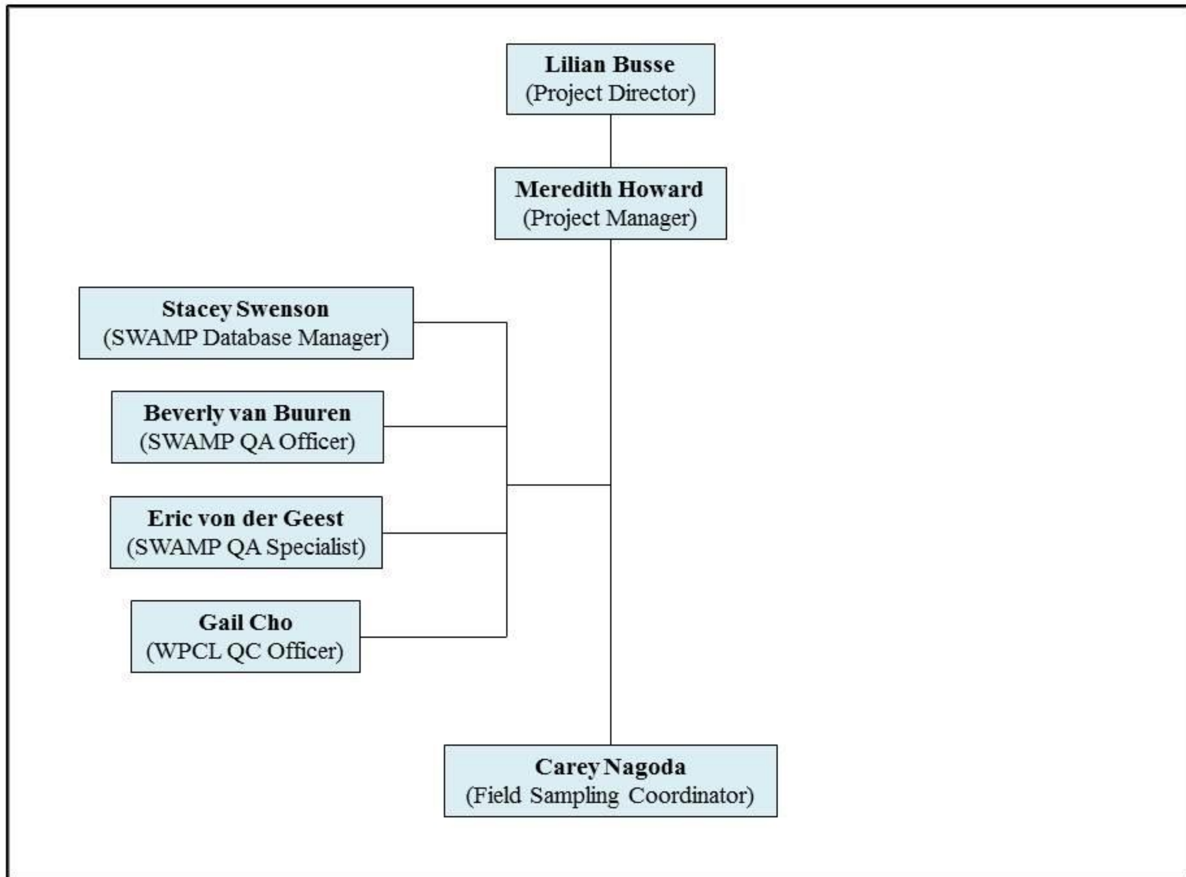
The Project Director (Lilian Busse) in association with the QA Specialist (Eric von der Geest) will be responsible for updating the QAPP. Lilian Busse will submit drafts for review, distribute updates and/or changes to the project team, and submit the final copy for signatures.

4.4 Organization chart and responsibilities

Figure 4-1 shows the organization of staff participating in the cyanotoxin screening of lakes/reservoirs and coastal wetlands in the San Diego region. The project team, responsible for the deliverable items, includes Lilian Busse, Meredith Howard, and Carey Nagoda. The parties

responsible for playing an advisory role for the project include Stacey Swenson, Beverly van Buuren, Eric von der Geest, and Gail Cho.

Figure 4-1 Organizational chart



5. Problem Definition/Background

5.1 Problem statement

Toxic cyanobacteria have been reported in freshwater, brackish, and marine environments all over the world (World Health Organization (WHO), 1999). Cyanobacterial blooms, often caused by anthropogenic eutrophication of surface waters, represent a major ecological and human health problem. When cyanobacteria die, a cell breaks, or a benthic mat detaches, cell membranes rupture and can release toxins into the water. Humans can be exposed to these toxins through recreational activity and drinking water. Pets and wildlife may ingest the toxins through water consumption or when eating crusts (dried mats) of cyanobacteria. Toxins released by various cyanobacteria species include neurotoxins (affect nervous system), hepatotoxins (affect liver), and dermatoxins (affect skin).

Besides releasing toxins when blooms die, the decaying process of cyanobacteria consumes oxygen, can cause taste and odor problems for drinking water, and may destroy fishery habitats. Presence of high levels of cyanotoxins in recreational or drinking water can cause symptoms in humans that include: fever, headaches, muscle and joint pain, blisters, stomach cramps, diarrhea, vomiting, mouth ulcers, and allergic reactions. In the most severe cases, effects can include seizures, liver failure, respiratory arrest, and (rarely) death. Harmful cyanobacteria and their toxins are a class of contaminants of emerging concern and were placed on the Candidate Contaminant List (CCL) by the United States Environmental Protection Agency in July 2012 (USEPA, 2012). Included on the list are microcystin-LR, anatoxin-a, and cylindrospermopsin.

Currently, a cyanobacteria and cyanotoxin monitoring program does not exist in the region, or in the State of California. We propose to conduct cyanotoxin screening in lakes/reservoirs and coastal wetlands in the San Diego Region in 2013. This screening data will be used in conjunction with screening data collected in 2012 for streams and depressional wetlands in the San Diego Region.

5.2 Decisions or outcomes

The proposed screening for the presence of cyanotoxins in the lakes/reservoirs and coastal wetlands in the San Diego Region will provide data to address the following assessment questions:

a. In which lakes/reservoirs and coastal wetlands in the San Diego Region are cyanotoxins present?
b. Which toxins (e.g., microcystin variants, anatoxin-a, nodularin) are found in these water bodies?
c. Are there correlations between cyanotoxin presence and specific conditions (e.g., dissolved nutrients, temperature, etc.) at the sites?

Information gained through this initial investigation of lakes/reservoirs and coastal wetlands will provide insight for planning future, larger-scale cyanobacteria and cyanotoxin monitoring efforts.

5.3 Water quality or regulatory criteria

Despite the widespread occurrence of cyanobacteria blooms in water bodies throughout the United States, and the potential health risks they present to humans and animals, there are currently no cyanobacteria or cyanotoxin monitoring programs in the region, or in the State of California. However, the WHO established preliminary guidelines for *Microcystis* dominated samples for recreational activities that have served as a foundation for monitoring programs. They established a Tolerable Daily Intake (TDI) and Guideline Values (GVs) for microcystin toxin in water. The TDI for microcystin-LR toxin in water is 0.04 µg/kg body weight. The guideline values (GVs) are defined at three concentration levels: mild or low, moderate, and high probability of risk for adverse health impacts. The GVs are calculated values, derived from the TDI (Table 4-2). The WHO also established a provisional value for microcystin-LR in drinking water of 1 µg/L.

Table 5-1 WHO Guidelines for Algae and Cyanobacteria in Fresh Water

Guidelines for Algae and Cyanobacteria in Fresh Water (from WHO Guidelines for Safe Recreational Water Environments, Table 8.3, Guidelines for Safe Practice in Managing Recreational Waters, page 150 (WHO, 2003))				
Probability of adverse health effects	**Guidance level or situation**	**How guidance level derived**	**Health Risks**	**Typical Actions**
Relatively Low	20,000 cyanobacterial cells/mL or 10 µg/chlorophyll-a/L with dominance of cyanobacteria	From human bathing epidemiological study	Short-term adverse health outcomes (e.g., skin irritations, gastrointestinal illness)	Post on-site risk advisory signs Inform relevant authorities
Moderate	100,000 cyanobacterial cells/mL or 50 µg/chlorophyll-a/L with dominance of cyanobacteria	From provisional drinking-water guideline value for microcystin-LR [= 1 µg/L] and data concerning other cyanotoxins	Potential for long-term illness with some cyanobacterial species Short-term adverse health outcomes (e.g., skin irritations, gastrointestinal illness)	Watch for scums or conditions conducive to scums Discourage swimming and further investigate hazard Post on-site risk advisory signs Inform relevant authorities
High	Cyanobacterial scum formation in areas where whole-body contact and/or risk of ingestion/aspiration occur	Inference from oral animal lethal poisonings Actual human illness case histories	Potential for acute poisoning Potential for long-term illness with some cyanobacterial species Short-term adverse health outcomes (e.g., skin irritations, gastrointestinal illness)	Immediate action to control contact with scums; possible prohibition of swimming and other water contact activities Public health follow-up investigation Inform public and relevant authorities
*Actual action taken should be determined in light of extent of use and public health assessment of hazard.				

In 2010, the Blue Green Algae Work Group, comprised of members from the State Water Resources Control Board (SWRCB), the California Department of Public health (CDPH), and Office of Environmental Health and Hazard Assessment (OEHHA), developed voluntary statewide guidance for educating and notifying the recreating public about blue-green algae blooms of non-marine water bodies in the state of California (SWRCB et al., 2010). The following guidance values are used when determining whether action (i.e., advisory posting or closures) should be taken to limit exposure to cyanobacteria and associated toxins during recreational activities:

- 40,000 to 100,000 cells/mL
- Microcystin ≥ 8µg/L
- Scum associated with toxigenic species

In May 2012, OEHHA finalized a report that provides calculated health-based water concentration levels (action levels) of microcystins (LA, LR, RR, and YR), anatoxin-a, and cylindrospermopsin for people, pets, and livestock exposed to the cyanotoxins through various scenarios. Health-based concentrations in sport fish and shellfish were also calculated (OEHHA, 2012). These action levels may be applied as needed by local, regional, state or tribal entities and are shown in Table 4-3.

Table 5-2 Action levels for cyanotoxins under selected scenarios (OEHHA, 2012)

	Microcystins (LA, LR, RR, and YR)	Anatoxin-a	Cylindrospermopsin	Media (units)
Human recreational uses[1]	0.8	90	4	Water (µg/L)
Human fish consumption	10	5000	70	Fish (ng/g) ww[2]
Subchronic water intake (dog)[3]	2	100	10	Water (µg/L)
Subchronic crust and mat intake (dog)	0.01	0.3	0.04	Crusts and Mats (mg/kg) dw[4]
Acute water intake (dog)[5]	100	100	200	Water (µg/L)
Acute crust and mat intake (dog)	0.5	0.3	0.5	Crusts and Mats (mg/kg) dw[4]
Subchronic water intake (cattle)[6]	0.9	40	5	Water (µg/L)
Subchronic crust and mat intake (cattle) [6]	0.1	3	0.4	Crusts and Mats (mg/kg) dw[4]
Acute water intake (cattle) [6]	50	40	60	Water (µg/L)
Acute crust and mat intake (cattle) [6]	5	3	5	Crusts and Mats (mg/kg) dw[4]

[1] The most highly exposed of all the recreational users were 7- to 10-year-old swimmers. Boaters and water-skiers are less exposed and therefore protected by these action levels. This level should not be used to judge acceptability of drinking water concentrations.
[2] Wet weight (ww) or fresh weight
[3] Subchronic refers to exposure over multiple days.
[4] Based on sample dry weight (dw).
[5] Acute refers to exposures in a single day.
[6] Based on small breed dairy cows because their potential exposure to cyanotoxins is greatest.

6. Project/Task Description

6.1 Work statement and produced products

Because a monitoring program does not currently exist for cyanobacteria and cyanotoxins, we are proposing a plan for an initial screening lakes/reservoirs and coastal wetlands in the San Diego Region for this class of contaminants of emerging concern. The proposed efforts will complement the cyanotoxin screening that was completed in 2012 on streams and depressional wetlands. Information from the screenings will be combined and used to determine the extent and occurrence of cyanotoxins in the various water bodies in the San Diego Region.

Specifically, the proposed screening efforts detailed in the monitoring plan will be used to address the following assessment questions:

a. In which lakes/reservoirs and coastal wetlands in the San Diego region are cyanotoxins present?
b. Which toxins (e.g., microcystin variants, anatoxin-a, nodularin) are found in these water bodies?
c. Are there correlations between cyanotoxin presence and specific conditions (e.g., dissolved nutrients, temperature, etc.) at the sites?

Information gained from this assessment will be used to plan future monitoring needs and regulatory actions, and may be used by water quality managers to determine where management actions could be implemented to reduce sources and improve water quality. This screening will be conducted in accordance with the Framework for Monitoring and Assessment in the San Diego Region, recently adopted by the Board, considering the following beneficial use questions:

- Is the water safe to drink?
- Are the fish and shellfish safe to eat?
- Is water quality safe for swimming and other recreational activities?
- Are habitats and ecosystems healthy?

Monitoring will include obtaining samples using discrete (i.e., grab samples) and passive, continuous (i.e., Solid Phase Adsorption Toxin Tracking (SPATT) bag) methods. SPATT bags are sampling devices constructed of resins that adsorb specific toxins, which are deployed in a water body for a fixed amount of time (Kudela, 2011). SPATT provide an integrated sample to supplement the grab samples, which are subject to variability due to special and temporal heterogeneity in toxin expression in water bodies. SPATT are useful, inexpensive screening tools that robustly detect microcystins.

Sampling sites -
The SWAMP funding that is available for the proposed cyanotoxin screening will allow for ten (10) samples collected from lakes/reservoirs and ten (10) samples collected from coastal wetlands. It is anticipated that sampling will occur at the following lakes/reservoirs and coastal wetlands shown in Table 6-1. Several (2-3) samples shall be taken at different locations in the San Diego Bay.

Table 6-1 List of potential lakes/reservoirs and coastal wetlands for cyanotoxin sampling

	LAKES / RESERVOIRS
1	O'Neill Lake
2	Diamond Valley Lake
3	Lake Hodges
4	Sutherland Lake
5	Miramar Reservoir
6	Lake Murray
7	El Capitan Reservoir
8	Cuyamaca Reservoir
9	Lower Otay Reservoir
10	Morena Reservoir

	COASTAL WETLANDS
1	Tijuana River Estuary
2	San Diego Bay – Full/Muted/Marina
3	Mission Bay
4	Los Peñasquitos Lagoon
5	San Elijo Lagoon
6	Buena Vista Lagoon
7	Santa Margarita Estuary
8	San Mateo Lagoon

The water bodies chosen for sampling in this targeted design include those that are listed as impaired for nutrients, provide a variety of uses, and are most likely accessible for sample collection. The lakes/reservoirs chosen for sampling have (1) drinking water use, (2) fish use, and (3) recreational use. The coastal wetlands chosen for sampling included those in the region which are more heavily used for recreation purposes. Water bodies with threatened and endangered species and/or critical or sensitive habitats that would limit or prohibit sampling were avoided.

If it is not feasible (e.g., access is not granted) to conduct sampling at any of the water bodies listed above, alternative sampling sites will be chosen from the lists shown in Table 6-2.

Table 6-2 Alternate lakes/reservoirs and coastal wetlands for cyanotoxin sampling

LAKES / RESERVOIRS
Vail Lake
Lake Skinner
Turner Lake
Lake Henshaw
Olivenhain Reservoir
Lake Dixon
Lake Wohlford
Lake Poway
Lake Jennings
Sweetwater Reservoir
Loveland Reservoir
Upper Otay Reservoir
Lake Barrett

COASTAL WETLANDS
Sweetwater Channel
Famosa Slough
San Diego River Estuary
San Dieguito Lagoon
Batiquitos Lagoon
Agua Hedionda
Loma Alta Slough
Las Pulgas Creek
San Juan Creek

Deliverable Products –
A technical report will be produced to present the findings of the screening effort outlined in this monitoring report. The report will also include data from the streams and depressional wetlands screenings that were completed in 2012. The technical report will be finalized by December 31, 2014 and made available to the public on the San Diego Water Board website by January 31, 2015.

6.2 Constituents to be monitored and measurement techniques

Monitoring will include measurements of in-situ water quality parameters, discrete grab samples, and continuous passive sampling devices (SPATT).

Parameters measured in-situ during each sampling event include:

1. Dissolved oxygen
2. Temperature
3. Conductivity
4. pH
5. Salinity (for coastal wetlands)
6. Secchi depth

These data will be obtained using a Quanta multi-probe, salinity refractometer, and Secchi disk.

Grab samples will be collected to obtain data on the following parameters:

1. Water Column Chlorophyll-a
2. Cyanotoxins (particulate)
3. Nutrients (Particulate Nitrogen, Particulate Phosphorous, Total Nitrogen, Total Phosphate, Dissolved Inorganic Nutrients (Nitrate+Nitrite, Ammonium, Phosphate, Silicate))
4. Pigments

The procedures used for collecting and preparing the field samples will follow the Fetscher et al. (2012) Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1, with minor modifications to the grab sample procedure. The SOP is provided in Appendix B. An integrated sample will be collected by combining ten (10) surface grabs of 300 mL each into a large bottle. The grabs will be taken from an area in the water body that contains or is most-likely to contain cyanobacteria. In lakes/reservoirs, the grab samples will be collected in the wind-blown shallow areas open to sunlight, where algae accumulate. In coastal wetlands, the grab samples will be collected close to the freshwater inflow and/or shallow, still areas where algae is known to accumulate.

A portion of the grab sample will also be used to run an alkalinity (digital titration) test in the field. All samples will be field filtered, as necessary, to prepare for proper analyses. Chlorophyll-a will be analyzed using fluorescence. Cyanotoxin samples will be analyzed using liquid-chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) techniques. Nutrients will be analyzed using various methods (see Table 13-3), and pigments will analyzed using high-performance liquid chromatography (HPLC) techniques.

The passive (SPATT) samplers will be deployed in each water body. After a deployment period of 4 weeks, the SPATT samplers will be retrieved and replaced with a second SPATT. The second SPATT will be deployed for 4 weeks*. The SPATT samplers will be analyzed for dissolved cyanotoxins (microcystins) using liquid chromatography-mass spectrometry (LC-MS) methods.
*Funding limitations may not allow for a second deployment at every water body in the study.

All field sampling and measurements will be conducted as outlined in “SWAMP Bioassessment Procedures 2010: Standard operating procedures for collecting stream algae samples and associated physical habitat and chemical data for ambient bioassessments in California” and in the SWAMP Quality Assurance Program Plan (November 19, 2008).

Additional details about sample collection, handling, and laboratory procedures are provided in Section 13.

6.3 Project schedule

Task 1 – Conduct reconnaissance and determine a list of sampling sites for the cyanotoxin screening, with GPS locations. Deliverable date: 06/30/2013.

Task 2 – Conduct sampling at the lakes/reservoirs and coastal wetland sites. Samples will be sent to the laboratories on weekly or bi-weekly bases. Dates: 07/01/2013 – 10/31/2013.

Task 3 – Enter field data into SWAMP database. Deliverable date: 11/30/2013.

Task 4 – Laboratory analyses of samples and enter data into SWAMP database. Deliverable date: 03/13/2014.

Task 5 – Analyses of all data produced in the cyanotoxin screening studies and write final report. Deliverable date: 12/31/2014.

Task 6 – Final report posted online. Deliverable date: 01/31/2015.

Figure 6-1 Project schedule

TASK	MAR 2013	APR 2013	MAY 2013	JUN 2013	JUL 2013	AUG 2013	SEP 2013	OCT 2013	NOV 2013	DEC 2013	JAN 2014	FEB 2014	MAR 2014	APR 2014	MAY 2014	JUN 2014	JUL 2014	AUG 2014	SEP 2014	OCT 2014	NOV 2014	DEC 2014	JAN 2015
1 - Reconaissance Sample site selection																							
2 - Field collection Samples sent to labs																							
3 - Field data entered into SWAMP																							
4 - Laboratory analyses SWAMP data entry																							
5 - Data analyses Report writing																							
6 - Final report posted online																							

6.4 Geographical setting

The geographic scope of data collection for this project include samples collected in lakes/reservoirs and coastal wetlands in the San Diego Region. The San Diego Region, located in the southwest corner of California, occurs within the Peninsula Range Physiographic Province (Figure 6-2). It encompasses most of San Diego County, and portions of Orange and Riverside Counties. The Pacific Ocean coastline is the Region's western boundary, extending 85 miles north from the United States and Mexico Border. The eastern boundary of the Region is formed by the Laguna Mountains and other mountain ranges located in the Cleveland National Forest. The northern boundary of the San Diego Region is formed by the hydrologic divide near Laguna Beach and extends eastward into the Cleveland National Forest. The southern boundary of the Region is formed by the United States and Mexico border.

Figure 6-2 San Diego Region location map

The proposed project includes collecting ten (10) samples in lakes/reservoirs and ten (10) samples in coastal wetlands in the San Diego region. The water bodies chosen for sampling represent a range of hydrologic units (HUs) and hydrologic subareas (HSAs) (Table 6-3).

Table 6-3 Hydrologic units and subareas of the water bodies chosen for cyanotoxin sampling

Lakes/Reservoirs	HU	HSA	Coastal Wetlands	HU	HSA
O'Neill Lake	902	2.13	San Mateo Lagoon	901	1.40
Diamond Valley Lake	902	2.35, 2.36	Santa Margarita Estuary	902	2.11
Lake Hodges	905	5.21	Buena Vista Lagoon	904	4.21
Sutherland Lake	905	5.53	San Elijo Lagoon	904	4.61
Miramar Reservoir	906	6.10	Los Peñasquitos Lagoon	906	6.10
Lake Murray	907	7.11	Mission Bay	Multiple	Multiple
El Capitan Reservoir	907	7.31	San Diego Bay	Multiple	Multiple
Cuyamaca Reservoir	907	7.43	Tijuana River Estuary	911	11.11
Lower Otay Reservoir	910	10.31			
Morena Reservoir	911	11.50			

Figures 6-3 and 6-4 show the locations of the lakes/reservoirs and coastal wetlands, respectively, proposed for cyanotoxin sampling efforts.

Figure 6-3 Proposed cyanotoxin sampling locations of lakes/reservoirs in the San Diego Region

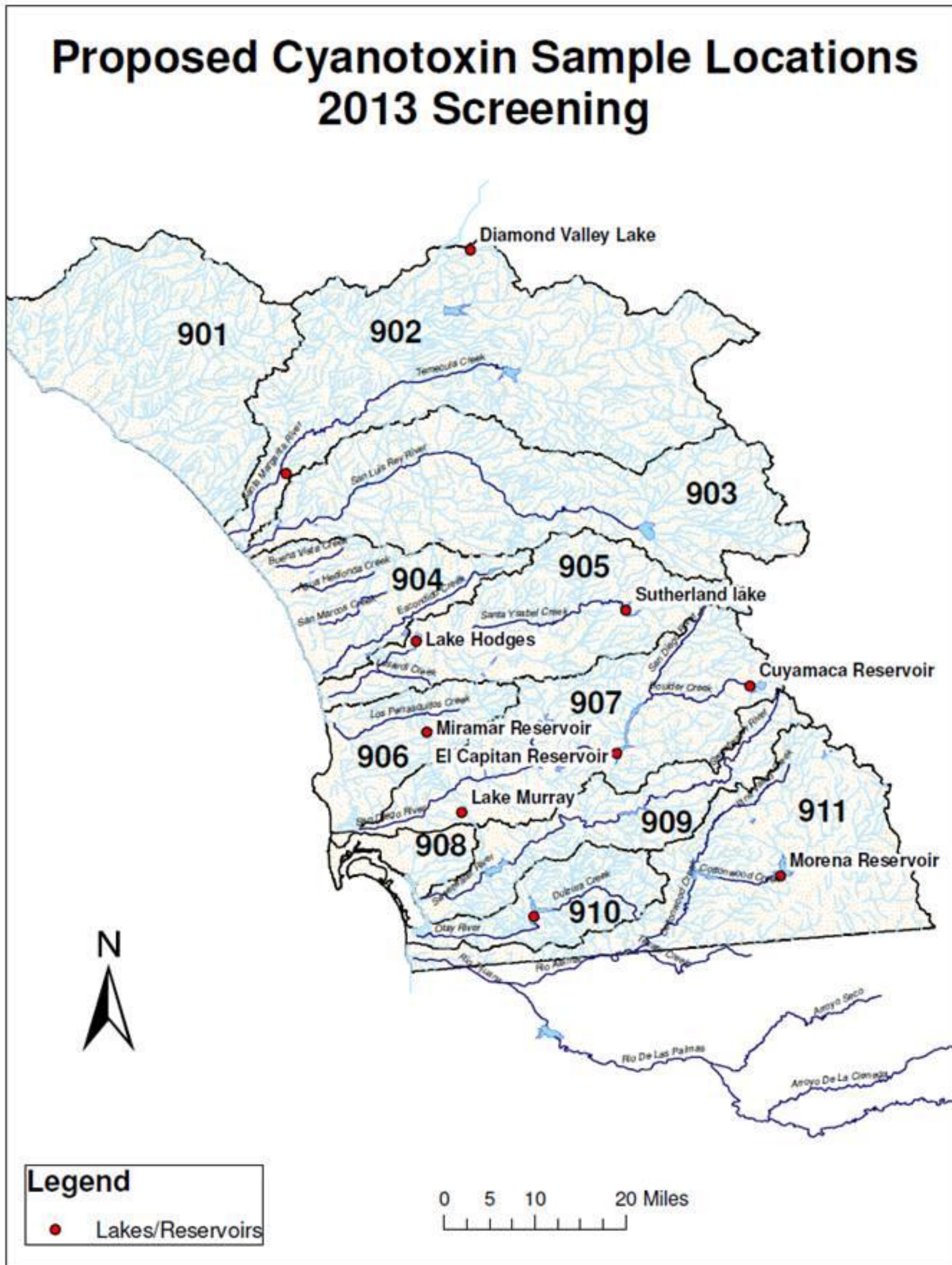
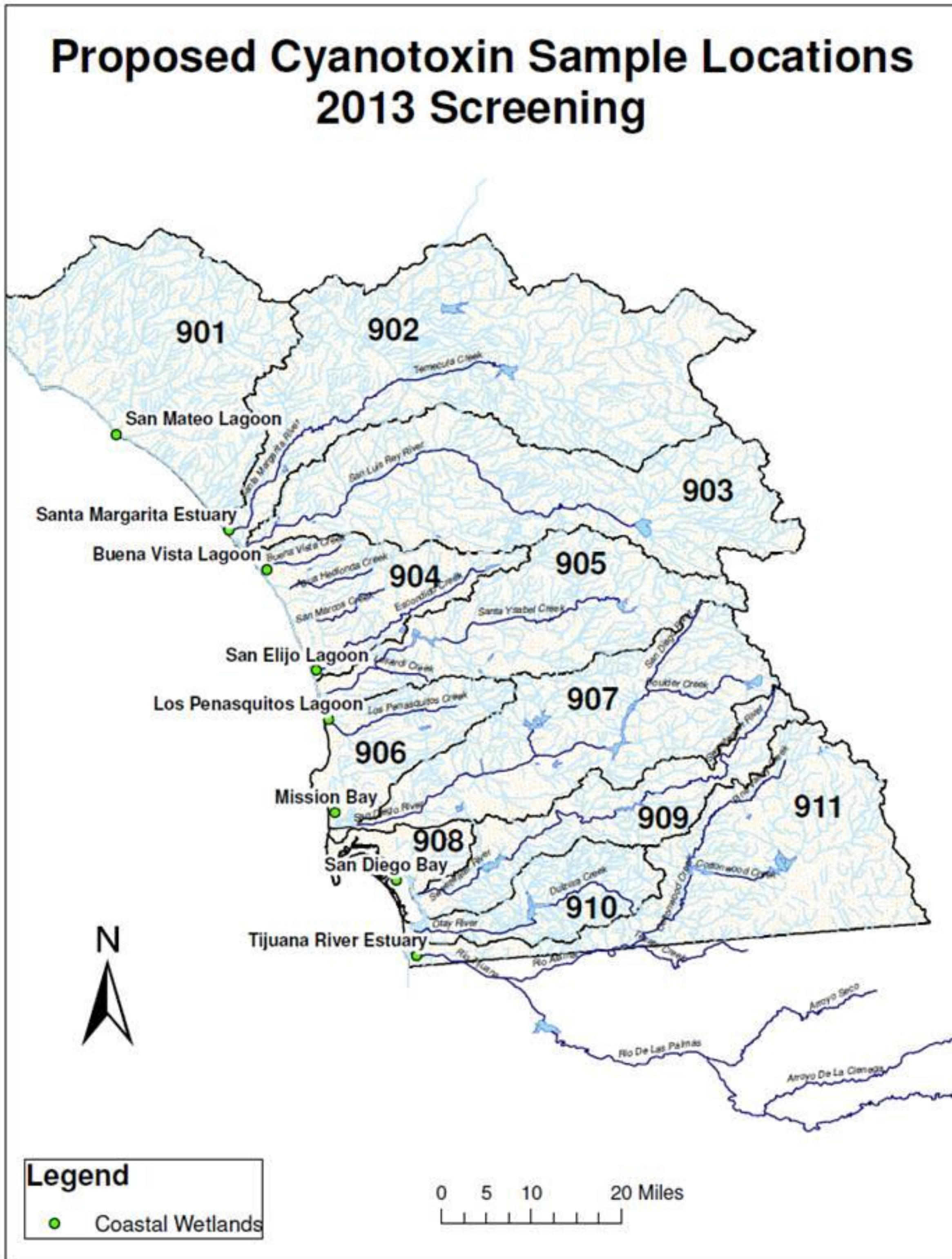


Figure 6-4 Proposed cyanotoxin sampling locations of coastal wetlands in the San Diego Region



6.5 Constraints

The proposed sampling sites were chosen to minimize constraints. Privately-owned water bodies and those with species of special concern were avoided due to potential access issues. A list of alternate sites was prepared in case sampling cannot be conducted at certain water bodies that were chosen for this screening. Varying water flow throughout the watersheds may become a constraining factor for determining proper SPATT bag placement. SPATT bags must be placed within the water column at a depth that will remain submerged during the entire deployment period. Consideration must be given to anticipated fluctuation of water surface levels.

7. Quality Objectives and Criteria for Measurement Data

7.1 Data quality indicators

Data acquisition activities will include both field measurements and laboratory analyses. The following indicators are used to assess data quality: accuracy, precision, representativeness, comparability, and completeness. These indicators and data quality objectives (DQOs) are used to determine the level of error considered to be acceptable in the data produced by the sampling program. Table 7-1 provides the data quality indicators for the parameters that will be measured in this project. A brief discussion of the objectives for the indicators used in this study is provided below.

Table 7-1 Measurement or analyses type and applicable data quality indicators

Measurement or Analyses Type	***Applicable Data Quality Indicators***
Field Measurement, Dissolved Oxygen	Accuracy, Precision, Completeness
Field Measurement, Temperature	Accuracy, Precision, Completeness
Field Measurement, Conductivity	Accuracy, Precision, Completeness
Field Measurement, pH	Accuracy, Precision, Completeness
Field Measurement, Salinity	Accuracy, Precision, Completeness
Field Testing, Alkalinity	Accuracy, Precision, Completeness
Laboratory Analyses, Water Column Chlorophyll-a	Accuracy, Precision, Completeness
Laboratory Analyses, Cyanotoxins (particulate)	Accuracy, Precision, Completeness
Laboratory Analyses, Nutrients	Accuracy, Precision, Completeness, Representativeness
Laboratory Analyses, Pigments	Accuracy, Precision, Completeness
Laboratory Analyses, Cyanotoxins (dissolved)	Accuracy, Precision, Completeness

<u>Accuracy</u>

Accuracy (bias) measures the conformity between measured and true values.

Field Measurements

To achieve accuracy in measurements of pH, dissolved oxygen, and conductivity, the portable measuring device (i.e., Quanta multi-probe) will be calibrated before every sampling event. Salinity measurement accuracy will be achieved by calibrating the refractometer before each use.

Laboratory Analyses

To determine accuracy of nutrients, chlorophyll-a, pigment and cyanotoxin data, certified quality control references of known concentrations will be analyzed with each batch of samples. The reference values for the QC samples must be within 80-120% of the true concentrations for the batch of samples to be immediately accepted. A reference must be analyzed per 20 samples or each analytical batch (whichever is more frequent).

The LC-MS and LC-MS/MS will be calibrated with a full range of calibration standards each time samples are run. The linear regression for the calibration must meet or exceed $r^2 = 0.990$.

Field and laboratory blanks (5% of total samples) will be prepared and analyzed for each parameter measured to demonstrate freedom of contamination. For each round of sample collection, it is anticipated that a total of 20 samples will be collected per parameter. Therefore, one field and one laboratory blank will be prepared for each type of sample collected during each round of sampling.

Matrix spikes will be analyzed with each batch of samples or per 20 samples analyzed (whichever is more frequent). The percent recovery for spiked samples must be 80-120% for the sample batch to be accepted.

$R = (Cs - C) / S * 100$

Where:

R = percent recovery, Cs = spiked sample concentration, C = sample background concentration, and S = concentration of analyte added to the sample.

Precision

Precision measures how closely repeated measurements of a given sample agree with each other.

Field Measurements

Field duplicates will be collected to determine the precision of the field sampling. Duplicates must be collected for at least 5% of all samples. It is anticipated that a total of 20 samples per parameter will be collected during each round of sampling. Therefore, one duplicate will be collected for each type of sample, during every round of sampling.

Laboratory Analyses

Laboratory duplicates will be analyzed to assess laboratory precision. As required by the SWAMP QAMP, at least one duplicate of a field sample should be performed per 20 samples or one per batch, whichever is more frequent. Following analysis, the results from the duplicate samples are evaluated by calculating the relative percent difference (RPD).

$RPD = [X_1 - X_2] / [(X_1 + X_2) / 2] * 100$

Where X_1 = the larger of the two values, and X_2 = the smaller of the two values

Laboratory batches with RPDs < 25% are acceptable.

Laboratory batches with RPDs > 25% are classified as estimated.

Representativeness

Representativeness describes the degree to which the results of analyses represent the samples collected, and the samples in turn represent the environment from which they are taken. Determining appropriate locations for grab and passive sampling, utilizing integrated water samples, and the use of only approved/documented SOPs and analytical methods will ensure that the field conditions are represented to the best extent possible.
Because site conditions (e.g., cyanobacteria bloom formations) may be affected by inflow, tidal cycles, weather conditions, etc., the site conditions will be noted during each sampling event. Sampling schedules will be designed with respect to locations and methodologies to maximize representativeness, where possible.

Completeness

Completeness is the percentage data available for use compared to the potential amount of data identified in the monitoring plan. Ideally, 100% of the data should be available. However, possibilities exist for issues to arise that could result in incomplete data sets. These include unexpected field conditions, laboratory error, and shipment complications that cause samples to experience temperatures outside of the acceptable preservation range. Therefore, 90% data completeness is required for this project.

7.2 Data quality objectives

The data quality objectives for field and laboratory measurements of this study are provided in Tables 7-2 and 7-3.

It should be noted that while data quality objectives are listed below for nutrient and pigment measurements, the nutrient and pigment analyses for this study are not funded by SWAMP. The following data will not be entered into the SWAMP database:

- Particulate Nitrogen
- Particulate Phosphorous
- Total Nitrogen
- Total Phosphorous
- Orthophosphorous
- Total Dissolved Nitrogen
- Ammonia
- Nitrate + Nitrite
- Pigments

Table 7-2 Data quality objectives for field measurements

Parameter	Measurement Device / Method	Accuracy	Precision	Completeness
pH	Glass Electrode	± 0.2 pH units	± 0.2 pH units	90%
Conductivity	6-Electrode Cell	± 2 µS/cm	± 10% or ± 10 µS/cm, whichever is greater	90%
Dissolved oxygen	Polarographic Sensor	± 0.2 mg/L	± 5%	90%
Temperature	Thermistor	± 0.15 ^{o}C	± 0.5 ^{o}C	90%
Salinity	Refractometer	± 2 ‰	± 2 ‰	90%
Alkalinity	Digital Titration (Hach Method 8203)	1 mg/L	1 mg/L	90%

Table 7-3 Data quality objectives for laboratory measurements

Group	Parameter	Method	SWAMP Target Reporting Limits	Accuracy	Precision	Recovery	Completeness
Conventional Constituents (Nutrients)	Particulate Nitrogen	EPA 440.0	165 mg/kg N in 10 mg sample	Standard Reference Materials	Laboratory Duplicate, Blind Field Duplicate 25% RPD	Matrix Spike 80% - 120%	90%
	Particulate Phosphorous	EPA 365.5	100 mg/kg P in 10 mg sample				
	Total Nitrogen	USGS I-2650-03	0.03 mg/L				
	Total Phosphorous	EPA 365.2/365.4 USGS I-2650-03	0.014 mg/L				
	Orthophosphate (dissolved)	EPA 365.3	0.01 mg/L				
	Total Dissolved Nitrogen	USGS I-2650-03	0.03 mg/L				
	Ammonia	EPA 350.1	0.02 mg/L				
	Nitrate + Nitrite	EPA 353.2	0.01 mg/L				
Cyanobacteria	Chlorophyll-a	EPA 445.0	0.002 mg/L	No SWAMP requirements - Recommend 30% of standard reference material	No SWAMP requirements - Recommend Duplicate 25% RPD	N/A	90%
	Pigments	Van Heukelem and Thomas, 2001	No SWAMP requirement				
	Cyanotoxins LC-ESI-MS/MS	Mekebri et al., 2009	No SWAMP requirement - Recommend 1µg/L Microcystin				
	Cyanotoxins (SPATT) - LC-MS	Kudela, 2011					

As mentioned above, the frequency at which laboratory blanks, reference materials, matrix spikes, matrix spike duplicates, and laboratory duplicates must be analyzed is per 20 samples analyzed or per analytical batch, whichever is more frequent. The frequency at which field duplicates and field blanks must be collected is at least 5% of the total project sample count.

7.3 Project action limits

Cyanotoxins are the parameter of interest for this screening study. The levels and guidelines provided by WHO (2003) and OEHHA (2012) will be used (See Section 5). If cyanotoxin concentrations are found above the (Microcystin) limits listed for human recreation or drinking water, the Department of Public Health will be notified and the corresponding appropriate measures will be taken.

8. Special Training Needs/Certification

Personnel assigned to perform field sampling and laboratory analyses have prior experience and training in the type of water quality monitoring proposed for this project. However, no special certification is required for the field and laboratory tasks. The QA and QC Officers are responsible for ensuring that all necessary standard operating procedures (SOPs) and the most up-to-date QAPP are distributed to the project team, understood, and followed for the duration of this study.

9. Documents and Records

9.1 Field and laboratory records

Field Records –
All field results will be recorded by SD RWQCB staff at the time of completion using standard field data sheets. The data sheets will be reviewed for obvious omissions and outliers prior to leaving the sample site. The following information will be recorded at each sampling site:

- Project ID
- Site code
- Names of individuals collecting the samples
- Date and time
- Location of sampling event
- Field observations/Site conditions
- GPS coordinates
- Field data collected
- Number and types of samples collected
- Additional information that may affect the integrity of the samples

Field data will be entered into the SWAMP database. It will also be kept in the California Environmental Data Exchange Network (CEDEN). Field data sheets (hard copies) will be indexed and stored by SD RWQCB staff for 5 years. Electronic copies will be provided to the

project team. In addition, electronic copies will be stored with the SD RWQCB on the S:drive and in Paperless Office (ECM) for a minimum of 5 years.

Each sample that is collected will be labeled with the following information:

- Project ID
- Site code
- Date and time
- Volume
- Sample type
- Collector's names

Chain-of-custody (COC) and Analysis Authorization (AA) forms will be generated weekly or bi-weekly to meet sample shipment needs to each participating laboratory. Electronic copies of all COC and AA forms will be provided to the SWAMP Database Manager and SWAMP QA Officer. Electronic copies of these forms will also be kept by the SD RWQCB staff for 5 years.

Laboratory Records-
Laboratory personnel are responsible for documenting all analyses performed. Reporting shall include:

- Type of analysis performed
- Method(s) used
- Date of analysis
- Summary of analytical results
- Summary of QA/QC data

Laboratory personnel are responsible for working with the SWAMP Officers to upload all acceptable laboratory data into the SWAMP database. Table 19.1 provides information on the specific parties responsible for data entry.

Copies of this QAPP will be distributed to all parties involved with the project. Any future amendments to the sampling plan will be held and distributed in the same fashion. All originals of the first and subsequent documents will be held at the Regional Board Office. Copies of versions, other than the most current, will be discarded so as not to create confusion.

Copies of this QAPP and the project's monitoring plan (Nagoda and Busse, 2013) will be stored on the SD RWQCB S:drive and in Paperless Office (ECM) for 5 years.

A final report on the findings of this study, in combination with the cyanobacteria and cyanotoxin screenings that were conducted in 2011 and 2012 for depressional wetlands and streams, will be developed by the project team and finalized by January 2015. The report will answer the questions proposed in Section 5.2. An electronic version (PDF) of this document will be stored on the SD RWQCB S:drive and in Paperless Office (ECM) for 5 years.

Group B: Data Generation and Acquisition

10. Sampling Process Design

The work performed under this QAPP includes a cyanotoxin screening of lakes/reservoirs and coastal wetlands in the San Diego Region. This data will be used in conjunction with 2012 cyanotoxin screening data from streams and depressional wetlands in the San Diego Region. Cyanotoxin monitoring will be accomplished using discrete and continuous methods, as described below, throughout the duration of the study. Sampling efforts will also include gathering data on site conditions, in-situ water quality measurements, and surface water grab samples analyzed for chlorophyll-a, nutrients, and pigments.

Available funding will allow for a total of 20 sampling sites (10 located in lakes/reservoirs and 10 located in coastal wetlands). A targeted approach was used to choose the sample sites from a variety of hydrologic units. Sites were chosen to include those that are listed as impaired for nutrients on the Clean Water Act Section 303(d) list, provide a variety of beneficial uses, and are most likely accessible for sample collection. The lakes/reservoirs chosen for sampling have (1) drinking water use, (2) fish use, and (3) recreational use. The coastal wetlands that were chosen for this study are those in the region that are more heavily used. A list of alternate sampling sites was developed for use in the event that sampling is not feasible at a particular site. Reconnaissance site visits will be conducted throughout May and June to each water body (See Table 6-1) to determine the appropriate sample site location at each lake/reservoir and coastal wetland. Sample locations will be chosen where cyanobacteria concentrations are expected to be greatest (i.e., upper extents of coastal wetlands, near freshwater inputs and shallow, wind-blown areas in lakes/reservoirs). A target GPS point and site description will be recorded, and photos will be taken to aid the field sampling team.

One integrated grab sample will be collected from the surface water at each of the twenty (20) sampling sites for this study during each sampling round. An integrated grab, composed of 10 smaller grabs evenly spaced throughout the sample site, will be used to obtain a representative sample. One sampling round will occur in July, one in August, and one in September. The water collected for the integrated grab will be processed immediately on-site to create samples for each of the seven (7) parameters listed below in Table 11-1. Therefore, a sampling event per site will produce a total of seven (7) samples, each for a different parameter. Each sampling round (20 sites) will produce a total of 140 samples. Three sampling rounds will occur for each site in this study throughout the summer, creating a grand total of 420 samples. In addition, two (2) SPATT samplers will be deployed at each site, once in July and once in August. They will be retrieved in August and September. A total of forty (40) SPATT samplers will be collected during the course of this study. The critical information collected in this screening study includes the cyanotoxin analyses (SPATT and cyanotoxin filters). The remaining nutrient, cholorphyll, and pigment data (See Table 11-1) collected will be used as supporting information.

As mentioned above in Section 6.2 and shown in Figure 6-1, site reconnaissance will be completed by June 30, 2013. The first round of sampling at each sample site will occur in July. The second round will occur in August, and the third will occur in September. If scheduling logistics or unforeseen circumstances arise, sampling may extend into October. Samples will be

sent to the labs on a weekly basis, starting the first week of July 2013 for DFW-WPCL and SCCWRP, and starting the first week of August 2013 for the Kudela Laboratory at UC Santa Cruz.

Additional information is available in the Surface Water Ambient Monitoring Program (SWAMP) Monitoring Plan for Cyanotoxins in Lakes/Reservoirs and Coastal Wetlands, Region 9, FY 2012/2013(Nagoda and Busse, 2013). This document is available online at: http://www.swrcb.ca.gov/water_issues/programs/swamp//docs/workplans/r9_cmplan1213.pdf.

11. Sampling Methods

At each water body selected for cyanotoxin screening, the field team will conduct sampling up to three times throughout the duration of the study. During the first visit, in-situ measurements will be taken, grab samples will be collected, and a SPATT sampler will be deployed. After 4 weeks, the sampling team will return to the site to retrieve the SPATT sampler, take in-situ measurements, collect another set of grab samples, and deploy a second SPATT sampler. The second SPATT sampler will be deployed for 4 weeks; after which, the field team will return to retrieve the SPATT sampler, take in-situ measurements, and collect grab samples. Depending on funding availability and site conditions, SPATT samplers may not be deployed twice at each water body chosen for this study. However, SPATT will be deployed at a minimum of one time (for 4 weeks) at each water body.

11.1 In-situ measurements

Temperature, pH, dissolved oxygen, and conductivity will be measured at each site (in the vicinity of the grab sampling location) using a Quanta Hydrolab multi-parameter water quality instrument. Measurements will be taken at a depth of about 0.1 meter. The instrument will be calibrated each day and rinsed with distilled water following use at each site.
In lakes/reservoirs, profile measurements will also be taken at the deepest point. In-situ readings will be taken at the surface (depth ~0.1 meter) and at 1-meter increments until a depth of 5 meters is reached. Below 5 meters, readings will be taken at 5-meter increments until the bottom is reached.

11.2 Grab samples

The procedures used for collecting and preparing the field samples will follow the Fetscher et al. (2012) Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1 (Appendix B), with minor modifications to the grab sample collection method. Integrated samples will be collected by combining ten (10) grab samples from the surface waters (depth of 0.1 meter) at each site. The samples will be collected in the portions of the water bodies that are most likely to contain cyanobacteria. In lakes/reservoirs, samples will be taken in shallow regions in the wind-blown direction. The coastal wetland samples will be taken close to the inflow.

All water samples will be aliquoted into appropriate sample containers in the field immediately following collection. Glass-fiber filters (47-mm diameter, 0.7-µm pore size) will be prepared for chlorophyll-a, cyanotoxins, total nitrogen, total phosphorous, particulate nitrogen, particulate phosphorous, and pigments. Each parameter requires filtering a volume of 250 mL of water, except cyanotoxins, which require 500 mL of sample water. The filters will be folded and placed into petri dishes. Some petri dishes will be covered with aluminum foil, and all will be placed into plastic Whirlpak bags and sealed. The dissolved nutrient samples will be passed through a clean, plastic syringe fitted with a 0.45-micron filter and into a HDPE bottle. Total nitrogen and total phosphorous sample water will be placed directly into HDPE bottles. All samples will be frozen immediately (i.e., placed on dry ice inside of a cooler). Table 11-1 provides sample preparation information for the parameters measures at each site.

Table 11-1 Sample preparation requirements per site

Sample	# of Samples	Filter	Volume Required	Container Type	Storage
FILTERS					
Chlorophyll-a	1	GF/F filter	250 mL	Petri dish; cover with foil; insert into Whirlpak	Immediately frozen
Chlorophyll-a (**DUPLICATE**)	1 sample per 20	GF/F filter	250 mL	Petri dish; cover with foil; insert into Whirlpak	Immediately frozen
Cyanotoxin	2	GF/F filter	500 mL	Petri dish; cover with foil; insert into Whirlpak	Immediately frozen
Pigments	1	GF/F filter	250 mL	Petri dish; cover with foil; insert into Whirlpak	Immediately frozen
Particulate Nitrogen	1	Combusted GF/F filter 25mm; use syringe	100 mL	Petri dish; insert into Whirlpak	Immediately frozen
Particulate Nitrogen (**FIELD BLANK**)	1 per 20 sites	Combusted GF/F filter 25mm; use syringe and **DI water**	100 mL	Petri dish; insert into Whirlpak	Immediately frozen
Particulate Phosphorous	1	Combusted GF/F filter	100 mL	Petri dish; insert into Whirlpak	Immediately frozen
Particulate Phosphorous (**FIELD BLANK**)	1 per 20 sites	Combusted GF/F filter 25mm; use syringe and **DI water**	100 mL	Petri dish; insert into Whirlpak	Immediately frozen
UNFILTERED WHOLE WATER					
Total Nitrogen and Total Phosphate	1	N/A Whole water (unfiltered)	Fill bottle 2/3 full	HDPE bottles	Immediately frozen
Total Nitrogen and Total Phosphate (**FIELD BLANK**)	1 per 20 sites	N/A Unfiltered **DI water**	Fill bottle 2/3 full	HDPE bottles	Immediately frozen
FILTERED WATER					
Dissolved Inorganic Nutrients	1	Filter using syringe	Fill bottle 2/3 full (not less than 20 mL)	HDPE bottles	Immediately frozen
Dissolved Inorganic Nutrients (**FIELD BLANK**)	1 per 20 sites	Filter **DI water** using syringe	Fill bottle 2/3 full (not less than 20 mL)	HDPE bottles	Immediately frozen

The sample bottle and lid may only come into contact with surfaces known to be clean, or the water sample. If the performance requirements for specific samples are not met, the sample will

be recollected. If contamination of the sample container is suspected, a fresh sample container will be used.

A portion of the grab sample collected will be used to measure salinity (for coastal wetlands only) and alkalinity in the field. Salinity will be measured using a refractometer. Alkalinity requires a sample volume of 100 mL and will be measured using digital titration (Hach Method 8203). All equipment used for these measurements will be calibrated according to manufacturer's specifications and rinsed with distilled water following use at each site.

If the field crew is unable to collect the integrated grab sample and/or process the samples at a specific site, the Project Director will be notified. If the reason for failure is equipment related, it will be noted and the equipment will be repaired or replaced, as necessary. If the reason for failure is field condition related, the Project Director will determine whether it is best to re-schedule the field sampling or choose a site from the alternate list instead (for first visit only).

11.3 Continuous samples

SPATT samplers (or SPATT bags) will be affixed to a pole, buoy, or other structure that will ensure the sampler is submerged in the water body for the duration of the sampling period (4 weeks) until it is retrieved. When the SPATT sampler is retrieved, larger debris (e.g., twigs, grasses, chunks of dirt) shall be removed. The SPATT sampler will be placed into a plastic bag and labeled with the station, date/time in, and date/time out of the water. The bag will be stored immediately on dry ice inside of a cooler.

11.4 Decontamination

To reduce the potential of spreading aquatic invasive species, field equipment used in this screening will be decontaminated between watersheds and/or water bodies. Decontamination Protocols for Field Activities (CA Department of Fish and Wildlife, February 2012 revision) will be followed.

12. Sample Handling and Custody

12.1 Sample collection and initial preservation

Staff from the San Diego Regional Water Quality Control Board (SD RWQCB) will collect and field process all of the samples. All caps, petri dishes, and Whirlpak bags will be checked for tightness prior to storing. Sample containers will be clearly labeled with all required information. The samples will be stored on dry ice inside of a cooler until they are brought back to the SD RWQCB laboratory. To ensure that no bottles are lost in transport, SD RWQCB staff will check-off each sample bottle on the field log sheet upon returning to the field room/laboratory. All of the samples will be stored in the laboratory freezer until they are prepared for shipping. Table 12-1 provides additional information on the initial preservation and storage required for all samples collected in this study.

Table 12-1 Sample preservation and storage requirements

*Of the parameters listed below, only Chlorophyll-a and Cyanotoxin data will be entered into the SWAMP database.

Parameter	Container	Volume	Initial Preservation	Holding Time
Chlorophyll-a	Petri dish covered with foil stored in plastic Whirlpak	**250 mL** passed through GF/F filter	Immediately frozen	28 days, frozen
Cyanotoxin (Filters)	Petri dish covered with foil stored in plastic Whirlpak	**500 mL** passed through GF/F filter	Immediately frozen	28 days, frozen (No SWAMP requirements available)[a]
Pigments	Petri dish covered with foil stored in plastic Whirlpak	**250 mL** passed through GF/F filter	Immediately frozen	28 days, frozen (No SWAMP requirements available)[b]
Particulate Nitrogen	Petri dish stored in plastic Whirlpak	**100 mL** passed through 25mm GF/F filter	Immediately frozen	28 days, frozen (No SWAMP requirements available)
Particulate Phosphorous	Petri dish stored in plastic Whirlpak	**100 mL** passed through 25mm GF/F filter	Immediately frozen	28 days, frozen (No SWAMP requirements available)
Total Nitrogen and Total Phosphorous	HDPE bottle	Whole water (unfiltered) **Fill bottle 2/3 full**	Immediately frozen	28 days, frozen (New SWAMP requirements $\leq 6^{o}C$, H_2SO_4 $pH<2$)[c]
Dissolved Inorganic Nutrients (Nitrate+Nitrite, Ammonium, Ortho-Phosphate, Silicate)	HDPE bottle	Filtered water **Fill bottle 2/3 full**	Immediately frozen	Nitrate+Nitrite: 28 days – refrigerated, acidified[c] Ammonium: 28 days - refrigerated, acidified[c] Ortho-Phosphate: 7 days, refrigerated[d] Silicate: 28 days, frozen
Cyanotoxin (SPATT Bags)	Plastic bag	N/A	Immediately frozen	28 days, frozen (No SWAMP requirements available)[e]

[a]Based on Mioni (2011) for whole water samples. We will apply the holding times to our filters because they will be analyzed for the same analytes.
[b]Based on EPA Method 447.0. The data will not be entered into the SWAMP database.
[c]Samples will be frozen but not acidified. This data will not be entered into SWAMP. Because it is ancillary data, it can still be used in this study.
[d]Holding time (48 hours) for ortho-phosphate will not be met in this study. Preliminary data show that ortho-phosphate remains almost unchanged if refrigerated over 7 days (Ode, personal communication). The data will not be entered into SWAMP database. Because it is ancillary data, it can still be used in this study.
[e]Based on Mioni (2011).

The SD RWQCB field crew is responsible for keeping a field log and data sheets for each sampling event. The field crew has custody of the samples during field sampling until they are sent to the various laboratories for analyses.

Samples will be shipped in groups each week or every other week to the appropriate laboratories. Chain-of-custody (COC) forms will be used to track the samples from collection through analysis (See Appendix A for COC form). Analysis Authorization (AA) forms will be prepared for each shipment group. Electronic copies of these forms will be sent to the SWAMP QA Officer and Database Manager.

12.2 Transport to laboratory

All samples will be transported or shipped overnight on dry ice inside of coolers to the laboratories running the analyses. Hard copies of the COC forms will accompany the samples for signature. Electronic copies of the COC and AA forms will be sent to the laboratories for each shipment. The SPATT bags will be shipped to UC Santa Cruz for microcystin analysis. All chlorophyll-a, nutrient, and pigment samples will be shipped to Southern California Coastal Water Research Project (SCCWRP) for analysis.

When the SPATT analyses result in a positive hit for microcystin at a site, the cyanotoxin filter will be sent to the Department of Fish and Wildlife, Water Pollution Control Laboratory (WPCL), where the samples will be analyzed for microcystins, anatoxin-a, and nodularin using LC-ESI-MS/MS methods.

13. Analytical Methods and Field Measurements

13.1 Field water quality measurements

Temperature, pH, dissolved oxygen, and conductivity will be determined using a Quanta Hydrolab multi-parameter water quality instrument. It will be calibrated and used according to the manufacturer's instructions. The in-situ measurements will be achieved by submerging the Quanta Hydrolab multi-parameter water quality instrument just below the water surface and waiting until all readings have equilibrated (approximately 5-10 minutes) before recording the data.

Table 13-1 Specifications for water quality parameters measured with Quanta Hydrolab

Parameter	Range	Accuracy	Resolution
Temperature	+23 to +120º F -5 to +50º C	± 0.15º C	0.01º C
pH	0 to 14 units	± 0.2	0.01
Conductivity	0 to 100 mS/cm	± 1% range	4 digits
Dissolved Oxygen	0 to 20 mg/L	± 0.2 mg/L	0.01 mg/L

The Quanta multi-probe will be stored and maintained according to the users' manual. Probes will never be stored dry or in DI water, and the protective cap will be used when taking field readings. This will prevent fouling of the probes.

Alkalinity will be measured in the field using digital titration, following Hach Method 8203 (Appendix O). The waste from the alkalinity test is non-toxic and slightly acidic. It will be stored in a bottle and brought back to the RWQCB lab, where the pH will be neutralized before pouring down the drain. Salinity will be measured using a General® portable refractometer according to the manufacturer's operation manual.

Table 13-2 Specifications for alkalinity and salinity field measurements

Parameter	Method	Detection Range	Accuracy
Alkalinity	Hach Method 8203	10 to 4,000 mg/L as $CaCO_3$	Use accuracy check specified in method
Salinity	General® refractometer	0 to 10% salinity	± 0.10% (1 ppt)

13.2 Laboratory analytical measurements

Water samples collected for nutrient, chlorophyll-a, pigment, and cyanotoxin evaluation, and SPATT samplers will be analyzed using three different laboratories. These include Southern Coastal California Water Research Project (SCCWRP), Department of Fish and Wildlife - Water Pollution Control Laboratory (WPCL), and the Kudela Laboratory at UC Santa Cruz. Table 13-3 provides a summary of the types of analyses and methods that will be employed for this project. SPATT sampler results shall be reported to the RWQCB each week as the samples are analyzed, as positive hits for microcystin in the SPATT samplers triggers the particulate sample to be sent to WPCL for analyses. All analyses and SWAMP data entry must be completed by March 2014.

Table 13-3 Laboratory analytical methods

Analyte	Laboratory	Method		SWAMP Target Reporting Limits
		Type	Reference	
Particulate Nitrogen	SCCWRP	Elemental Analysis	EPA 440	165 mg/kg N in 10 mg sample
Particulate Phosphorous	SCCWRP	Colorimetric	EPA 365.5	100 mg/kg P in 10 mg sample
Total Nitrogen	SCCWRP	Alkaline Persulfate Digestion	USGS I-2650-03	0.03 mg/L
Total Phosphorous	SCCWRP	Colorimetric/Block Digestion, Colorimetric/Ascorbic Acid, Alkaline Persulfate Digestion	EPA 365.2 EPA 365.4 USGS I-2650-03	0.014 mg/L
Orthophosphate (dissolved)	SCCWRP	Colorimetric, Ascorbic Acid	EPA 365.3	0.01 mg/L
Total Dissolved Nitrogen	SCCWRP	Alkaline Persulfate Digestion	USGS I-2650-03	0.03 mg/L
Ammonia	SCCWRP	Colorimetry	EPA 350.1	0.02 mg/L
Nitrate + Nitrite	SCCWRP	Cadmium Reduction, Colorimetry	EPA 353.2	0.01 mg/L
Chlorophyll-a	SCCWRP	Fluorescence	EPA 445.0	0.002 mg/L
Pigments	SCCWRP	High-performance Liquid Chromatography	Van Heukelem and Thomas, 2001	No SWAMP requirement
Microcystins, Anatoxin, Nodularin (particulates)	WPCL	Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry	Mekebri et al., 2009	No SWAMP requirement
Microcystins (dissolved)	Kudela (UC Santa Cruz)	Liquid Chromatography-Mass Spectrometry	Kudela, 2011	No SWAMP requirement

13.3 Failures

If failures occur during field work or laboratory analyses, the Project Director must be notified. The Project Director will document the incident and determine the appropriate corrective actions to be taken. These records will be kept on file with the project report for 5 years.

14. Quality Control

To ensure high quality data, all study participants will adhere to: 1) the standard operating procedures and methods listed above in Table 13-3, and 2) all quality assurance and quality control actions outlined in Section 7.

Table 14-1 provides a list of the quality control checks that will be used in this project. Additional details are included below in Sections 14.1 and 14.2.

Table 14-1 Quality control checks and information provided

QC Check	Information Provided
Blanks	
Bottle blank	Cleanliness
Field blank	Transport, storage, and field handling bias
Equipment blank	Contaminated equipment
Method blank	Response of an entire laboratory system
Reagent blank	Contaminated reagent
Replicates	
Field replicate	Precision of all steps after acquisition
Laboratory replicate	Analytical precision
Analysis replicate	Instrument precision
Spikes	
Matrix spike	Analytical (preparation + analysis) bias
Matrix spike replicate	Analytical bias and precision
Analysis matrix spike	Instrument bias
Surrogate spike	Analytical bias
Calibration checks	
Zero check	Calibration drift and memory effect
Span check	Calibration drift and memory effect
Mid-range check	Calibration drift and memory effect

14.1 Field sampling

Field blanks and field replicates will be used for quality control of sampling activities. The total number of blanks and replicates utilized in this study shall equal 5% of all samples collected. They will be collected and prepared at randomly selected sampling sites.

Field blanks are used to assess whether a source(s) of contamination exists in the collection process, from sample bottle cleaning to handling and transport. The blank sample is prepared using the same equipment and procedures used for sample collection, but DI water is used in lieu of the sample water.

Field replicates are used to evaluate the precision of the sampling techniques and to assess short-term environmental variability at the sample site. Replicates will be prepared in the field by duplicating the sample collection procedures, either at the same time or in rapid sequence. As described in Section 7, the relative percent difference (RPD) will be calculated for duplicate samples. The calculation and criteria are as follows:

$RPD = [X_1 - X_2] / [(X_1 + X_2) / 2] * 100$
Where: X_1 = the larger of the two values, and X_2 = the smaller of the two values

RPD < 25% » acceptable
RPD > 25% » classify as estimated

14.2 Laboratory

Laboratory quality control checks will include the use of method blanks, matrix spikes, duplicates, and laboratory control samples. The required frequency of each type of these QC samples is one (1) per every twenty (20) samples or analytical batch, whichever is more frequent. Corrective actions will be taken if control limits are exceeded. Any issues will be documented and reported to the contract manager. Any suspicious sample or control will be rerun.

As described in Section 7, the precision of the laboratory measurements will be determined by calculating the relative percent difference (RPD) between duplicate samples as shown in Section 14.1. Percent recovery is calculated for matrix spike samples as follows:

$R = (Cs - C) / S * 100$

Where: R = percent recovery, Cs = spiked sample concentration, C = sample background concentration, and S = concentration of analyte added to the sample

$80\% \leq R \leq 100\%$ » acceptable

14.3 Review and corrective action

Field and laboratory data generated for this project will be reviewed using the data quality objectives outlined in this QAPP throughout the duration of the study, from the point of collection through laboratory analysis and reporting.

Corrective actions will be taken when analysis is deemed suspect for some reason. The corrective action typically involves the following:

- Check procedures
- Review documentation and calculations to identify possible errors
- Correct errors
- Re-analyze sample, if available

15. Instrument/Equipment Testing, Inspection, and Maintenance

15.1 Field equipment

RWQCB staff will test and maintain field equipment in accordance with the manufacturers' specifications. Maintenance will be provided on an as-needed basis. All probes will be inspected during calibration (prior to each sampling event). Any deficiencies will be reported, and corrective action will be taken as soon as problems are identified. Spare probes for the Quanta Hydrolab multi-parameter water quality instrument and extra sampling equipment and containers will be kept in the SD RWQCB field room and with the field sampling crew on each sampling trip.

15.2 Laboratory instruments

The contract laboratories (WPCL, SCCWRP, and UC Santa Cruz) maintain equipment in accordance with their SOPs, includes those specified by the manufacturer and those specified by the analytical methods used. As part of the daily calibration procedure, all equipment is tested for appropriate responses and behavior prior to analysis. If equipment fails to behave properly as specified in the operating manual, the QA Officer will be notified immediately. Corrective actions will be taken with the guidance of technical support from the manufacturer until the equipment functions properly. All problems and corrective actions will be recorded.

16. Instrument/Equipment Calibration and Frequency

All equipment used by the RWQCB field crew will be calibrated in accordance with the SOPs, including manufacturer's recommendations or those specified by the method. Calibration will be done on a daily basis and in compliance with SWAMP requirements. The Quanta multi-probe will be calibrated daily, and the refractometer will be calibrated before each use. If calibration is unsuccessful, the instrument will be cleaned and parts will be replaced until a successful calibration occurs. If calibration I still unsuccessful, then the Project Director will be notified and data will either not be collected with that instrument, or it will be collected and flagged.

The contract laboratories (WPCL, SCCWRP, and UC Santa Cruz) are responsible for calibrating equipment in accordance with the manufacturers' SOPs. The LC-MS, HPLC, and LC-ESI-MS/MS will be calibrated each time a set of samples are analyzed. If calibration is unsuccessful or other malfunctions occur, the Project Director must be notified, and analyses must be postponed until functional equipment is available. Any affected data will be flagged. Samples may be re-run once equipment is functioning properly. All problems and corrective actions will be recorded by the contract laboratory, and the Project Director will be notified.

17. Inspection/Acceptance of Supplies and Consumables

All supplies and consumables used for this project (under SWAMP funding) were purchased through the Moss Landing Marine Laboratory and the Department of Fish and Wildlife. They will inspect the necessary supplies and consumables according to their SOPs. All supplies must be inspected prior to use, and examined for any damage.

18. Non-Direct Measurements (Existing Data)

This study will mainly use data that is collected during the screening. However, it will be combined with data collected in the cyanotoxin screenings (streams and depressional wetlands) conducted in 2012 and presented in a single report. The 2012 screenings were conducted through a SWAMP-funded project (depressional wetlands) and the Southern California Stormwater Monitoring Coalition (streams), all following SWAMP protocols and therefore are of high quality and considered valid.

There is a possibility that related data, available in CEDEN or that has been collected at the water bodies sampled in this study, may be used to inform sampling decisions for this study. These data will not be analyzed together with data collecting in this project. It will solely be

used as background information, and noted as such, in the report. The validity of this data will be checked by locating the source and reviewing the SOP and QAPP used for the collection and analysis. If the validity is suspect, then the data will not be considered.

19. Data Management

The SWAMP Database Manager and support staff (Moss Landing Marine Laboratory (MLML)) will maintain a file of all data records, including field generated data and laboratory data. Table 19-1 lists the parties responsible for submitting data to SWAMP Temp/Entry database. MLML will follow their SOPs for data management, including record keeping and tracking, document control, and data handling. They will perform all QA/QC on data before entering into the SWAMP Permanent database.

Table 19-1 Parties responsible for entering data into SWAMP

Data	Responsible for submitting to SWAMP
Site information	SD RWQCB
Dissolved Oxygen	SD RWQCB
Temperature	SD RWQCB
Conductivity	SD RWQCB
pH	SD RWQCB
Salinity	SD RWQCB
Alkalinity	SD RWQCB
Water Column Chlorophyll-a	SCCWRP
Pigments	No submittal (not accepted by SWAMP)
Cyanotoxins (particulate)	WPCL or SD RWQCB (TBD)
Cyanotoxins (dissolved/SPATT)	SCCWRP
Particulate Nitrogen	No submittal (not SWAMP funded)
Particulate Phosphorous	No submittal (not SWAMP funded)
Dissolved Inorganic Nutrients	No submittal (not SWAMP funded)
Total Nitrogen & Phosphorous	No submittal (not SWAMP funded)

Appendix P provides the field sheets that will be used to record data and document sample collection. It also contains the checklist that will be used to keep record of sample shipments. The entities listed in the table above will be responsible for keeping track of which data has been entered into the SWAMP database.

Group C: Assessment and Oversight

20. Assessment & Response Actions

Due to the nature and scope of this small screening study, audits will not be conducted as a part of this project. However, self-assessments will be used by the field crew and laboratories for the duration of this study.

The field crew will review appropriate SOPs before going out to the sites to collect samples to ensure all methods are understood and the necessary equipment and supplies are present and ready for use. Checklists on the field sheets will be reviewed before leaving the sampling sites to verify that all samples were collected. If the field crew encounters any issues that cannot be immediately corrected at the sample site, they will notify the Project Director. Either a re-visit to the sample site will be scheduled to complete data collection, or the error will be noted with the data in the SWAMP database.

The laboratory technicians are responsible for providing a summary of the QA/QC data to the SWAMP QA staff to verify that the performance criteria of the QAPP were met. If it is determined that the precision and accuracy objectives were not met, all samples will be re-analyzed, and the QA Officer will review laboratory techniques to minimize errors. The Project Director will be notified.

All corrective actions required over the reporting period will be recorded and reported to the QA Officer.

21. Reports to Management

Drafts and final reports (Word and PDF versions) will be provided for review. In addition, a fact sheet with recommendations for decision-makers will be developed. The findings of this study will be presented to the San Diego Regional Water Quality Control Board management and the SWAMP roundtable.

Group D: Data Validation and Usability

22. Data Review, Verification, and Validation Requirements

This section of the QAPP addresses the quality assurance activities that occur following the completion of sampling activities, including data review, verification and validation. Data generated by project activities will be reviewed against the data quality objectives cited in Element 7 and the quality assurance/quality control practices cited in Elements 14, 15, 16, and 17. Data will be separated into three categories: 1) data meeting all data quality objectives as specified in this document and those specified by SWAMP, 2) data failing precision or recovery criteria, and 3) data failing to meet accuracy criteria. Data meeting all data quality objectives, but with failures of quality assurance/quality control practices will be set aside until the impact of the failure on data quality is determined. Once determined, the data will be moved into either the first category or the last category. These data meet the criteria as specified in this document and where appropriate all SWAMP criteria.

The field and laboratory personnel as well as the QA and QC Officers will be responsible for verifying that the sample collection, handling, and analysis procedures were in accordance with the approved QAPP. In addition, the QA Officer will be primarily responsible for reviewing the data. Data falling in the first category is considered usable by the project. Data falling in the last category is considered not usable. Data falling in the second category will have all aspects assessed. If sufficient evidence is found supporting data quality for use in this project, the data will be moved to the first category, but will be flagged with a “J” as per EPA specifications. Any data resulting from procedures in conflict with QAPP requirements will be rejected.

23. Verification and Validation Methods

Data verification will be performed to evaluate the completeness, correctness, and conformance of the datasets against the methods and procedural requirements. Data quality indicators will be continuously monitoring throughout the duration of this screening study by field crews and laboratory personnel, and any issues will be noted. Any corrections require an agreement with the Regional Board that the correction is appropriate.

Data verification and validation for sample collection and handling activities will consist of the following tasks:

- Verification that the sampling activities (i.e., sample locations, number of samples collected,
- and type of analysis performed) are performed in accordance with QAPP requirements;
- Documentation of any field changes or discrepancies;
- Verification that the field activities (including sample location, sample type, sample
- date and time, name of field personnel, etc.) were properly documented;
- Verification of proper completion of sample labels and secure storage of samples; and
- Verification that all samples recorded in the field log were received by the laboratory.

Checklists are available in Appendix P, which include field sheets that are used to record which samples are collected and a spreadsheet that will be used to record when samples are shipped to the consulting laboratories.

Data verification and validation for the sample analysis activities will consist of the following tasks:

- Appropriate methodology has been followed;
- Instrument calibrations are correct;
- QC samples meet performance criteria;
- Analytical results are complete and correct; and
- Documentation is complete.

Verification and validation of data entry includes:

- Reviewing data to identify missing or mistyped (too large or too small) values; and
- Double-checking all typed values.

24. Reconciliation with User Requirements

The data quality will be evaluated according to this document, with respect to the sampling design, sampling methods, field and laboratory analyses, quality control, and maintenance. By properly following the guidelines in this document and documents referenced (e.g., equipment manuals), the data quality will be validated. If samples or procedures used in this study fail to meet the guidelines listed in this document, the data will be flagged and reported to the Project Director. Any flagged data will be carefully scrutinized to determine whether the data can be used in the final analyses. Data that is not rejected after being scrutinized will be documented in the final reports.

The data quality objectives of this study were created with the data's intended use in mind. The goal of this study is to provide an initial screening of lakes/reservoirs and coastal wetlands in the San Diego region for the presence of cyanotoxins. Regulatory agencies like the Regional Board can use this information to identify water bodies where cyanobacteria and their toxins may be an issue of concern and where preventative measures would be most beneficial. Information gained from this study could be used to plan further investigation and analyses (e.g., plankton taxonomic analysis), direct future studies, and to aid the development of regional or state-wide monitoring programs.

References

Fetscher, E.A., K. Lunde, E. Stein, and J. Brown. 2012. Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1. Southern California Coastal Water Research Project (SCCWRP). Costa Mesa, CA.

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APPENDIX A

Chain of Custody Form

Fiscal Year: 1213 | **Group:** Toxins | Contact Person: Carey Nagoda
Region: 9 | **Date:** | Phone: 858-627-3933
ProjectCode: RWB9_CyanoBac_2013 | **EventType:** WQ | email: carey.nagoda@waterboards.ca.gov
Funding: | **ProtocolCode:** San Diego Cyanotoxin QAPP 2013 | Mailing Address: 9174 Sky Park Court, Suite 100
AgencyCode: RWQCB9 | **Field Crew:** C. Nagoda, C. Loflen | San Diego, CA 92123-4340

StationCode	Sample Type Code	Location Code	Sample Date	Collection Time	CollectionDepth wat (m) sed (cm) integrated (-88)	Salinity (ppt) EC (ppt)	Field Preparation Preservation	Sample Volume (ml)	Water Toxins/Harmful Algal Blooms filter
FIELDQA	Integrated				0.1		frozen		1
902TV0111	Integrated				0.1		frozen		1
903PLH214	Integrated				0.1		frozen		1
904AGHDLG	Integrated				0.1		frozen		1
904SNELLG	Integrated				0.1		frozen		1
904TB0047	Integrated				0.1		frozen		1
905PLH070	Integrated				0.1		frozen		1
905PLS198	Integrated				0.1		frozen		1
90606MISS	Integrated				0.1		frozen		1
906LSPNLG	Integrated				0.1		frozen		1
906PLM142	Integrated				0.1		frozen		1
907CUYRES	Integrated				0.1		frozen		1
907LKMURR	Integrated				0.1		frozen		1
907PEC062	Integrated				0.1		frozen		1
907SDRVES	Integrated				0.1		frozen		1
908SDBYSS	Integrated				0.1		frozen		1
908SDBYSW	Integrated				0.1		frozen		1
908SDBNTC	Integrated				0.1		frozen		1
910PLO182	Integrated				0.1		frozen		1
911PMR110	Integrated				0.1		frozen		1
911TJRVES	Integrated				0.1		frozen		1
							TOTAL		21

Comments:

Samples Relinquished by:		**Samples Received by:**	
Name (Print and Sign)	**Date & Time**	**Name (Print and Sign)**	**Date & Time**

APPENDIX B

Fetscher et al., 2012

Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1

Standard Operating Procedures (SOP) for Collection of Macroinvertebrates, Algae, and Associated Physical Habitat Data in California Depressional Wetlands v1

10 May 2012

Prepared by:

A. Elizabeth Fetscher, Kevin Lunde, Eric Stein, and Jeff Brown

The following individuals provided valuable input on the SOP (alphabetical):

Billy Jakl, Nathan Mack, Shawn McBride, Sean Mundell, Bill Patterson, Glenn Sibbald, Marco Sigala, Wendy Willis, Jennifer York

NOTE:

This SOP has been designed specifically for use in 2012. It is anticipated that this first year of sampling will provide insights triggering minor modifications to the procedures. As such, changes to the SOP should be expected before it may be considered final and eligible for adoption as a SWAMP protocol.

General description of sampling activities

This sampling procedure is intended for use in any depressional wetland, per the definition of depressional wetlands that has been set forth by the California Rapid Assessment Method (CRAM; cramwetlands.org). To date, it has been used in wetlands ranging from 150m^2 to 5 hectares.

Macroinvertebrates (MIs) (i.e., benthic (bottom dwelling), nekton (swimming), and neuston (surface)) and benthic algae are each subsampled from sets of 10 transects evenly distributed within the wetland. The same number of transects is always used, regardless the wetland size. The 10 subsamples of the MI

assemblage are combined into a composite sample that is preserved in 95% ethanol in the field. The 10 subsamples of the algae assemblage are also combined into a composite sample. From the algae composite, two aliquots are prepared and fixed with preservative for lab IDs: one for diatom analysis using formalin, and one for soft-bodied algae analysis using glutaraldehyde. A qualitative sample of soft algae is also collected and, *unlike the case with stream algae samples, it is kept in a plastic bottle and* ***fixed*** *with glutaraldehyde* to facilitate long-term storage*.*

Integrated water chemistry samples are also collected from 10 transects for analysis of several chemical constituents as well as suspended chlorophyll *a* and cyanotoxins (incl. microcystin). Field probes are used to collect data on water temperature, conductivity, pH, and dissolved oxygen at two locations that are a good distance apart within the wetland. An integrated sediment sample is collected for chemistry and toxicity analyses.

Macroalgal and macrophyte cover are visually estimated at each of the transects associated with collections of MIs and algae. Other “PHab” (physical habitat) parameters are also recorded.

In addition to sampling of biota, water, and sediment as described in this SOP, general condition of each wetland is assessed using CRAM. In 2012, this will be carried out during distinct site visits, which will occur after the biotic/water chemistry/sediment sampling has taken place at each site.

The index period for sampling depressional wetland invertebrates (the time frame during which sampling should occur) is similar to the SWAMP perennial stream index period for each region. The goal is to sample invertebrates when they are large enough to identify, yet before they emerge as adults. For example, in southern California, the recommended index period is May 1st to June 30th. The index period may need to be moved earlier in the year if the study goal is to sample a large number of non-perennial wetlands (i.e., those that dry out seasonally), or if the spring is hot and dry, thus accelerating pond desiccation and insect emergence. Sampling should not be conducted less than a week after the last significant rain event.

Habitat observations at the level of the wetland

Data on physical characteristics of the wetland (that could serve as explanatory factors for the biotic community composition data) are collected at the level of the wetland as a whole, as well as associated with each of the MI and algae sampling spots. The latter data types are discussed in detail within the MI and algae sampling sections. All of the data under "Habitat Observations" portion on the first page of the field sheets are meant to characterize the wetland as a whole. Some of the wetland characteristics should be determined beforehand during an "office assessment", and then confirmed in the field. These include the wetland's origin (natural or artificial), its age and function (if created), whether or not vector control activities currently occur at the wetland (and what kind), and what the wetland's hydroperiod is believed to be.

Wetland trophic status is a general classification of three categories, based on observed algae and herbaceous plant levels, from oligotrophic (little or no algae or plant biomass), mesotrophic (moderate algae and/or plant biomass), to eutrophic (very high algae and/or plant biomass). Other data to record include wetland length and width, which are assessed using a rangefinder or using Google Earth/Bing imagery after knowing the current water surface elevation, and observations relating to weather conditions during the assessment period (e.g., precipitation, cloud cover, and wind conditions). The number of paces[1] required to walk the perimeter of the water are also recorded and translated into meters for the database, as are wetland maximum depth and average depth. Both depth assessments are estimates based on the topography of the pond bottom. In a wetland that is 100% wadeable, the average depth will be close to the average of the near/mid/far sample depths. However, in a system where only a few meters of the littoral fringe is wadeable, then the field crew will need to make a best-faith estimate of the depth. Depth information can also be obtained from landowners, when possible. Depth information is

[1] A "pace" is defined as a single step when walking with a gait that is normal for the individual. Ideally, the same individual who enumerates the number of paces around the wetland perimeter should be the one to measure off spacing between adjacent nodes/transects.

measured in meters in the field and converted to cm for entry into the SWAMP database.

The percent cover of vegetation across the pond, compartmentalized into categories for emergent, submerged, and surface (floating) vegetation, is recorded for the pond as a whole. Within the latter two categories, separate percentages are given for algal vs. non-algal ("other") plant material. This is analogous to the categories used for the MI and algae transect-specific PHab (see below). The percent of the pond's surface area that is wadeable (littoral) is estimated from the transects and recorded. Likewise, the percent value of the "% Surface area of maximum" on the field sheet is calculated by estimating the current surface area size as a fraction of the total surface area the pond would have at "high water" stage (e.g., this would be 100% if the pond happened to be assessed at high-water stage). High water marks around the pond are observed and used to make this estimate. Perennial wetlands will tend to be closer to 100%, but non-perennial wetlands may be just a small fraction of their winter maximum size according to how much evaporation has occurred since the last rain event.

In addition to wetland physical characteristics, wildlife use is also noted by marking what types of animals are observed at the wetland during the assessment. These observations are best made when first approaching the wetland, before the actual transect laying and sampling begin, which may scare wildlife away. Some species-specific information is recorded in the case of amphibians, for which major invasives (e.g., bullfrog, African clawed frog) are noted, as well as native species (e.g., California newt, spadefoot toad, red-legged frog). An excellent amphibian resource for identification of eggs, larvae, and adults is *California Herps* (http://www.californiaherps.com/). For birds, the number of individuals falling into various guilds (i.e., raptors, waterfowl, shorebirds, passerines) is recorded. Raptors are birds-of-prey, such as hawks, kestrels, kites, terns, and osprey. Examples of waterfowl are ducks, geese, loons, and grebes. Shorebirds include herons and egrets, and passerines are songbirds, such as red-winged blackbirds and grackles. Record in the comments section any birds that you are unable to classify into these guilds.

Additional stressors are recorded on the field sheet. Note if any fish are present at the site, as they can alter MI communities. Most fish species found in wetlands will be invasive, warmwater fish, such as bass, sunfish, and mosquitofish. Fish information can be obtained by asking property owners, or individuals at the site (e.g., people who are fishing). Also note the presence of crayfish, which can alter both physical and botanic conditions at wetlands and lakes. Early life stages of crayfish are likely to show up in the MI samples, or they may be detected onsite by looking for evidence of their burrows around the wet terrestrial margins of the wetland. “Recent grazing” is loosely defined as cattle activity within the past year. Note that grazing of seasonal ponds may occur when the pond is dry. In the field, look for active cow presence, cow patties from the current season (still somewhat moist), muddy hoof prints around the pond margin, or by asking the landowners. If there is cow activity, but you are not certain if it is recent, still select YES. If the site was not grazed in the past year but was grazed within the past 2-10 years, note that in the comments section.

Bank slope is measured at four representative locations above the water’s edge using an "angle finder", and all four measurements are recorded on the field sheet.

Identification of sampling nodes

The goal of this step is to set up 10 evenly spaced sampling “nodes”, the distance between which is based on the circumference of the wetland (Figure 1). Circumference data can either be collected before visiting the site by using remote photography, or it can be determined in the field. Upon occupying the site, identify a starting point (it could be where the wetland is first accessed). From this point, walk along the periphery, always maintaining a constant distance from the edge of the surface water, and count how many paces are required to walk the entire perimeter. During this time, take note of the wetland and make observations of any species of birds and amphibians and record them on your field sheet (see below). Divide the total number of paces by 10 to yield the distance between adjacent sampling nodes along the wetland’s edge (Figure 1). Gather 10 orange transect flags and a roll of orange flagging tape. Begin

pacing the perimeter of the wetland again, using the same path as before, and stop to place a flag (or tie flagging tape) in a highly visible spot at each node where sampling transects will be located. Water samples can be collected concurrently with placement of the orange flags.

Order of data collection

Sampling should always be carried out in the following order: 1) water chemistry measurements (turbidity, probe measurements) and water-column grabs for laboratory chemistry analyses, 2) MI samples, 3) the quantitative algae sample, and then a qualitative algae sample, and 4) sediment. Each of the 10 sampling “nodes” will have an associated water collection transect, an MI transect, and an algae transect. The water sample will have been collected corresponding directly to where each orange flag was placed (and turbidity and probe measurements will also be collected from transects located adjacent to the water collection transects associated with nodes 1 and 5). The MIs will be collected from transects positioned 3 paces beyond (to the right of) each of the orange flags (as one walks around the perimeter of the wetland) at the appropriate near, mid, or far positions (see below). The quantitative algae samples will be collected from transects positioned 6 paces beyond each of the orange flags. Sediment and qualitative algae samples will be collected throughout the wetland, where applicable (see below).

To facilitate placement of the sampling array, the MI collector will hang a yellow flag where the MI collection was made, so that the algae collector, who comes along subsequently, will know to collect 3 paces beyond that. In order to keep track of where each sample type is to be collected, the collectors should always walk around a given wetland in the same, counterclockwise, direction, and under no circumstances shall any sample be collected where the sediment/water column has been stirred up by previous sampling activities.

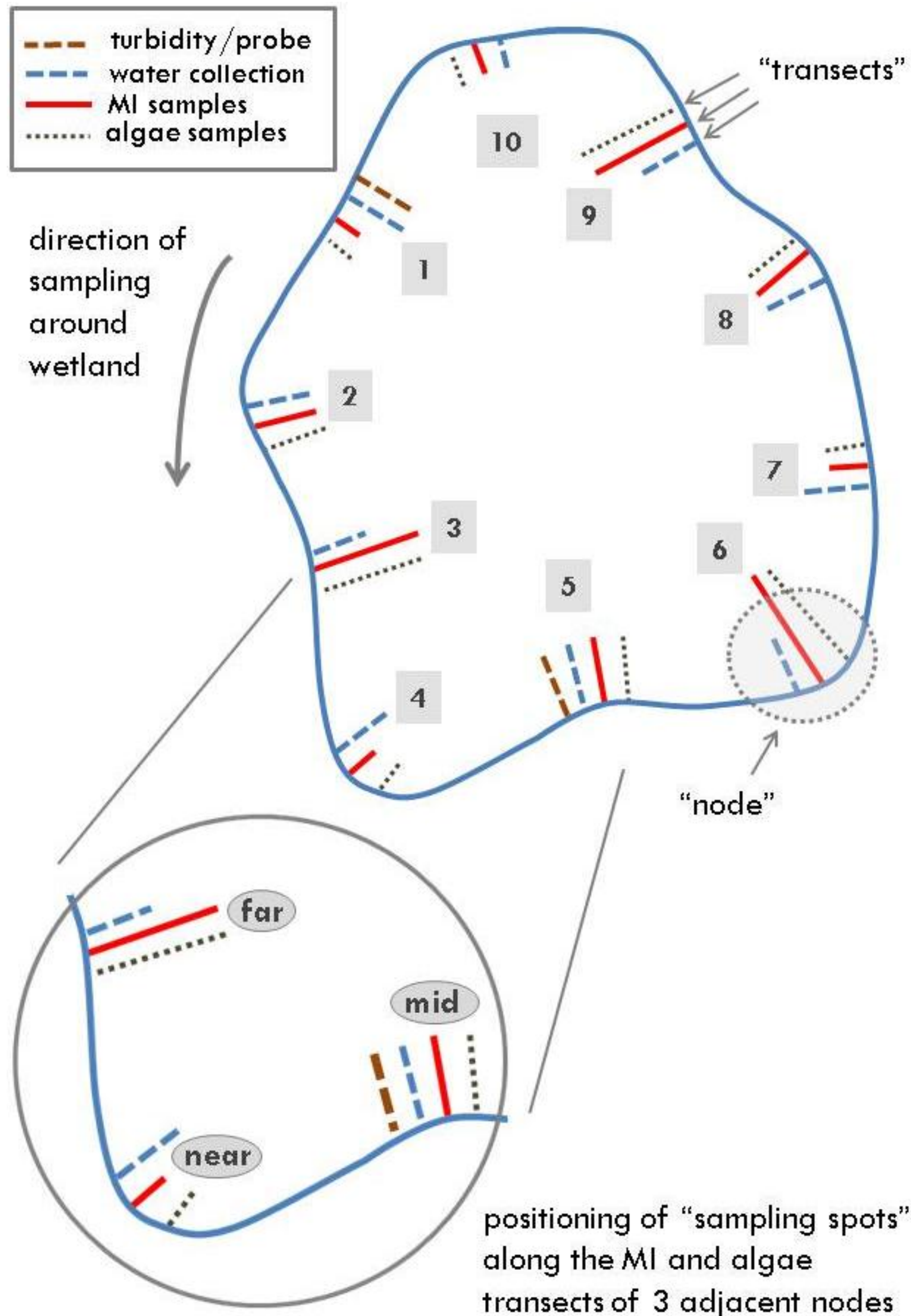


Figure 1. Placement of sampling transects for collection of water, MI, and algae samples at each of the 10 nodes around the edge of the wetland's surface water, and collection of turbidity and probe (conductivity, temperature, pH, DO, etc.) data at nodes 1 and 5. Transects are nested within nodes, and sampling

spots are contained within transects. Sediment is collected in appropriate spots where no other sampling has taken place, after all other samples have been collected (see below).

Water sample collection and field measurements overview

Water chemistry field measurements and water samples are collected prior to any other sampling at each node. Water samples are collected for the following analytes: suspended chlorophyll *a*, cyanotoxins (including microcystin), turbidity, alkalinity, hardness, and nutrients (total Kjeldahl nitrogen (TKN), nitrate, nitrite, ortho-phosphate, total nitrogen, and total phosphorus). All water samples are aliquoted into appropriate sample containers from one or the other of two large, integrated samples that have been composited across the water collection transects associated with the10 sampling nodes. The chlorophyll *a* and cyanotoxin samples come from one of these bottles and are filtered in the field, stored temporarily on wet ice, and frozen within 4 hours of filtering (which may require that dry ice be brought to the field). All of the other water chemistry samples are aliquoted from the other bottle (and the dissolved nutrients sample is filtered before being aliquoted). Appendix 1 provides details on sample preparation.

Field measurements

Before collecting water samples for laboratory chemistry analyses, turbidity and probe measurements [water temperature (deg C), pH, specific conductivity (uS/cm), salinity (ppt), dissolved oxygen (mg/L and % saturation)] are recorded at two nodes at roughly opposite ends of the wetland: Node 1 and Node 5. At each of these two nodes, the turbidity/probe measurement are collected at the "mid" sampling spot along transects placed 3 paces to the left of the orange flags associated with each node (i.e., they are placed on the opposite side of the orange flag from which the biotic samples will later be collected; Fig 1). For the purposes of this SOP, "mid" is always defined as ¼ of the way across the wetland along the transect at hand, when sighting perpendicularly from the shore upon which the collector is standing. Note, however, that under no circumstances shall sampling transects within a node, or entire nodes, be

allowed to cross one another. If such a situation were to present itself, the samplers should be cognizant of this and should either shorten, or longitudinally shift, transects as needed in order to prevent them from overlapping.

Avoid kicking up sediments that will interfere with turbidity readings by paying close attention to the sediment plume resulting from wading into the wetland. Turbidity can be taken with a YSI multi-probe or a Hach 2100P Turbidimeter, or it can be measured in the laboratory. Alkalinity (mg/L) can be measured with a field test kit (e.g. Hach AL-AP #2444301) or in the laboratory. If using a portable meter, collect approximately 250 mL of water for turbidity measurements approximately 10 cm below the water surface and take two separate readings from subsamples of the same grab sample.

Once the turbidity sample has been collected at a given transect, the probe measurements (water temperature, pH, specific conductivity, salinity, dissolved oxygen (mg/L and % saturation)) can be taken. Measurements are made 10 cm below the water surface according to the MPSL-DFG SOP for Conducting Field Measurements and Field Collections of Water and Bed Sediment Samples in the Surface Water Ambient Monitoring Program (http://swamp.mpsl.mlml.calstate.edu/wp-content/uploads/2009/04/swamp_sop_field_measures_water_sediment_collection_v1_0.pdf). Using a YSI or similar device, measure specific conductivity (set to 25°C; uS/cm), salinity (ppt), temperature (°C), dissolved oxygen (in % and mg/L), and pH.

Water sample collection

Collect two integrated water samples across the 10 water-collection transects. At each point where there is an orange flag, wade into the wetland perpendicularly from the water's edge. This is the water sampling transect. Hold the water sample grabber extended in front of you and walk to a distance where the end of the grabber hovers over the point corresponding to the "mid" sampling spot for that transect. ***<The following instructions will change somewhat based on what the final sampling device ends up being>.*** Dip the grabber's cup face-down into the water slowly and gently in such a way that avoids disturbing the sediment on the wetland's bottom, and that avoids allowing any surface scum that might be present on the water to enter the cup (use the air bubble inside the cup as a barrier to prevent this). Aim to collect water in the stratum 10 – 15 cm below the water's surface. Once underwater, "burp" the cup to allow water to slowly enter. Then, maintaining the water grabber underwater, pull it toward you slowly and place a clean cover over the top of the cup. Lift it out of the water vertically, with the cup opening facing straight up and with the cover in place. Immediately return to the shore and discard all but 250 mL from the cup. Then pour the 100 mL of this into a clean, foil-covered 1- or 2 L bottle (for the chlorophyll *a* and cyanotoxins samples), and pour the remaining 150 mL into the other (2 L) bottle (for the other water chemistry samples). Repeat this procedure at each of the remaining 9 flags. If possible, all water samples should be collected from the same spot for each transect. Once all 10 water collection transects have been sampled, mix each of the two integrated samples by capping the bottles and turning each upside-down and right-side-up for 5 cycles, slowly and gently.

Two types of samples are prepared from the smaller-volume integrated water sample by concentrating the plankton via filtration: chlorophyll *a* and cyanotoxins. Other samples are taken from the larger-volume integrated sample and transferred to the appropriate types of bottles. The sample for dissolved nutrients (i.e., for nitrate, nitrite, and orthophosphate analysis) is field-filtered using a 0.45 μm aqueous filter, whereas TN, TP and TKN are not filtered. TKN is acidified. Field filtering for dissolved nutrients and freezing of

nutrient samples are carried out in order to permit longer holding times[2]. Project holding times, field preparation, preservation (if applicable), bottle types, and recommended volumes for each water chemistry analyte can be found in Appendix 1. Please consult the Marine Pollution Studies Laboratory – Department of Fish and Game (MPSL-DFG) Standard Operating Procedures (SOPs) for Conducting Field Measurements and Field Collections of Water and Bed Sediment Samples in the Surface Water Ambient Monitoring Program (SWAMP) (http://swamp.mpsl.mlml.calstate.edu/wp-content/uploads/2009/04/swamp_sop_field_measures_water_sediment_collection_v1_0.pdf) for more information.

DO NOT use Sparquat 256, Quat 128, or any other related agent to decontaminate the water grabber for invasive species/pathogens, as this could chemically contaminate the grabber, thus affecting the quality of water chemistry data. Bleach may be used, instead (see decontamination section at the end of this document).

[2] It should be noted that freezing of nutrients constitutes a deviation from current procedures per the SWAMP QAPrP.

Preparation of suspended chlorophyll a and cyanotoxin sample filters

To prepare the chlorophyll *a* sample, filter 250 mL of the well-mixed sample from the integrated sample bottle. ***Prepare duplicate filters for the chlorophyll* a *sample AT EVERY SITE***. For the cyanotoxins sample, filter 500 mL. Aim to filter the prescribed volumes for each sample type, but always be sure to record the volumes that were actually filtered on the field sheets and the sample labels.

To reduce the likelihood of pigment (chlorophyll *a*, etc.) degradation as much as possible during the sampling, try to move rapidly from sampling node to sampling node when collecting water, and filter the samples as quickly as possible once sampling is complete. Do not leave the sample out in the sun or otherwise exposed to heat. Shading of the sample material should be ensured during the entire filtering process. If no shade is available around the wetland, an umbrella can be used.

Use 47 mm diameter, 0.7 μm pore size, glass fiber filters to isolate the samples for laboratory analysis. During the filtering process, make sure that the pressure on the filter never exceeds 7 psi. Use a hand pump equipped with a gauge to ensure this limit is observed. Pump slowly, if necessary, to regulate the amount of pressure on the filter. Once most of the sample has passed through the filter, rinse the sides of the filter reservoir with a few mL of deionized (DI) water, and continue filtering until all the visible surface water is drawn down. The filter should not be sucked dry, but rather left slightly moist, in order to avoid applying excessive pressure to the sample, which could cause algal cells to burst. Make sure the filter reservoir is thoroughly cleaned with DI water between sites in order to avoid contaminating samples with residual cyanotoxins, if present, from previous sites.

After isolating the algal material on the filter, fold it in half, with the side coated with material on the inside, and place it into a clean, snapping Petri dish. Then wrap the Petri dish in aluminum foil and place into a 60 or 100 mL Whirl-Pak bag along with a filled-out sample label (outward-facing) that has been printed on Rite-in-the-Rain paper and filled in with either a pencil or waterproof pen.

Whirl the Whirl-Pak shut after pushing out as much air as possible, and then twist the wires together to seal shut. Do not store the filters in Ziploc® bags, as these will allow water to enter, if submerged in the wet ice chest.

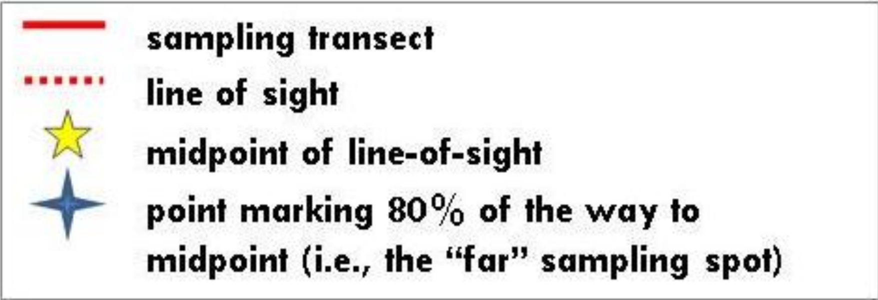
In the field, store the bagged filters in a wet ice chest (or if possible, on dry ice), and keep them well submerged (not floating atop the water in the chest) in order to keep them as cold as possible. Once back at the lab each afternoon, place the filters in the freezer. They must be kept very cold from the time of collection onward, and must be frozen within 4 hours of collection. Fig. 2 provides a label for the chlorophyll *a* and cyanotoxin samples.

Contract/ Billing Code: __________ circle one: chl *a* cyano
Project: __________ Date: _______ Time: ______
Site Code: __________ Sample ID: __________
Repl #: ________ Vol Filtered (mL): __________
Wetland Name: ____________________
County: ________ Collector: ______________

Figure 2. Sample label for chlorophyll *a* and cyanotoxin samples.
Specific instructions for collecting other water chemistry samples are provided in a separate document.

Macroinvertebrate collection

At a distance of 3 paces to the right of each orange flag (i.e., when walking counterclockwise around the perimeter of the wetland), a transect is placed for collection of MIs. As the collector progresses from transect to transect, the distance of the sampling spot from the shore of the wetland (i.e., the boundary where surface water appears) rotates through 3 positions: “near”, “mid”, and “far”, starting with “near”. “Near” is defined as 1.5 m inward from the water’s edge. “Far” is defined as either the closest distance from the edge at which the water is 1 m deep, or if the entire transect is wadeable, “far” is defined as 80% of the distance to the midpoint between the edge of the wetland you are standing at and the opposite shore, as you fix your gaze perpendicularly outward from the shore (Fig. 3). “Mid” is defined as one-quarter of the distance across the wetland along the transect in question. In deeper wetlands with a narrow littoral zone, the “mid” and “far” sampling spots may fall within the same distance from the shore.


sampling transect
line of sight
midpoint of line-of-sight
point marking 80% of the way to
midpoint (i.e., the "far" sampling spot)

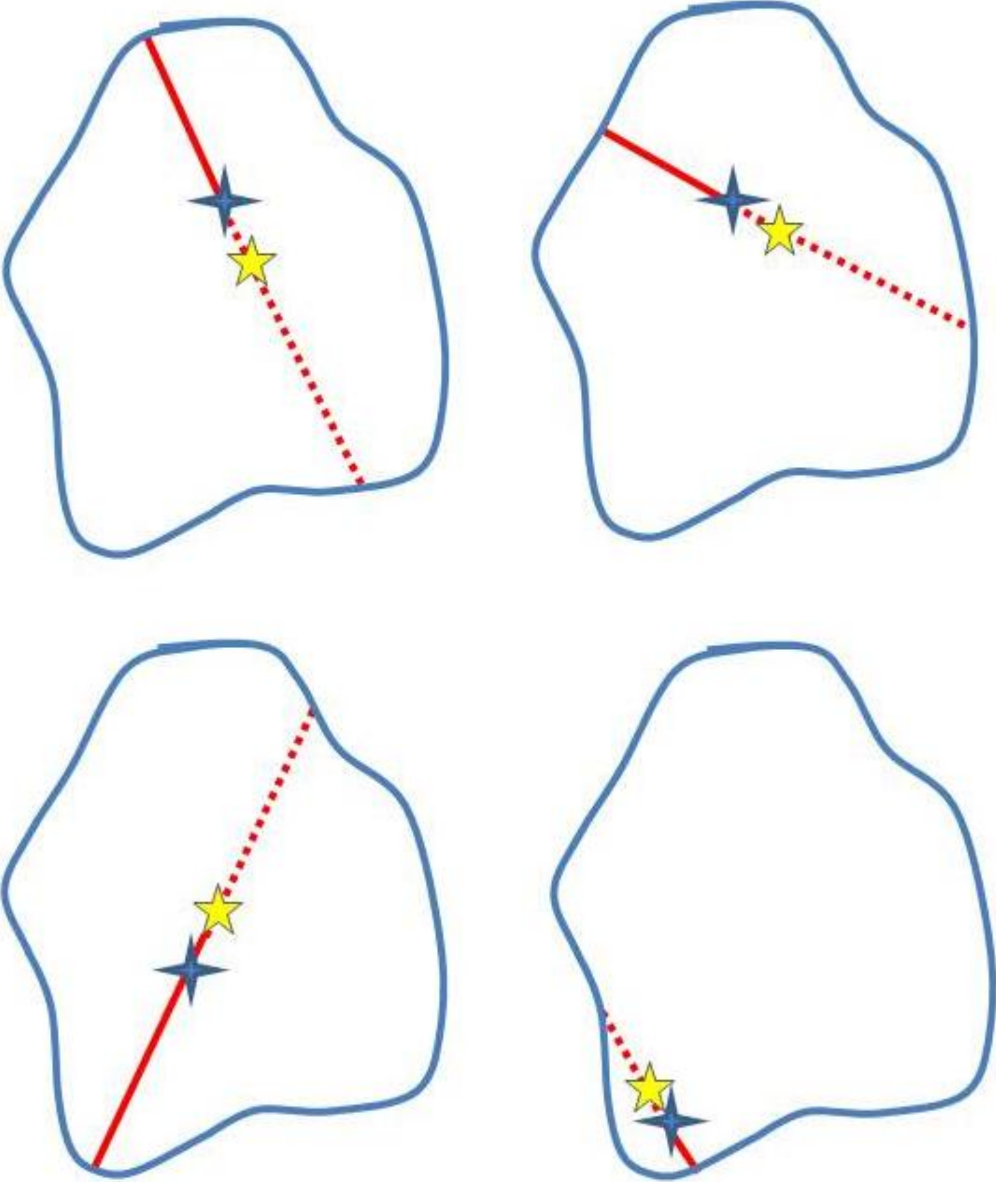


Figure 3. Examples of finding the point corresponding to 80% of the way to the midpoint, which is the definition for the "far" sampling spot. "Midpoint" in this context should not be confused with the "mid" sampling spot.

A summary of the definitions of near, mid, and far sampling spots is provided in Table 1.

Table 1. Definitions of sampling spots for collection of water collection, MI, and algae samples. Transect trajectory is always defined as perpendicular to the shore upon which the sample collector is standing.

Sampling spot location	Water collection/turbidity/probes	MIs	Algae
Near	N/A	Starting at a point 1.5 m from shore along transect trajectory, but no deeper than 1 m	0.5 m from shore along transect trajectory, but no deeper than 0.5 m
Mid	¼ way across wetland along transect trajectory	¼ way across wetland along transect trajectory, but no deeper than 1 m	¼ way across wetland along transect trajectory, but no deeper than 0.5 m
Far	N/A	80% of the way to wetland midpoint along transect trajectory, but no deeper than 1 m	80% of the way to wetland midpoint along transect trajectory, but no deeper than 0.5 m

Once the spot to be sampled is identified, the procedure for collecting the sample is as follows. Approach the sampling spot slowly with your D-frame kick net in the air (not dragging underwater), in order to avoid frightening the bugs away, or accidentally collecting organisms, as you draw nearer. When you have reached the spot, hold the net straight in front of you at arms' length, with the opening of the net facing down. Plunge the net into the water, pulling it through the water column towards the wetland bottom. Then pull it toward you, gently rubbing the pond bottom in an undulating motion that covers a swath about a meter long. The idea is to catch bugs that are in the water column, clinging to vegetation, and along the sediment benthos, all the while avoiding collecting a lot of sediment in the sample. Once your net has nearly reached your feet, quickly swivel it around 180 degrees, and push it back away from you for a second sweep in the opposite direction, using a similar undulating motion. At the mid and far sampling spots (when in water deeper than the height of the

net), pull the net back and forth a second time in the water column to sample swimming invertebrates and invertebrates associated with submerged vegetation. If sampling emergent vegetation, keep the sample area the same, but vigorously work the net against the vegetation and benthos while trying to keep the net in forward motion to prevent escape of captured organisms. Pull the net out of the water with its opening facing upward to keep the material inside, and then return to the shore by retracing the steps you took on your way in.

Add the material collected to a 5-gallon bucket that has been filled approximately halfway with pond water. Then carry out the same collection procedure at the appropriate spot (i.e., mid, far, near, mid…) at each of the remaining MI sampling transects around the wetland. Ideally, two people should work together to collect the MIs: one person to make the collections, and the other to carry equipment and record the required information on the field sheets. For example, at each collection point the sampler will record the information on vegetation cover, which "sampling spot" was sampled at that transect (near, mid far), the depth at which the sample was collected, distance from bank (wetted edge), and distance from wetted edge to winter inundation line (bankfull). For assessing the type of habitat (vegetation) associated with each sampling area, the recorder will record relative percent cover for each of the following categories: emergent vegetation (e.g., cattails tules, spikerushes), submerged vegetation (e.g., water milfoil, pondweed, ditch grass), floating vegetation (duckweed, mosquito fern, algae), and open areas (absence of vegetation).

Associated with each sampling area (which is equivalent to an imaginary 1m^2 area centered around where the MI sweep was made), a suite of habitat observations is recorded. The data recorder estimates the location of the boundaries of the 1m^2 area. Within each sampling area, the relative percentages of habitat type, based on vegetation, are recorded within the appropriate data field, using the categories of emergent, submerged, surface, and open. For each row, the values in the cells should add up to 100. The data collector will need to mentally "flatten" the image of habitat types within the 1m^2 square area to determine what types of habitat are contained within it, and reflect this in the

relative percentages. The submerged and surface vegetation categories are further divided into the algae vs. other (where “other” is non-algal vegetation). The depth (cm) of the water at the sampling area, as well as the distance from the bank (m) is recorded. The water line to bankfull (m) is also recorded. This is determined by measuring the distance from the wetted edge to the seasonal high water mark, which is often marked by a clear change in vegetation type from terrestrial to aquatic.

Whenever a sweep is completed, before moving on to the next transect, leave a yellow flag corresponding to the point where the wetland was entered for that MI sample. This will aid the algae collector in identifying where algae can be sampled.

Once all 10 transects have been sampled for MIs, release any larval amphibians or other non-target organisms (e.g., fish) that may have been captured during the sampling back to the wetland. A small aquarium net is useful to capture these organisms. It may be necessary to use one or more dish tubs as holding chambers to aid in the cleaning of debris from the sample.

Field elutriation and preservation of MI samples

Fill two large dishpan tubs (11qt) with surface pond water and use an aquarium net to remove any macroinvertebrates from this water. Inspect the D-frame sampling net and use forceps to remove any organisms clinging to the net and place these in the bucket. Take large clumps of debris (detritus, wood, live vegetation, macroalgal mats/filaments) that take up a lot of volume in the bucket and individually place them in the first tub. Gently rub the surfaces of the material to remove invertebrates, visually inspect the debris to make sure MIs were removed, and then place the piece into the second tub. Leave the piece in the second tub for a few minutes before discarding back to the wetland. Repeat this process for all large debris. When a majority of large debris has been removed, pour the tubs into a 500μm sieve held over an empty dish tub in case some of the sample misses the sieve. Then pour all of the remaining bucket material into the sieve. (A 4mm sieve can be helpful in trapping amphibian larvae first, if they are abundant at the site.) Transfer all of the

material in the sieve (invertebrates and organic matter) into the sample jar. Carefully inspect the gravel and debris remaining in the bottom of the bucket and tubs for any cased caddisflies, clams, snails, or other dense animals that might remain. Remove any remaining animals by hand and place them in the sample jar.

Place a completed date/locality label (Fig 4) on the inside of the jar (use pencil only, as most "permanent" inks dissolve in ethanol) and completely fill with 95% ethanol. Place a second label of the same type on the outside of the jar. Note that the target concentration of ethanol is 70%, but 95% ethanol is used in the field to account for dilution from water in the sample. Do not add more than 50% debris or material into your 1L jar. If there is a lot of organic and inorganic material, multiple jars might be necessary to store a single composite MI sample. Record the total number of jars for a single sample on the external labels and field sheets.

Funding/Billing code: __________ MI Sample
Project ________ Date: / / 2012 Time: ______
SWAMP ID: _____________ Sample ID (lab) _________
Site Name: ________________________________
Sample Type: Integrated Method: BMI_RWB_DW
County: ___________ Jar # ____ of _____
Collector: ____________Replicate # _____

Figure 4. Internal and external jar label printed on Rite-in-the-Rain or similar water-resistant paper and recorded in pencil.

Algae collection

Algae collection should happen after MI collection. There are two ways to coordinate MI and algae collection depending, in part, upon the number of people in the field crew. There can either be one team (ideally, of two people) for collecting MIs and a separate team (also ideally of two people) for collecting algae, or there can be a single team of two that collects both assemblages in succession. For the former option, the two teams can circle the wetland

concurrently to collect subsamples, but the MI collectors should go first at each node, in order to limit the amount of activity in the general sampling area, thus reducing the likelihood that the bugs will be flushed. For the latter option, the team of two collects MIs at all 10 nodes first, and then goes around the wetland again to collect algae samples.

Within each node, algae are sampled at transects 3 paces to the right of each of the yellow flags (which indicates where MIs had been collected), when walking counterclockwise around the wetland. The procedure for identifying the spots for sampling algae is very similar to that for MIs. The major differences are that sampling for algae should never occur at a spot deeper than 0.5 m, and the "near" spot is approximately 0.5 m from the edge of the surface water. The "far" spot is either the closest place where the water is 0.5 m deep or, if the wetland does not get that deep along that transect, it is defined as 80% of the distance to the midpoint between the edge of the wetland you are standing at and the opposite shore, when sighting perpendicularly from the shore. "Mid" is defined as one-quarter of the distance across the wetland along the transect in question (and is also never deeper than 0.5 m).

Once the general area for the sampling spot is identified at each transect, the sample should be collected from the dominant substratum type in that area, and from a "representative" spot therein. ***<u>*Note that it is necessary to take TWO adjacent grabs*</u>** for algae samples at *each* sampling node (that is, collect two algae subsamples at each of the 10 nodes around the wetland for a total of 20 subsamples composited into the sample container). This is to double the volume of material collected, thus facilitating the preparation of both a diatom sample and a soft-bodied algae sample. Be careful never to collect a sample from a spot that has already been sampled from or otherwise disturbed (Remember: *"Don't sample where you trample!"*) Indicate on your field sheet what substratum type(s) you collected from at each transect. For instance, if you collected one of your 2 subsamples (grabs) from soft sediment and the other from a live cattail stem, then you would mark a "1" in the box corresponding to "soft sediment" and another "1" in the box corresponding to "plant (live)" for that transect. If both subsamples had been collected from soft sediment, then you would mark a "2" in the box corresponding to "soft

sediment". The other boxes would be populated with zeros. Only "0", "1", or "2" can be filled in any box for substratum type for a given replicate.

Unlike with MIs, for which all collections are made using the same device, there are two different sampling devices for collecting algae. The most commonly employed one is a "sediment corer" (5.3 cm^2), which is used both for soft sediments and for delineating a sampling area on plant material and hard substrata that can be removed from the pond. The other device is a "syringe scrubber" (5.3 cm^2), which is used for sampling hard substrata that cannot be removed from the pond, and is identical to what is used for sampling underwater in wadeable streams. Keep a tally of the number of times each sampling device is used, and when sampling is complete, record this value on the field sheets and sample labels.

If the sampling spot encountered falls on an area of soft sediment, the following procedure applies. Use the "sediment corer", which is a plastic 60 mL syringe with the tip of the syringe cut off near the base where the "0 mL" mark is, and with a line (preferably made with a bright pink or silver marker) drawn around the perimeter of the barrel for a distance corresponding to 5 mL up the barrel from the location of the cut. For example, if you cut the syringe barrel at the "5 mL", then the line you draw around the barrel should be positioned at the "10 mL" mark. The open end of the corer must also be filed so as to make it as sharp as possible for cutting into sediment and macroalgal mats.

Submerge the coring device in the pond water to moisten it; then move the plunger up and down a few times to loosen it. Adjust the plunger so that its pointed rubber tip is near the open end of the syringe barrel, but not protruding. Submerge the corer in the water and slowly press the barrel vertically downward into the sediment at the sampling spot, at the same time pulling slowly on the plunger to aid insertion into the sediment to a depth of about 5 cm. Make sure as you make contact between the device and the sediment that you do not cause a current of water that will flush the top surface of the sediment away. You want to keep this intact, as it is where most of the algae will be.

After the core has been taken up into the device, carefully remove the device from underwater and hold it with the open end facing upward. Slowly push on the plunger to move the core out of the syringe barrel. Once the seal ring (the one closest to the end of the barrel) of the black rubber tip on the plunger reaches the mark you have drawn around the syringe barrel, stop pushing the plunger and cut the end of the core off and discard it. What remains within the barrel (~5 mL of material) is the sample. Hold this over the sample bottle and resume pushing on the plunger to release the core into the bottle. Then rinse the end of the plunger into the sample bottle, trying to capture all the sediment particles. An intact (uncut) 60 mL syringe filled with water from the pond being sampled can be used to squirt the end of the corer to dislodge any residual sediment and collect this rinsate into the sample container.

If the sediment is too loose to form a cohesive core inside the coring device, then take another grab from an undisturbed adjacent spot of the same substratum type. Start with the seal ring of the plunger positioned at the mark you had drawn around the syringe barrel, insert the barrel down into the sediment, up to that mark. Then gently slide a spatula beneath it, and pull the device out of the water with the spatula in place. Remove excess sediment (outside of the syringe barrel) from the spatula; then dump the sampled material into the sample bottle. Rinse any residual sediment off the corer and spatula into the sample container.

If plant material is the substratum type encountered, use a soft-bristled toothbrush to ***gently*** remove biofilm coating from the plant within a 5.3 cm^2 area. Do this by clipping the plant at the base (if rooted) with a shears or scissors, then very slowly and gently removing it from the water in order to avoid shearing loosely attached algae from the plant as much as possible. Collect material from the lower portion of the plant, near where it had been rooted. The sediment corer, with the plunger retracted, can be used as a guide for how much area to brush. After brushing within that area, rinse the area over the sample bottle to catch the rinsate and then discard the cleaned substrate. Also thoroughly rinse the brush into the sample container. ***Before using the toothbrush at a given site, always make sure that it has been cleaned thoroughly and does not have any contaminating algae from a previous site***.

If a mat of macroalgae is the substratum type encountered, use the sediment corer to create a core of macroalgae (representing its full thickness) as the sample. This is done by “sandwiching” the entire thickness of the macroalgal mat between the corer and a spatula underwater and using a utility knife to cut the algae around the perimeter of the corer, then adding the resulting “circle” of algae to the sample bottle. This same general procedure can be used if the substrate type is a layer of dead leaves or other such organic debris resting on the pond bottom. In this case, sandwich the leaf between the corer and a spatula underwater, pull the device out of the water, and use a utility knife to isolate a circle of the leaf matter. Once the excess leaf material is cut away, hold the device over the sample bottle and pour all the water (which probably contains algae that had been loosely associated with the leaf) that is above the leaf inside the corer barrel, as well as the leaf piece, into the sample bottle. Then gently brush the algal biofilm off the leaf piece and rinse both the brushed part of the leaf and the brush itself into the bottle. Discard the cleaned leaf piece.

If a hard substratum is encountered at the sampling spot and it can be removed from the pond bottom, very slowly pull it out of the water, delineate the sample area using the sediment corer, and use a toothbrush and rinse water as was described above for sampling from plants. If the substratum cannot be removed from the pond, then use the syringe scrubber to collect the sample underwater. Refer to Fetscher et al. (2010) for instructions for constructing a syringe scrubber.
To use the syringe scrubber, submerge it in the pond and work the plunger a few times to loosen it up. Then affix a fresh, white scrubbing pad circle onto the bottom of the plunger by attaching it to the Velcro®. Press the plunger down so that the bottom of the scrubbing pad is flush with the bottom of the barrel. Then submerge the instrument, press the syringe firmly against a flat area of the substratum, and rotate the syringe scrubber 3 times in order to collect the biofilm from the substratum surface onto the scrubbing pad. If the surface of the substratum where your sampling spot fell is not flat enough to allow for a tight seal with the syringe barrel, objectively choose whatever sufficiently flat area on the exposed face of the substratum is closest to where

the original spot fell, and sample there. After sampling, and before removing the syringe scrubber from the substratum, gently retract the plunger just slightly, so it is not up against the substratum anymore, but not so much that it pulls a lot of water into the barrel. Carefully slide the spatula under syringe barrel (which should be pulled just slightly away from the substratum on one side to allow the spatula to slide under), trying not to allow too much water to rush into the barrel. Then pull the instrument back up out of the water with the spatula still firmly sealed against the syringe-barrel bottom.

Hold the syringe scrubber over the sample container and then remove the spatula, allowing any water to fall into the container. Carefully detach the pad from the plunger and hold the pad over the container. Using rinse water sparingly, remove as much algal material from the pad as possible by rinsing it off with the intact syringe filled with pond water, and wringing it into the sample container before discarding the used pad. Start this process by rinsing from the backside of the pad (the side that had been affixed to the plunger) to "push" the collected algae forward out of the front surface of the pad. It is recommended that a fresh (new) pad be used each time a sample is collected, even within the same pond. Under no circumstances should the same pad be used at more than one site.

Whenever rinsing substrata and sampling devices into the sample bottle, try to be sparing enough with the water to keep the final sample volume under 500 mL, if possible, and definitely under 1 L.

Note that, because two subsamples of algae will be collected from each algae transect (for a total of 20 across the wetland), it is possible to collect from two different substratum types within a single sampling-spot area. For instance, if plants are abundant within a given sampling spot area, one of the subsamples should be collected from the plant material, and another should be collected from sediment or whatever the dominant bed material is within that spot (i.e., organic debris, wood, rock). No more than half of the 20 samples for a wetland should be collected from live plants.

Once all the subsamples have been collected, cap and ***very gently*** agitate the sample container to mix the material well without breaking cells. Pour the entire contents of the container into a 1 L graduated cylinder to measure the volume of the sample. Wait awhile (in the shade) to allow the heavier sediment to settle to the bottom. Once a clear boundary between sediment and liquid is apparent, determine the approximate volume of the liquid portion of the sample as well as the volume of the whole sample (including sediment). Record ***the liquid portion as "composite volume"*** on your field sheet (but make note of the volume of the entire sample, including sediment, for later, as you will use this information to determine how much sample to pour into the diatom and soft-algae sample bottles). Pour all of the material back into the sample container. Gently pour back and forth between the graduated cylinder and the sample container a few times, if necessary, in order to get residual material out of the graduated cylinder. Do not use new water for the rinsing, as this would add volume, requiring that you record a new composite volume.

Very gently mix the sample in the container until it is homogeneous. Using a graduated cylinder, pour half the sample (sediment included) into a 500 mL plastic sample bottle and label it for "diatoms", and pour the remaining half of the sample into another 500 mL sample bottle and label it for "soft algae". Fig. 5 shows a label for algae taxonomy samples. Note that if the total volume of material collected was well under 500 mL, then 250 mL sample bottles could be used for the diatom and soft-bodied algae samples, as long as the volume of fixative that will need to be added to the bottles will not cause the final volumes to exceed 250 mL each.

If any macroalgal clumps are in the sample, use a pair of long forceps to "fish" the material out of the sample bottle. Cut each distinct clump in half, one-by-one, with shears or scissors, and add the resulting halves to each of the sample bottles (diatom and soft-algae).

Once the soft algae sample has been prepared, place the bottle in the wet ice cooler. It must be kept very cold (but not frozen) and in the dark. This sample should be fixed in the laboratory with glutaraldehyde to a final concentration of 2.5% as soon as possible, but no more than 4 days after collection. Diatoms are

fixed while still in the field, using enough 10% formalin to yield a final concentration of 2%. Be sure the wear appropriate protective gear and employ safe practices when handling this fixative. Fetscher et al. (2010) provides detailed guidance for this. ***The calculations used to determine the amount of fixative needed for both the diatom and soft samples can be based on the sample volumes for each being equivalent to ½ the measured <u>composite</u> volume (i.e., the liquid portion of the sample, excluding the sediment).***

Contract/ Billing Code: ________ circle one: **diatoms soft**
Project: __________ Date: ______ Time: ______
Site Code: __________ Sample ID: __________
Repl #: ________ Vol Aliquotted (mL): ________
Composite Vol (mL): ________________
# of Grabs: Sediment Corer: [ ] Syringe: [ ]
Fixative Added: ________________
Wetland Name: ________________
County: ________ Collector: ____________

Figure 5. Label for algae taxonomy samples.

Just as with MIs, associated with each sampling spot (which is equivalent to an imaginary 1m^2 area centered around where the algae subsample grabs were made for each transect), a suite of habitat observations is recorded. The data recorder estimates the location of the boundaries of the 1m^2 area. Most of the habitat observations are the same for both algae and MIs, but a few are associated strictly with one or the other. At each algae sampling spot, the relative percentages of habitat type, based on vegetation, are recorded within the appropriate data field, just as with MIs, again using the categories of emergent, submerged, surface, and open. For each row, the values in the cells should add up to 100. The data collector will need to mentally "flatten" the image of habitat types within the 1m^2 square area to determine what types of habitat are contained within it, and reflect this in the relative percentages. As mentioned above, the submerged and surface vegetation categories are further divided into the algae vs. other (where "other" is non-algal vegetation). The depth (cm) of the water at the sampling point, as well as the distance from the

bank (m) is recorded. For algae, the estimated % shading within the 1m^2 area is also recorded. In addition, the type of pond substratum from which each sample was collected (soft sediment, plant (live/dead) including wood, or rock (including concrete and consolidated sediment)) is also recorded.

Qualitative sampling of macroalgae

The purpose of the qualitative soft-algal sample is to capture the taxonomic diversity of this assemblage in the wetland and to aid in identification of ambiguous specimens collected in the quantitative sample. For qualitative soft-bodied algal samples, collect specimens of all obviously different types of macroalgal filaments and mats, as well as microalgae (in the forms of scrapings using a utility knife), and depositional samples (suctioned from along the surface of sediments using a clean turkey baster). If you suspect something may be algae, but are not sure, it is always preferable to collect some of it for the qualitative sample. The laboratory will determine whether it qualifies for inclusion in the species list. Collect from as many distinct locations as possible throughout the wetland so as to capture as much of the apparent diversity as you can. Also, when possible, try to grab part of the holdfast structures that attached the macroalgae to the substrate (if there are any attached algae in the wetland) as these structures can be useful for taxonomic identification. An attempt should be made to be as exhaustive as possible in sampling the various taxa present. Add all material collected to a labeled (Fig. 6), 250-mL container. Keep the bottle in the dark on wet ice while in the field. ***Upon returning to the laboratory, fix the material to a final concentration of 2.5% glutaraldehyde and store in the refrigerator in the dark (Note: this is a deviation from the stream protocol, in which the qualitative sample is not fixed, but kept fresh and delivered to the lab as soon as possible for analysis.)***

Contract/ Billing Code: ________ **qualitative (soft)**
Project: __________ Date: _______ Time:______
Site Code: __________Sample ID: ___________
Site Name: ____________________________
Fixative added: _________________________
Wetland Name: _________________________
County: _______ Collector: _______________

Figure 6. Label for soft-algae qualitative sample.

Sediment sample collection

An integrated sediment sample is collected within each wetland after all other sampling has been carried out, and prior to removal of the flags. The sediment samples will provide information about potential sources of toxicity. Collect 2-cm deep subsamples of sediment from various spots dispersed throughout the wetland, wherever fine-grained sediment is available. Sample only from places that have not been trampled by previous sampling/data collection, and which appear to be reasonably representative of the overall wetland. Detailed sediment sampling instructions can be found in MPSL-DFG SOP for Conducting Field Measurements and Field Collections of Water and Bed Sediment Samples in the Surface Water Ambient Monitoring Program (http://swamp.mpsl.mlml.calstate.edu/resources-and-downloads/standard-operating-procedures). After the sediment samples have been collected, all of the transect flags can be retrieved.

Collecting field duplicate samples

At 10% of sites, a field duplicate should be collected for each of the sample types: water chemistry, MI, algae, and sediment. Ideally, duplicates of all sample types are collected at the same set of sites. In the case of both the water chemistry and the algae samples, duplicates can be taken from within the same general sampling spot areas within their respective transects. In other words, for algae, instead of collecting 2 subsamples within the sample spot area, 4 are taken, with 2 of them being deposited into one sample bottle, and

the other 2 deposited into a second bottle. (The collector must be careful never to resample the same exact spot.) For the MIs, it is not possible to use the same sampling area because of the wide swath the net makes in the course of capturing the MIs. Instead, the duplicate samples should be taken concurrently, from adjacent sampling areas along the same transect (i.e., with two collectors standing next to each other, both positioned at the near, mid, or far sampling spot, depending upon which spot is being sampled from at the transect in question). The two MI collectors should be working concurrently, side-by-side, rather than in succession, so that neither is likely to scare MIs away from the other's sampling area.

The vegetation cover, distance, and depth measures should be recorded separately for the two duplicate MI samples, because the imaginary 1m^2 areas associated with the net sweeps to collect the duplicates are distinct. Conversely, for the algae, because the replicate samples can all be collected within the same 1m^2 area at each transect, it is not necessary to record separate PHab data for the replicates (and the same information could be used to populate that component of the algae PHab data for both replicates 1 and 2 in the database). However, with respect to recording substratum type associated with the algal replicate subsamples, this must be done separately for replicates 1 and 2. Information on recording PHab data for duplicate samples of MIs and algae is summarized in Table 2.

Table 2. Recording PHab data when duplicate samples are collected.

	PHab Data Type		
Assemblage	**Vegetation Cover**	**Depth/Dist. from Bank, Shading, etc.**	**Recording of Substrata Types**
MIs	Distinct for Reps 1 & 2	Distinct for Reps 1 & 2	N/A
Algae	Same for Reps 1 & 2	Same for Reps 1 & 2	Distinct for Reps 1 & 2

Photo documentation of site

At least one photo should be taken of each wetland sampled. If it is a small wetland, a single photo may be sufficient, as long as it captures the wetland area in its entirety. For large wetlands, several photos will be necessary. Take the photos from whatever vantage points best allow the wetland to be captured photographically. Record the photo numbers on the field sheet.

Decontamination of equipment

In depressional wetlands, the primary concern for aquatic invasive species (AIS) and disease is chytrid fungus, which has been decimating amphibian populations worldwide, including causing declines in mountain yellow-legged frogs in the Sierra Nevada. Consult the decontamination SOP from USFS Region 4, which has an excellent summary table of viable methods for multiple types of AIS
http://www.fs.fed.us/r4/resources/aquatic/guidelines/2011techguidelines_fire_AIS.pdf.
A 5% Quat 128 solution requires 30 seconds of soak time to kill chytrid fungus. However, New Zealand mud snail (NZMS), although unlikely to be found in wetlands, could nonetheless be present, and requires 10 minutes of soak time at the same concentration. Because lakes and reservoirs may have NZMS and be sampled with this protocol, a 10 min soak time for all gear in 5% Quat 128 solution or similar Quat related product should prevent movement of all potential invasive species, including aquatic diseases. If a non-chemical solution is preferred, waders can be fully cleaned of mud and debris, then exposed to sun for three hours and allowed to rest completely dry for 48 hrs. Please refer to the USFS guidance for specifics. *Note: freezing gear alone will not kill chytrid fungus, so this is not an acceptable method of decontamination for wetland sampling.*

An alternative method for decontaminating gear for chytrid fungus is via the use of a bleach solution, along with freezing to kill any NZMS that may be present. In consideration of the difficult logistics of field decontamination, a potential approach would be to have 5 pairs of waders available for each crew member, who will use a different pair of waders each day (one site/day). At the end of the week, all of the waders can be cleaned with a brush, rinsed, and

treated with the appropriate concentration of bleach for the prescribed time (http://www.fs.fed.us/r4/resources/aquatic/guidelines/2011techguidelines_fire_AIS.pdf), dried, and then frozen. After each site visit, the water grabber can be thoroughly scrubbed and then treated with bleach (as described above for waders), rinsed well, and allowed to dry to promote evaporation of any residual bleach.

Equipment List – MI sampling and PHab data collection

- 500 µm D-frame custom all mesh net with 52-inch handle (Wildco 425-JD52-SPE)
- 500 µm sieve
- 4 mm (4000 µm) sieve (optional)
- two 500 ml polyethylene squirt bottles (one for water and one for ethanol)
- flexible and hard forceps
- 1 L Nalgene bottles (assume 1-2 bottles per site)
- 5 gallon bucket with lid
- 3 dish pans (approx 3 gallon or 11 qt) for field elutriation
- plastic funnel (best if 1/2 or 1 inch diameter spout)
- 2 L of 95% (190 proof) ethyl-alcohol per site to preserve sample
- test strips to examine % alcohol in samples
- small aquarium net (approx 2x2 inches with 1 mm mesh)
- MI Rite-in-the-Rain labels and pencil
- transect tape
- meter stick (in cm)
- “angle finder”
- 10 yellow flags
- Yellow flagging tape

Equipment List – Algae sampling and PHab data collection

Chlorophyll a and cyanotoxin sampling

- either 1, 1L bottle or 1, 2L bottle for integrated sample

- water grabber
- 250 mL, 500 mL, and 1 L plastic graduated cylinders
- bottle brush
- 250 and 500 mL capacity filtering apparati
- glass fiber filters (47 mm diameter, 0.7 μm pore size)
- hand-held vacuum pump with pressure gauge marked at 7 psi or equivalent
- deionized (DI) water
- clean squirt bottle
- flat-tipped filter forceps
- 47 mm snapping Petri dishes
- aluminum foil
- 60 or 100 mL Whirl-Pak bags
- sample labels
- wet ice cooler
- umbrella
- dry ice cooler or field freezer (if going to be in field all day)

Algae specimen sampling

- sediment corer
- silver or hot-pink marker
- syringe scrubber
- spatula
- shears
- utility knife or pocket knife (for cutting sediment core and macroalgal mats)
- white scrubber pads cut into circles for syringe scrubber
- intact 60 mL syringes (to use for rinsing sample into container)
- sample labels
- 250 mL, 500 mL, and 1 L HDPE bottles (**wide mouth**)
- clear plastic tape
- scissors
- clean, soft-bristled toothbrushes
- long, blunt-ended forceps for grabbing algal clumps out of sample bottle

- 10% formalin
- 25% glutaraldehyde (for use in the laboratory; only if sampling soft-bodied algae)
- waterproof meter stick
- meter tape
- turkey baster

Algae PHab and misc field supplies

- pencil/permanent markers
- clipboard
- field sheets printed on Rite-in-the-Rain
- rangefinder
- GPS unit
- clean plastic tarp
- orange and yellow transect flags and flagging tape

Decontamination for waders and equipment

- Sparquat 256 or Quat 128[3], or bleach
- test strips to confirm appropriate Quat concentration
- scrub brushes
- large washing bin

Water chemistry data and sample collection

- 1, 2L bottle for integrated water sample
- YSI or comparable probe
- Field turbidimeter
- Field test kit for alkalinity (e.g. Hach AL-AP #2444301)
- 3 x 250 ml HDPE wide mouth jars
- 1 x 500 ml wide mouth jar
- 0.45μm filters for dissolved nutrients and 60 mL syringes

---

[3] ***DO NOT*** **use Sparquat 256 or Quat 128 on ANY gear that could potentially result in contamination of water chemistry sampling equipment (e.g., the water grabber/pole). See SOP text for alternative methods.**

- 10 orange flags
- Orange flagging tape

Sediment toxicity samples

- 2 x 1 L HDPE wide mouth jars

Sediment chemistry samples

- 2 x 4 oz glass jars
- 1 x 22 oz wire bag/Whirl-Pak

Appendix 1. Constituent containers, holding times and conditions.

	Container	Number of containers	Holding time	Holding condition	Destination Lab
Water Chemistry					
Turbidity	Field measurement	-	-	-	-
Alkalinity, hardness	250 mL HDPE	1	14 d	4 °C	Physis
TKN	500 mL HDPE	1	28 d	H_2SO_4	Physis
Nitrate, nitrite, ortho-phosphate	250 mL HDPE	1	28 d	Filtered, frozen	Physis
Total phosphorus, Total nitrogen (direct measurement)	250 mL HDPE	1	28 d	Frozen	Physis
Chlorophyll *a*	Glass fiber filter in snapping Petri dish wrapped in aluminum foil	2	28 d	-20 °C[4], dark	SCCWRP
Cyanotoxins (incl. microcystin)	Glass fiber filter in snapping Petri dish wrapped in aluminum foil	1	weeks	-20 °C[2], dark	SCCWRP
Sediment Chemistry					
TOC	4 oz glass	1	6 mo	frozen	Physis
Grain size	Whirlpak is fine; 10-15 g sediment	1	6 mo	4 °C	Physis

[4] -80 °C is preferable, if possible

Metals (Al, Sb, As, Ba, Be, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mo, Ni, Se, Ag, Sr, Tl, Sn, Ti, V, Zn), pyrethroids (bifenthrin, cyfluthrin, cypermethrin, danitol, deltamethrin, esfenvalerate, fenvalerate, fluvalinate, L-cyhalothrin, permethrin, prallethrin, allethrin)	4 oz glass	1	1 year	frozen	SCCWRP
Sediment Toxicity	1 L HDPE	2	2 weeks	4 °C	SCCWRP
Algae					
Diatoms	250 or 500 mL HDPE bottle	1	Months (once fixed)	Formalin	EcoAnalysts
Soft algae	250 or 500 mL HDPE bottle	1	Months (once fixed; store at 4 °C)	Glutaraldehyde	EcoAnalysts
Macroinvertebrate	Wide mouth HDPE recommended	Dependent on site conditions	Months	EtOH	ABL

APPENDIX C

EPA Method 440.0

Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis

Method 440.0

Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis

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Method 440.0

Determination of Carbon and Nitrogen in Sediments and Particulates of Estuarine/Coastal Waters Using Elemental Analysis

1.0 Scope and Application

1.1 Elemental analysis is used to determine particulate carbon (PC) and particulate nitrogen (PN) in estuarine and coastal waters and sediment. The method measures the total carbon and nitrogen irrespective of source (inorganic or organic).

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Carbon	7440-44-0
Nitrogen	1333-74-0

1.2 The need to qualitatively or quantitatively determine the particulate organic fraction from the total particulate carbon and nitrogen depends on the data-quality objectives of the study. Section 11.4 outlines procedures to ascertain the organic/inorganic particulate ratio. The method performance presented in the method was obtained on particulate samples with greater than 80% organic content. Performance on samples with a greater proportion of particulate inorganic versus organic carbon and nitrogen has not been investigated.

1.3 Method detection limits (MDLs)[1] of 10.5 µg/L and 62.3 µg/L for PN and PC, respectively, were obtained for a 200-mL sample volume. Sediment MDLs of PN and PC are 84 mg/kg and 1300 mg/kg, respectively, for a sediment sample weight of 10.00 mg. The method has been determined to be linear to 4800 µg of C and 700 µg of N in a sample. Multilaboratory study validation data are in Section 13.

1.4 This method should be used by analysts experienced in the theory and application of elemental analysis. A minimum of 6 months experience with an elemental analyzer is recommended.

1.5 Users of the method data should set the data-quality objectives prior to analysis. Users of the method must document and have on file the required initial demonstration of performance data described in Section 9.2 prior to using the method for analysis.

2.0 Summary of Method

2.1 An accurately measured amount of particulate matter from an estuarine water sample or an accurately weighed dried sediment sample is combusted at 975°C using an elemental analyzer. The combustion products are passed over a copper reduction tube to convert the oxides of N into molecular N. Carbon dioxide, water vapor and N are homogeneously mixed at a known volume, temperature and pressure. The mixture is released to a series of thermal conductivity detectors/traps, measuring in turn by difference, hydrogen (as water vapor), C (as carbon dioxide) and N (as N_2). Inorganic and organic C may be determined by two methods which are also presented.

3.0 Definitions

3.1 ***Sediment Sample --*** A fluvial, sand, or humic sample matrix exposed to a marine, brackish or fresh water environment. It is limited to that portion which may be passed through a number 10 sieve or a 2-mm mesh sieve.

3.2 ***Material Safety Data Sheet (MSDS) --*** Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.3 ***Instrument Detection Limit (IDL) --*** The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc.

3.4 ***Method Detection Limit (MDL) --*** The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

3.5 ***Linear Dynamic Range (LDR) --*** The absolute quantity over which the instrument response to an analyte is linear.

3.6 ***Calibration Standard (CAL) --*** An accurately weighed amount of a certified chemical used to calibrate the instrument response with respect to analyte mass.

3.7 ***Conditioner --*** A standard chemical which is not necessarily accurately weighed that is used to coat the surfaces of the instrument with the analytes (water vapor, carbon dioxide, and nitrogen).

3.8 ***External Standards (ES) --*** A pure analyte(s) that is measured in an experiment separate from the experiment used to measure the analyte(s) in the sample. The signal observed for a known quantity of the pure external standard(s) is used to calibrate the instrument

response for the corresponding analyte(s). The instrument response is used to calculate the concentrations of the analyte(s) in the sample.

3.9 Response Factor (RF) -- The ratio of the response of the instrument to a known amount of analyte.

3.10 Laboratory Reagent Blank (LRB) -- A blank matrix (i.e., a precombusted filter or sediment capsule) that is treated exactly as a sample including exposure to all glassware, equipment, solvents, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.11 Field Reagent Blank (FRB) -- An aliquot of reagent water or other blank matrix that is placed in a sample container in the laboratory and treated as a sample in all respects, including shipment to the sampling site, exposure to sampling site conditions, storage, preservation, and all analytical procedures. The purpose of the FRB is to determine if method analytes or other interferences are present in the field environment.

3.12 Laboratory Duplicates (LD1 and LD2) -- Two aliquots of the same sample taken in the laboratory and analyzed separately with identical procedures. Analyses of LD1 and LD2 indicate precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.13 Field Duplicates (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.14 Laboratory Fortified Blank (LFB) -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the method is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.15 Laboratory Fortified Sample Matrix (LFM) -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.16 Standard Reference Material (SRM) -- Material which has been certified for specific analytes by a variety of analytical techniques and/or by numerous laboratories using similar analytical techniques. These may consist of pure chemicals, buffers or compositional standards. These materials are used as an indication of the accuracy of a specific analytical technique.

3.17 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

4.0 Interferences

4.1 There are no known interferences for estuarine/coastal water or sediment samples. The presence of C and N compounds on laboratory surfaces, on fingers, in detergents and in dust necessitates the utilization of careful techniques (i.e., the use of forceps and gloves) to avoid contamination in every portion of this procedure.

5.0 Safety

5.1 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 to these compounds should be as low as reasonably achievable. Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method.[2-5] A reference file of material safety data sheets (MSDS) should also be made available to all personnel involved in the chemical analysis.

5.2 The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be done in a fume hood.

5.3 All personnel handling environmental samples known to contain or to have been in contact with human waste should be immunized against known disease causative agents.

5.4 Although most instruments are adequately shielded, it should be remembered that the oven temperatures are extremely high and that care should be taken when working near the instrument to prevent possible burns.

5.5 It is the responsibility of the user of this method to comply with relevant disposal and waste regulations. For guidance see Sections 14.0 and 15.0.

6.0 Apparatus and Equipment

6.1 Elemental Analyzer

6.1.1 An elemental analyzer capable of maintaining a combustion temperature of 975°C and analyzing particulate samples and sediment samples for elemental C and N. The Leeman Labs Model 240 XA Elemental Analyzer was used to produce the data presented in this method.

6.2 A gravity convection drying oven. Capable of maintaining 103-105°C for extended periods of time.

6.3 Muffle furnace. Capable of maintaining 875°C ± 15°C.

6.4 Ultra-micro balance. Capable of accurately weighing to 0.1 µg. Desiccant should be kept in the weighing chamber to prevent hygroscopic effects.

6.5 Vacuum pump or source capable of maintaining up to 10 in. Hg of vacuum.

6.6 Mortar and pestle.

6.7 Desiccator, glass.

6.8 Freezer, capable of maintaining -20°C ± 5°C.

6.9 47-mm or 25-mm vacuum filter apparatus made up of a glass filter tower, fritted glass disk base and 2-L vacuum flask.

6.10 13-mm Swinlok filter holder.

6.11 Teflon-tipped, flat blade forceps.

6.12 Labware -- All reusable labware (glass, quartz, polyethylene, PTFE, FEP, etc.) should be sufficiently clean for the task objectives. Several procedures found to provide clean labware include washing with a detergent solution, rinsing with tap water, soaking for 4 hr or more in 20% (v/v) HCl, rinsing with reagent water and storing clean. All traces of organic material must be removed to prevent C-N contamination.

6.12.1 Glassware -- Volumetric flasks, graduated cylinders, vials and beakers.

6.12.2 Vacuum filter flasks -- 250 mL and 2 L, glass.

6.12.3 Funnel, 6.4 mm i.d., polyethylene.

6.12.4 Syringes, 60-mL, glass.

7.0 Reagents and Standards

7.1 Reagents may contain elemental impurities which affect analytical data. High-purity reagents that conform to the American Chemical Society specifications[6] should be used whenever possible. If the purity of a reagent is in question, analyze for contamination. The acid used for this method must be of reagent grade purity or equivalent. A suitable acid is available from a number of manufacturers.

7.2 Hydrochloric acid, concentrated (sp. gr. 1.19)-HCl.

7.3 Acetanilide, 99.9% + purity, $C_8H_{19}NO$ (CASRN 103-84-4).

7.4 Blanks -- Three blanks are used for the analysis. Two blanks are instrument related. The instrument zero response (ZN) is the background response of the instrument without sample holding devices such as capsules and sleeves. The instrument blank response (BN) is the response of the instrument when the sample capsule, sleeve and ladle are inserted for analysis without standard or sample. The BN is also the laboratory reagent blank (LRB) for sediment samples. The LRB for water samples includes the capsule, sleeve, ladle and a precombusted filter without standard or sample. These blanks are subtracted from the uncorrected instrument response used to calculate concentration in Sections 12.3 and 12.4.

7.4.1 Laboratory fortified blank (LFB) -- The third blank is the laboratory fortified blank. For sediment analysis, add a weighed amount of acetanilide in an aluminum capsule and analyze for PC and PN (Section 9.3.2). For aqueous samples, place a weighed amount of acetanilide on a glass fiber filter the same size as used for the sample filtration. Analyze the fortified filter for PC and PN (Section 9.3.2)

7.5 Quality Control Sample (QCS) -- For this method, the QCS can be any assayed and certified sediment or particulate sample which is obtained from an external source. The Canadian Reference Material, BCSS-1, is just such a material and was used in this capacity for the data presented in this method. The percent PC has been certified in this material but percent PN has not.

8.0 Sample Collection, Preservation and Storage

8.1 Water Sample Collection -- Samples collected for PC and PN analyses from estuarine/coastal waters are normally collected from a ship using one of two methods; hydrocast or submersible pump systems. Follow the recommended sampling protocols associated with the method used. Whenever possible, immediately filter the samples as described in Section 11.1.1. Store the filtered sample pads by freezing at -20°C or storing in a desiccator after drying at 103-105° C for 24 hr. No significant difference has been noted in comparing the two storage procedures for a time period of up to 100 days. If storage of the water sample is necessary, place

the sample into a clean amber bottle and store at 4°C until filtration is done.

8.1.1 The volume of water sample collected will vary with the type of sample being analyzed. Table 1 provides a guide for a number of matrices of interest. If the matrix cannot be classified by this guide, collect 2 x 1L of water from each site. A minimum filtration volume of 200 mL is recommended.

8.2 Sediment Sample Collection -- Estuarine/coastal sediment samples are collected with benthic samplers. The type of sampler used will depend on the type of sample needed by the data-quality objectives.[7] Store the wet sediment in a clean jar and freeze at -20°C until ready for analysis.

8.2.1 The amount of sediment collected will depend on the sample matrix and the elemental analyzer used. A minimum of 10 g is recommended.

9.0 Quality Control

9.1 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 continued analysis of laboratory reagent blanks, laboratory duplicates, field duplicates and calibration standards analyzed as samples as a continuing check on performance. The laboratory is required to maintain performance records that define the quality of data thus generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (MDLs, linear dynamic range) and laboratory performance (analysis of QC samples) prior to the analyses conducted by this method.

9.2.2 Linear dynamic range (LDR) -- The upper limit of the LDR must be established by determining the signal responses from a minimum of three different concentration standards across the range, one of which is close to the upper limit of the LDR. Determined LDRs must be documented and kept on file. The LDR which may be used for the analysis of samples should be judged by the analyst from the resulting data. The upper LDR limit should be an observed signal no more than 10% below the level extrapolated from the lower standards. Determined sample analyte concentrations that are 90% and above the upper LDR must be reduced in mass and reanalyzed. New LDRs should be determined whenever there is a significant change in instrument response and for those analytes that periodically approach the upper LDR limit, every 6 months or whenever there is a change in instrument analytical hardware or operating conditions.

9.2.3 Quality control sample (QCS) (Section 7.5) -- 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 analyses of a QCS. If the determined concentrations are not within ± 5% 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 analyses.

9.2.4 Method detection limits (MDLs) -- MDLs should be established for PC and PN using a low level estuarine water sample, typically three to five times higher than the estimated MDL. The same procedure should be followed for sediments. To determine MDL values, analyze seven replicate aliquots of water or sediment and process through the entire analytical procedure (Section 11). These replicates should be randomly distributed throughout a group of typical analyses. Perform all calculations defined in the method (Section 12) and report the concentration values in the appropriate units. Calculate the MDL as follows:[1]

$$MDL = (t) \times (S)$$

where, S = Standard deviation of the replicate analyses.

t = Student's t value for n-1 degrees of freedom at the 99% confidence limit; t = 3.143 for six degrees of freedom.

MDLs should be determined whenever a significant change in instrumental response, change of operator, or a new matrix is encountered.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 Laboratory reagent blank (LRB) -- The laboratory must analyze at least one LRB (Section 3.10) with each batch of 20 or fewer samples of the same matrix. LRB data are used to assess contamination from the laboratory environment. LRB values that exceed the MDL indicate laboratory or reagent contamination. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates of the samples must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained. For aqueous samples the LRB is a precombusted filter of the same type and size used for samples.

9.3.2 Laboratory fortified blank (LFB) -- The laboratory must analyze at least one LFB (Section 7.4.1) with each batch of samples. Calculate accuracy as percent recovery. If the recovery of any analyte falls outside the required control limits of 85-115%, that analyte is judged out of control, and the source of the problem should be identified and resolved before continuing analyses.

9.3.3 The laboratory must use LFB analyses data to assess laboratory performance against the required control limits of 85-115% (Section 9.3.2). 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:

$$\text{Upper Control Limit} = x + 3S$$

$$\text{Lower Control Limit} = x - 3S$$

The optional control limits must be equal to or better than the required control limits of 85-115%. After each five to ten 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 ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery and Data Quality

9.4.1 Percent recoveries cannot be readily obtained from particulate samples. Consequently, accuracy can only be assessed by analyzing check standards as samples and quality control samples (QCS). The use of laboratory fortified matrix samples has not been assessed.

10.0 Calibration and Standardization

10.1 Calibration -- After following manufacturer's installation and temperature stabilization procedures, daily calibration procedures must be performed and evaluated before sample analysis may begin. Single point or standard curve calibrations are possible, depending on instrumentation.

10.1.1 Establish single response factors (RF) for each element (C,H, and N) by analyzing three weighed portions of calibration standard (acetanilide). The mass of calibration standard should provide a response within 20% of the response expected for the samples being analyzed. Calculate the (RF) for each element using the following formula:

$$\text{Response factor } (\mu v/\mu g) = \frac{RN - ZN - BN}{WTN}$$

where,
RN = Average instrument response to standard (µv)
ZN = Instrument zero response (µv)
BN = Instrument blank response (µv)

and, $WTN = (M)(N_a)(AW/MW)$

where,
M = The mass of standard material in µg
N_a = Number of atoms of C, N or H, in a molecule of standard material
AW = Atomic weight of C (12.01), N (14.01) or H (1.01)
MW = Molecular weight of standard material (135.2 for acetanilide)

If instrument response is in units other than µv, then change the formula accordingly.

10.1.2 For standard curve preparation, the range of calibration standard masses used should be such that the low concentration approaches but is above the MDL and the high concentration is above the level of the highest sample, but no more than 90% of the linear dynamic range. A minimum of three concentrations should be used in constructing the curve. Measure response versus mass of element in the standard and perform a regression on the data to obtain the calibration curve.

11.0 Procedure

11.1 Aqueous Sample Preparation

11.1.1 Water Sample Filtration -- Precombust GF/F glass fiber filters at 500°C for 1.5 hr. The diameter of filter used will depend on the sample composition and instrument capabilities (Section 8.1.1). Store filters covered if not immediately used. Place a precombusted filter on fritted filter base of the filtration apparatus and attach the filtration tower. Thoroughly shake the sample container to suspend the particulate matter. Measure and record the required sample volume using a graduated cylinder. Pour the measured sample into the filtration tower, no more than 50 mL at a time. Filter the sample using a vacuum no greater than 10 in. of Hg. Vacuum levels greater than 10 in. of Hg can cause filter rupture. If less than the measured volume of sample can be practically filtered due to clogging, measure and record the actual volume filtered. **Do not** rinse the filter following filtration. It has been demonstrated that sample loss occurs when the filter is rinsed with an isotonic solution or the filtrate.[8] Air dry the filter after the sample has passed through by continuing the vacuum for 30 sec. Using Teflon-coated flat-tipped forceps, fold the filters in half while still on the fritted glass base of the filter apparatus. Store filters as described in Section 8.

11.1.2 If the sample has been stored frozen, place the sample in a drying oven at 103-105° C for 24 hr before analysis and dry to a constant weight. Precombust one nickel sleeve at 875° C for 1 hr for each sample.

11.1.3 Remove the filter pads containing the particulate material from the drying oven and insert into a pre-combusted nickel sleeve using Teflon-coated flat-tipped forceps. Tap the filter pad using a stainless steel rod. The sample is ready for analysis.

11.2 Sediment Samples Preparation

11.2.1 Thaw the frozen sediment sample in a 102-105°C drying oven for at least 24 hr before analysis and dry to a constant weight. After drying, homogenize the dry sediment with a mortar and pestle. Store in a desiccator until analysis. Precombust aluminum capsules at 550°C in a muffle furnace for 1.5 hr for each sediment sample being analyzed. Precombust one nickel sleeve at 875°C for 1 hr for each sediment sample.

11.2.2 Weigh 10 mg of the homogenized sediment to the nearest 0.001 mg with an ultra-micro balance into a precombusted aluminum capsule. Crimp the top of the aluminum capsule with the Teflon-coated flat-tipped forceps and place into a precombusted nickel sleeve. The sample is ready for analysis.

11.3 Sample Analysis

11.3.1 Measure instrument zero response (Section 7.4) and instrument blank response (Section 7.4) and record values. Condition the instrument by analyzing a conditioner. Calibrate the instrument according to Section 10 and analyze all preliminary QC samples as required by Section 9. When satisfactory control has been established, analyze samples according to the instrument manufacturer's recommendations. Record all response data.

11.3.2 Report data as directed in Section 12.

11.4 Determination of Particulate Organic and Inorganic Carbon

11.4.1 Method 1: Thermal Partitioning -- The difference found between replicate samples, one of which has been analyzed for total PC and PN and the other which was muffled at 550°C and analyzed is the particulate organic component of that sample. This method of thermally partitioning organic and inorganic PC may underestimate slightly the carbonate minerals' contribution in the inorganic fraction since some carbonate minerals decompose below 500°C, although $CaCO_3$ does not.[9]

11.4.2 Method 2: Fuming HCl -- Allow samples to dry overnight at 103-105°C and then place in a desiccator containing concentrated HCl, cover and fume for 24 hr in a hood. The fuming HCl converts inorganic carbonate in the samples to water vapor, CO_2 and calcium chloride. Analyze the samples for particulate C. The resultant data are particulate organic carbon.[10]

12.0 Data Analysis and Calculations

12.1 Sample data should be reported in units of µg/L for aqueous samples and mg/kg dry weight for sediment samples.

12.2 Report analyte concentrations up to three significant figures for both aqueous and sediment samples.

12.3 For aqueous samples, calculate the sample concentration using the following formula:

$$\text{Concentration } (\mu g/L) = \frac{\text{Corrected sample response } (\mu v)}{\text{Sample volume (L)} \times \text{RF } (\mu v/\mu g)}$$

where, RF = Response Factor (Section 10.1.1)
Corrected Sample Response (Section 7.4)

12.4 For sediment samples, calculate the sample concentration using the following formula:

$$\text{Concentration } (mg/kg) = \frac{\text{Corrected sample response } (\mu v)}{\text{Sample weight (g)} \times \text{RF } (\mu v/\mu g)}$$

where, RF = Response Factor (Section 10.1.1)
Corrected Sample Response (Section 7.4)

Note: Units of µg/g = mg/kg

12.5 The QC data obtained during the analyses provide an indication of the quality of the sample data and should be provided with the sample results.

13.0 Method Performance

13.1 **Single Laboratory Performance**

13.1.1 Single laboratory performance data for aqueous samples from the Chesapeake Bay are provided in Table 2.

13.1.2 Single-laboratory precision and accuracy data for the marine sediment reference material, BCSS-1, are listed in Table 3.

13.2 **Multilaboratory Performance**

13.2.1 In a multilab study, 13 participants analyzed sediment and filtered estuarine water samples for particulate carbon and nitrogen. The data were analyzed

using the statistical procedures recommended in ASTM D2777-86 for replicate designs. See Table 4 for summary statistics.

13.2.2 Accuracy as mean recovery was estimated from the analyses of the NRC of Canada Marine Sediment Reference Material, BCSS-1. Mean recovery was 98.2% of the certified reference carbon value and 100% of the noncertified nitrogen value.

13.2.3 Overall precision for analyses of carbon and nitrogen in sediments was 1-11% RSD, while the analyses of both particulate carbon and nitrogen in estuarine water samples was 9-14% RSD.

13.2.4 Single analyst precision for carbon and nitrogen in sediment samples was 1-8% RSD and 4-9% for water samples.

13.2.5 Pooled method detection limits (p-MDLs) were calculated using the pooled single analyst standard deviations. The p-MDLs for particulate nitrogen and carbon in estuarine waters were 0.014 mg N/L and 0.064 mg C/L , respectively. The p-MDLs for percent carbon and nitrogen in estuarine sediments were not estimated because the lowest concentration sediment used in the study was still 20 times higher than the estimated MDLs. Estimates of p-MDLs from these data would be unrealistically high.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction,* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202) 872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Filter Diameter Selection Guide

Sample matrix	47mm	Filter diameter 25mm	13mm
	Sample matrix volume		
Open ocean	2000 mL	500 mL	100 mL
Coastal	1000 mL	400-500 mL	100 mL
Estuarine (low particulate)	500-700 mL	250-400 mL	50 mL
Estuarine (high particulate)	100-400 mL	75-200 mL	25 mL

Table 2. Performance Data--Chesapeake Bay Aqueous Samples

Sample	Measured nitrogen concentration (µg/L)	S.D.[A] (µg/L)	Measured carbon concentration (µg/L)	S.D.[A] (µg/L)
1	147	± 4	1210	± 49
2	148	± 11	1240	± 179
3	379	± 51	3950	± 269
4	122	± 9	1010	± 63

[A] Standard deviation based on 7 replicates.

Table 3. Precision and Accuracy Data - Canadian Sediment Reference Material BCSS-1

Element	T.V.[A]	Mean measured value (%)	%RSD[B]	%Recovery[C]
Carbon	2.19%	2.18	± 3.3	99.5
Nitrogen	0.195%	0.194	± 3.9	99.5

[A] True value. Carbon value is certified; nitrogen value is listed but not certified

[B] Percent relative standard deviation based on 10 replicates.

[C] As calculated from T.V.

Table 4. Overall and Single Analyst Precision Estimates from Collaborative Study

Analyte	Sample	N[1]	Mean[2] Conc.	Overall Std. Dev.	Overall %RSD	Analyst Std. Dev.	Analyst %RSD
Particulate Nitrogen (as N) in Estuarine Waters	A	11	0.0655	0.0081	12.4%	0.0050	7.6%
	B	12	0.0730	0.0076	10.3%	0.0057	7.7%
	C	12	0.0849	0.0110	12.9%	0.0060	7.1%
	D	12	0.126	0.0138	11.0%	0.0071	5.6%
	E	11	0.182	0.0245	13.5%	0.0157	8.6%
Nitrogen (as %N) in Estuarine Water	1	10	0.178	0.0190	10.7%	0.0131	7.3%
	2	10	0.295	0.0114	3.9%	0.0046	1.6%
	3	10	0.436	0.0178	4.1%	0.0104	2.4%
	4	10	0.497	0.0183	3.7%	0.0082	1.6%
	5	10	0.580	0.0207	3.6%	0.0150	2.6%
Particulate Carbon (as C) in Estuarine Waters	B	12	0.369	0.0505	13.7%	0.0222	6.0%
	A	12	0.417	0.0490	11.8%	0.0230	5.5%
	D	12	0.619	0..0707	11.4%	0.0226	3.6%
	C	12	0.710	0.0633	8.9%	0.0367	5.2%
	E	12	0.896	0.1192	13.3%	0.0569	6.4%
Carbon (as %C) in Estuarine Sediments	1	13	1.78	0.1517	8.5%	0.1346	7.6%
	2	13	2.55	0.0372	1.5%	0.0204	0.8%
	3	13	3.18	0.0435	1.4%	0.0348	1.1%
	4	13	4.92	0.1201	2.4%	0.0779	1.6%
	5	13	5.92	0.0621	1.1%	0.0547	0.9%

(1) N = Number of participants whose data was used.

(2) Concentration in mg/L or percent, as indicated.

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

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and

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Revision 1.2
September 1997

National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

APPENDIX D

EPA Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

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Method 365.5

Determination of Orthophosphate in Estuarine and Coastal Waters by Automated Colorimetric Analysis

1.0 Scope and Application

1.1 This method provides a procedure for the determination of low-level orthophosphate concentrations normally found in estuarine and/or coastal waters. It is based upon the method of Murphy and Riley[1] adapted for automated segmented flow analysis[2] in which the two reagent solutions are added separately for greater reagent stability and facility of sample separation.

Analyte	Chemical Abstracts Service Registry Numbers (CASRN)
Phosphate	14265-44-2

1.2 A statistically determined method detection limit (MDL) of 0.0007 mg P/L has been determined by one laboratory in 3 parts per thousand (ppt) saline water.[3] The method is linear to 0.39 mg P/L using a Technicon AutoAnalyzer II system (Bran & Luebbe, Buffalo Grove, IL).

1.3 Approximately 40 samples per hour can be analyzed.

1.4 This method should be used by analysts experienced in the use of automated colorimetric analyses, and familiar with matrix interferences and procedures for their correction. A minimum of 6-months experience under experienced supervision is recommended.

2.0 Summary of Method

2.1 An automated colorimetric method for the analysis of low-level orthophosphate concentrations is described. 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.

3.0 Definitions

3.1 ***Calibration Standard (CAL) --*** A solution prepared from the stock standard solution that is used to calibrate the instrument response with respect to analyte concentration. One of the standards in the standard curve.

3.2 ***Dissolved Analyte (DA) --*** The concentration of analyte in an aqueous sample that will pass through a 0.45-μm membrane filter assembly prior to sample acidification or other processing.

3.3 ***Laboratory Fortified Blank (LFB) --*** An aliquot of reagent water to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether method performance is within acceptable control limits. This is basically a standard prepared in reagent water that is analyzed as a sample.

3.4 ***Laboratory Fortified Sample Matrix (LFM) --*** An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.5 ***Laboratory Reagent Blank (LRB) --*** An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, and reagents that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or apparatus.

3.6 ***Linear Dynamic Range (LDR) --*** The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.7 ***Method Detection Limit (MDL) --*** The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

3.8 ***Reagent Water (RW) --*** Type 1 reagent grade water equal to or exceeding standards established by American Society of Testing Materials (ASTM). Reverse osmosis systems or distilling units that produce 18 megohm water are two examples of acceptable water sources.

3.9 Refractive Index (RI) -- The ratio of the velocity of light in a vacuum to that in a given medium. The relative refractive index is the ratio of the velocity of light in two different media, such as sea or estuarine water versus reagent water. The correction for this difference is referred to as the refractive index correction in this method.

3.10 Stock Standard Solution (SSS) -- A concentrated solution of method analyte prepared in the laboratory using assayed reference compounds or purchased from a reputable commercial source.

4.0 Interferences

4.1 Interferences caused by copper, arsenate and silicate are minimal relative to the orthophosphate determination because of the extremely low concentrations normally found in estuarine or 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.[4]

4.2 Sample turbidity is removed by filtration prior to analysis.

4.3 Refractive Index interferences are corrected for estuarine/coastal samples (Section 12.2).

5.0 Safety

5.1 Water samples collected from the estuarine and/or ocean environment are generally not hazardous. However, the individual who collects samples should use proper technique.

5.2 Good laboratory technique should be used when preparing reagents. A lab coat, safety goggles, and gloves should be worn when preparing the sulfuric acid reagent.

6.0 Equipment and Supplies

6.1 Continuous Flow Automated Analytical System Consisting of:

6.1.1 Sampler.

6.1.2 Manifold or Analytical Cartridge equipped with 37°C heating bath.

6.1.3 Proportioning pump.

6.1.4 Colorimeter equipped with 1.5 X 50 mm tubular flow cell and a 880 nm filter.

6.1.5 Phototube that can be used for 600-900 nm range.

6.1.6 Strip chart recorder or computer based data system.

6.2 Phosphate-Free Glassware and Polyethylene Bottles

6.2.1 All labware used in the determination must be low in residual phosphate to avoid sample or reagent contamination. Washing with 10% HCl (v/v) and thoroughly rinsing with distilled, deionized water was found to be effective.

6.2.2 Membrane or glass fiber filters, 0.45 μm nominal pore size.

7.0 Reagents and Standards

7.1 Stock Reagent Solutions

7.1.1 Ammonium Molybdate Solution (40 g/L) -- Dissolve 20.0 g of ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}$•$4H_2O$, CASRN 12027-67-7) 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.

7.1.2 Antimony Potassium Tartrate Solution (3.0 g/L) -- Dissolve 0.3 g of antimony potassium tartrate [$(K(SbO)C_4H_4O_6$•$1/2H_2O$, CASRN 11071-15-1] in approximately 90 mL of reagent water and dilute to 100 mL. This reagent is stable for approximately three months.

7.1.3 Ascorbic Acid Solution (18.0 g/L) -- Dissolve 18.0 g of ascorbic acid ($C_6H_6O_6$, CASRN 50-81-7) in approximately 800 mL of reagent water and dilute to 1 L. Dispense approximately 75 mL into clean polyethylene bottles and freeze. The stability of the frozen ascorbic acid is approximately three months. Thaw overnight in the refrigerator before use. The stability of the thawed, refrigerated reagent is less than 10 days.

7.1.4 Sodium Lauryl Sulfate Solution (30.0 g/L) -- Sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$, CASRN 151-21-3). Dissolve 3.0 g of sodium lauryl sulfate (SLS) 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.

7.1.5 Sulfuric Acid Solution (4.9 N) -- Slowly add 136 mL of concentrated sulfuric acid (H_2SO_4, CASRN 7664-93-9) to approximately 800 mL of reagent water. After the solution is cooled, dilute to 1 L with reagent water.

7.1.6 Stock Phosphorus Solution -- Dissolve 0.439 g of pre-dried (105°C for 1 hr) monobasic potassium phosphate (KH_2PO_4, CASRN 7778-77-0) in reagent water and

dilute to 1000 mL. (1.0 mL = 0.100 mg P.) The stability of this stock standard is approximately three months when kept refrigerated.

7.1.7 Low Nutrient Seawater -- Obtain natural low nutrient seawater (36 ppt salinity; <0.0003 mg P/L) or dissolve 31 g analytical reagent grade sodium chloride, (NaCl, CASRN 7647-14-5); 10 g analytical grade magnesium sulfate, ($MgSO_4$) CASRN 10034-99-8); and 0.05 g analytical reagent grade sodium bicarbonate, ($NaHCO_3$, CASRN 144-55-8), in 1 L of reagent water.

7.2 Working Reagents

7.2.1 Reagent A -- Mix the following reagents in the following proportions for 142 mL of Reagent A: 100 mL of 4.9 N H_2SO_4 (Section 7.1.5), 30 mL of ammonium molybdate solution (Section 7.1.1), 10 mL of antimony potassium tartrate solution (Section 7.1.2), and 2.0 mL of SLS solution (Section 7.1.4). Prepare fresh daily.

7.2.2 Reagent B -- Add approximately 0.5 mL of the SLS solution (Section 7.1.4) to the 75 mL of ascorbic acid solution (Section 7.1.3). Stability is approximately 10 days when kept refrigerated.

7.2.3 Refractive Reagent A -- Add 50 mL of 4.9 N H_2SO_4 (Section 7.1.5) to 20 mL of reagent water. Add 1 mL of SLS (Section 7.1.4) to this solution. Prepare fresh every few days.

7.2.4 Secondary Phosphorus Solution -- Take 1.0 mL of Stock Phosphorus Solution (Section 7.1.6) and dilute to 100 mL with reagent water. (1.0 mL = 0.0010 mg P.) Refrigerate and prepare fresh every 10 days.

7.2.5 Prepare a series of standards by diluting suitable volumes of standard solutions (Section 7.2.4) to 100 mL with reagent water. Prepare these standards daily. When working with samples of known salinity, it is recommended that the standard curve concentrations be prepared in low-level natural seawater (Section 7.1.7) diluted to match the salinity of the samples. Doing so obviates the need to perform the refractive index correction outlined in Section 12.2. 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 (Section 12.2). The following dilutions are suggested.

mL of Secondary Phosphorus Solution (7.2.4)	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

8.0 Sample Collection, Preservation and Storage

8.1 Sample Collection -- Samples collected for nutrient analyses from estuarine and coastal waters are normally collected using one of two methods: hydrocast or submersible pump systems. Filtration of the sample through a 0.45-μm membrane or glass fiber filter immediately after collection is required.

8.1.1 A hydrocast uses a series of sampling bottles (Niskin, Nansen, Go-Flo or equivalent) that are attached at fixed intervals to a hydro wire. These bottles are sent through the water column open and are closed either electronically or via a mechanical "messenger" when the bottles have reached the desired depth.

8.1.2 When a submersible pump system is used, a weighted hose is sent to the desired depth in the water column and water is pumped from that depth to the deck of the ship for processing.

8.1.3 Another method used to collect surface samples involves the use of a plastic bucket or large plastic bottle. While not the most ideal method, it is commonly used in citizen monitoring programs.

8.2 Sample Preservation -- After collection and filtration, samples should be analyzed as quickly as possible. If the samples are to be analyzed within 24 hr of collection, then refrigeration at 4°C is acceptable.

8.3 Sample Storage -- Long-term storage of frozen samples should be in clearly labeled polyethylene bottles or polystyrene cups compatible with the analytical system's automatic sampler (Section 6.1.1). If samples cannot be analyzed within 24 hr, then freezing at -20°C for a maximum period of two months is acceptable.[5-8]

9.0 Quality Control

9.1 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, the continued analysis of LRBs, laboratory duplicates, and LFBs as a continuing check on performance.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (MDLs and linear dynamic range) and laboratory performance (analysis of QC samples) prior to analyses of samples using this method.

9.2.2 MDLs should be established using a low-level estuarine water sample fortified to approximately five

times the estimated detection limit.[3] To determine MDL values, analyze seven replicate aliquots of 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:

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

where, S = the standard deviation of the replicate analyses.

t = the Student's t value for n-1 degrees of freedom at the 99% confidence limit. t = 3.143 for six degrees of freedom.

MDLs should be determined every six months or whenever a significant change in background or instrument response occurs or when a new matrix is encountered.

9.2.3 *Linear Dynamic Range (LDR)* -- The LDR should be determined by analyzing a minimum of five calibration standards ranging in concentration from 0.001 mg P/L to 0.20, mg P/L across all sensitivity settings of the autoanalyzer. Normalize responses by dividing the response by the sensitivity setting multiplier. 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 ± 10% of the known concentration, C, where $C_C = (R - b)/m$. That concentration defines the upper limit of the LDR for your instrument. Should samples be encountered that have a concentration that is $\geq$90% of the upper limit of the LDR, then these samples must be diluted and reanalyzed.

9.3 Assessing Laboratory Performance (Mandatory)

9.3.1 *Laboratory Reagent Blank (LRB)* -- A laboratory should analyze at least one LRB (Section 3.5) with each set of samples. LRB data are used to assess contamination from the laboratory environment. Should an analyte value in the LRB exceed the MDL, then laboratory or reagent contamination should be suspected. When LRB values constitute 10% or more of the analyte level determined for a sample, fresh samples or field duplicates of the samples must be prepared and analyzed again after the source of contamination has been corrected and acceptable LRB values have been obtained.

9.3.2 *Laboratory Fortified Blank (LFB)* -- A laboratory should analyze at least one LFB (Section 3.3) with each batch of samples. Calculate accuracy as percent recovery. If the recovery of the analyte falls outside the required control limits of 90-110%, the analyte is judged out of control and the source of the problem should be identified and resolved before continuing the analyses.

9.3.3 The laboratory must use LFB data to assess laboratory performance against the required control limits of 90-110% (Section 9.3.2). 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 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 to 30 data points. Also, the standard deviation (S) data should be used to establish an ongoing precision statement for the level of concentrations included in the LFB. These data must be kept on file and be available for review.

9.4 Assessing Analyte Recovery - Laboratory Fortified Sample Matrix

9.4.1 A laboratory should add a known amount of analyte to a minimum of 5% of the routine samples or one sample per sample set, whichever is greater. The analyte concentration should be two to four times the ambient concentration and should be at least four times the MDL.

9.4.2 Calculate the percent recovery of the analyte, corrected for background concentrations measured in the unfortified sample, and compare these values with the values obtained from the LFBs.

Percent recoveries may be calculated using the following equation:

$$R = \frac{(C_s - C)}{S} x\ 100$$

where,
R = percent recovery
C_S = measured fortified sample concentration (background + concentrated addition in mg P/L)
C = sample background concentration (mg P/L)
S = concentration in mg P/L added to the environmental sample.

9.4.3 If the recovery of the analyte falls outside the designated range of 90-110% recovery, but the laboratory performance for that analyte is in control, 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 matrix related, not system related.

10.0 Calibration and Standardization

10.1 Calibration (Refer to Sections 11.5 and 12.0).

10.2 Standardization (Refer to Section 12.2).

11.0 Procedure

11.1 If samples are frozen, thaw the samples to room temperature.

11.2 Set up manifold as shown in Figure 1. 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.

11.3 Allow both colorimeter and recorder to warm up for 30 min. 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 that 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 (Section 12.2) be employed.

11.4 A good sampling rate is approximately 40 samples/hr with a 9:1, sample:wash ratio.

11.5 Place standards (Section 7.2.5) in sampler in order of decreasing concentration. Complete filling the sampler tray with samples, LRBs, LFBs, and LFMs.

11.6 Commence analysis.

11.7 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). This “apparent” concentration due to coloration of the water should be subtracted from concentrations obtained with Reagent A pumping through the system.

12.0 Data Analysis and Calculations

12.1 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.

12.2 Refractive Index Correction for Estuarine/ Coastal Systems

12.2.1 Obtain a second set of peak heights for all samples and standards with Refractive Reagent A (Section 7.2.3) being pumped through the system in place of Reagent A (Section 7.2.1). Reagent B (Section 7.2.2) 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.[9]

12.2.2 Subtract the refractive index peak heights from the heights obtained for the orthophosphate determination. Calculate the regression equation using the corrected standard peak heights. Calculate the concentration of samples from the regression equation using the corrected sample peak heights.

12.2.3 When a large data set has been amassed 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 (Section 7.2.3) and Reagent B (Section 7.2.2) and the regression of orthophosphate standards obtained with orthophosphate Reagent A (Section 7.2.1) and Reagent B (Section 7.2.2) for each sample. 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 operator to subtract an apparent orthophosphate concentration when the salinity is known, as long as other matrix effects are not present. Thus, the operator 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. An example follows:

Salinity (ppt)	Apparent orthophosphate conc. due to refractive index (mg P/L)
1	0.0002
5	0.0006
10	0.0009
20	0.0017

12.2.4 An example of a typical equation is:

mg P/L apparent PO_4^{3-} = 0.000087 X Salinity (ppt) where, 0.000087 is the slope of the line.

where, 0.000087 is the slope of the line.

12.3 Results should be reported in mg PO_4^{3-}- P/L or µg PO_4^{3-}- P/L.

mg PO_4^{3-} - P/L = ppm (parts per million)

µg PO_4^{3-} - P/L = ppb (parts per billion)

13.0 Method Performance

13.1 Single Analyst Precision -- A single laboratory analyzed three samples collected from Chesapeake Bay, Maryland, and East Bay, Florida. Seven replicates of each sample were processed and analyzed randomly throughout a group of 75 samples with salinities ranging from 3 to 36 ppt. The results were as follows:

Sample	Salinity (ppt)	Concentration (mg P/L)	Percent Relative Standard Deviation
1	36	0.0040	6.5
2	18	0.0024	10
3	3	0.0007	24

13.2 Multilaboratory Testing

13.2.1 This method was tested by nine laboratories using reagent water, high salinity seawater from the Sargasso Sea (36 ppt) and three different salinity waters from Chesapeake Bay, Maryland (8.3 ppt, 12.6 ppt, and 19.5 ppt). The reagent water and the Sargasso Seawater were fortified at four Youden pair concentrations ranging from 0.0012 mg P/L to 0.1000 mg P/L.[10] The Chesapeake Bay waters were fortified at three Youden pair concentrations ranging from 0.0050 mg P/L to 0.0959 mg P/L with the highest salinity waters containing the lowest Youden pair and the lowest salinity waters containing the highest Youden pair. Analysis of variance (ANOVA) at the 95% confidence level found no statistical differences between water types indicating that the refractive index correction for different salinity waters is an acceptable procedure. Table 1 contains the linear equations that describe the single-analyst standard deviation, overall standard deviation, and mean recovery of orthophosphate from each water type.

13.2.2 Pooled Method Detection Limit (p-MDL) -- The p-MDL is derived from the pooled precision obtained by single laboratories for the lowest analyte concentration level used in the multilaboratory study. The p-MDLs using reagent water and Sargasso Sea water were 0.00128 and 0.00093 mg P/L, respectively.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better. Laboratory Chemical Management for Waste Reduction,* available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel,* available from the American Chemical Society at the address listed in Section 14.2.

16.0 References

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17.0 Tables, Diagrams, Flowcharts, and Validation Data

Table 1. Single-Analyst Precision, Overall Precision and Recovery from Multilaboratory Study

Reagent Water (0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.972C - 0.000018
Overall Standard Deviation	S_R = 0.033X + 0.000505
Single-Analyst Standard Deviation	S_r = 0.002X + 0.000448
Sargasso Sea Water (0.0012 - 0.100 mg P/L)	
Mean Recovery	X = 0.971C - 0.000002
Overall Standard Deviation	S_R = 0.021X + 0.000550
Single-Analyst Standard Deviation	S_r = 0.010X + 0.000249
Chesapeake Bay Water (0.005 - 0.100 mg P/L)	
Mean Recovery X = 1.019C - 0.000871	
Overall Standard Deviation	S_R = 0.066X + 0.000068
Single-Analyst Standard Deviation	S_r = 0.030X + 0.000165

C True value of spike concentration, mg P/L
X Mean concentration found, mg P/L, exclusive of outliers.
S_R Overall standard deviation, mg P/L, exclusive of outliers.
S_r Single-analyst standard deviation, mg P/L, exclusive of outliers.

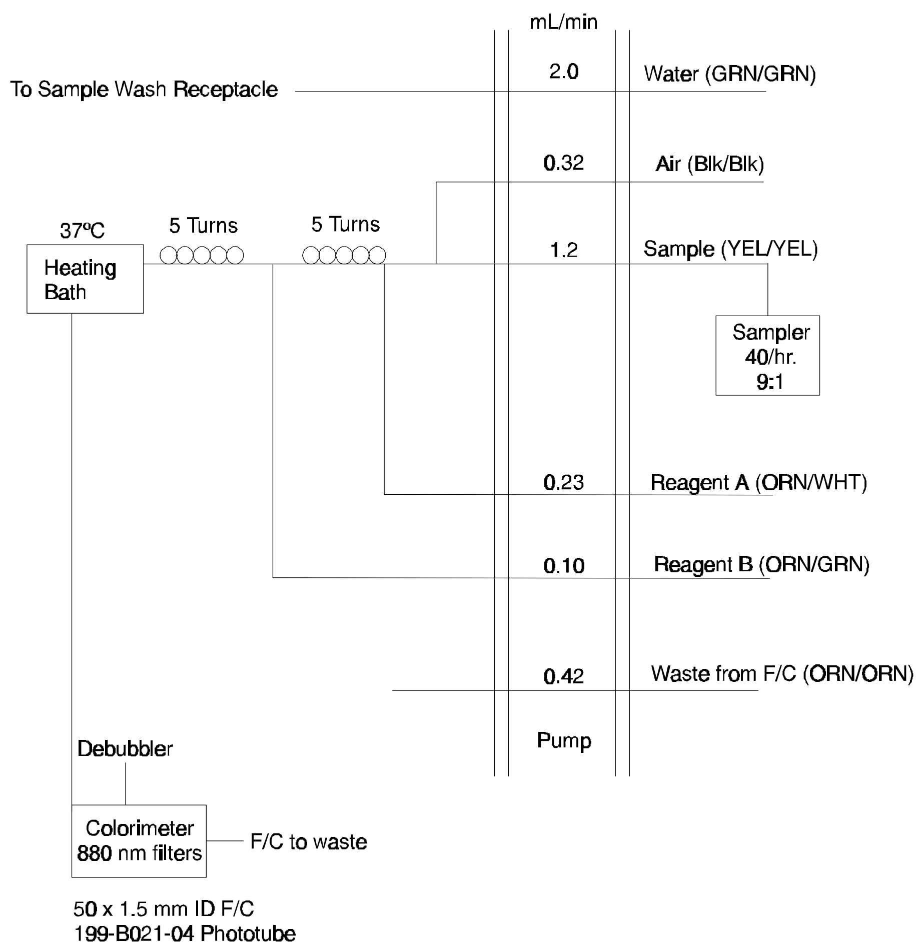

Figure 1. Manifold Configuration for Orthophosphate.

APPENDIX E

USGS Method I-2650-03

Methods of Analysis by the U.S. Geological Survey National Water Quality Laboratory. Evaluation of Alkaline Persulfate Digestion as an Alternative to Kjeldahl Digestion for Determination of Total and Dissolved Nitrogen and Phosphorous in Water

Available online: http://nwql.usgs.gov/Public/pubs/WRIR03-4174/WRIR03-4174.pdf

APPENDIX F

EPA Method 365.2

Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Single Reagent)

METHOD #: 365.2 Approved for NPDES (Issued 1971)

TITLE: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Single Reagent)

ANALYTE: CAS # P Phosphorus 7723-14-0

INSTRUMENTATION: Spectrophotometer

STORET No. See Section 4

1.0 Scope and Application

1.1 These methods cover the determination of specified forms of phosphorus in drinking, surface and saline waters, domestic and industrial wastes.

1.2 The methods are based on reactions that are specific for the orthophosphate ion. Thus, depending on the prescribed pre-treatment of the sample, the various forms of phosphorus given in Figure 1 may be determined. These forms are defined in Section 4.

1.2.1 Except for in-depth and detailed studies, the most commonly measured forms are phosphorus and dissolved phosphorus, and orthophosphate and dissolved orthophosphate. Hydrolyzable phosphorus is normally found only in sewage-type samples and insoluble forms of phosphorus are determined by calculation.

1.3 The methods are usable in the 0.01 to 0.5 mg P/L range.

2.0 Summary of Method

2.1 Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.

2.2 Only orthophosphate forms a blue color in this test. Polyphosphates (and some organic phosphorus compounds) may be converted to the orthophosphate form by sulfuric acid hydrolysis. Organic phosphorus compounds may be converted to the orthophosphate form by persulfate digestion[2].

3.0 Sample Handling and Preservation

3.1 If benthic deposits are present in the area being sampled, great care should be taken not to include these deposits.

3.2 Sample containers may be of plastic material, such as cubitainers, or of Pyrex glass.

3.3 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 mL conc. H_2SO_4 per liter and refrigeration at 4°C.

4.0 Definitions and Storet Numbers

4.1 Total Phosphorus (P)--all of the phosphorus present in the sample, regardless of form, as measured by the persulfate digestion procedure. (00665)

4.1.1 Total Orthophosphate (P, ortho)--inorganic phosphorus $[(PO_4)^{-3}]$ in the sample as measured by the direct colorimetric analysis procedure. (70507)

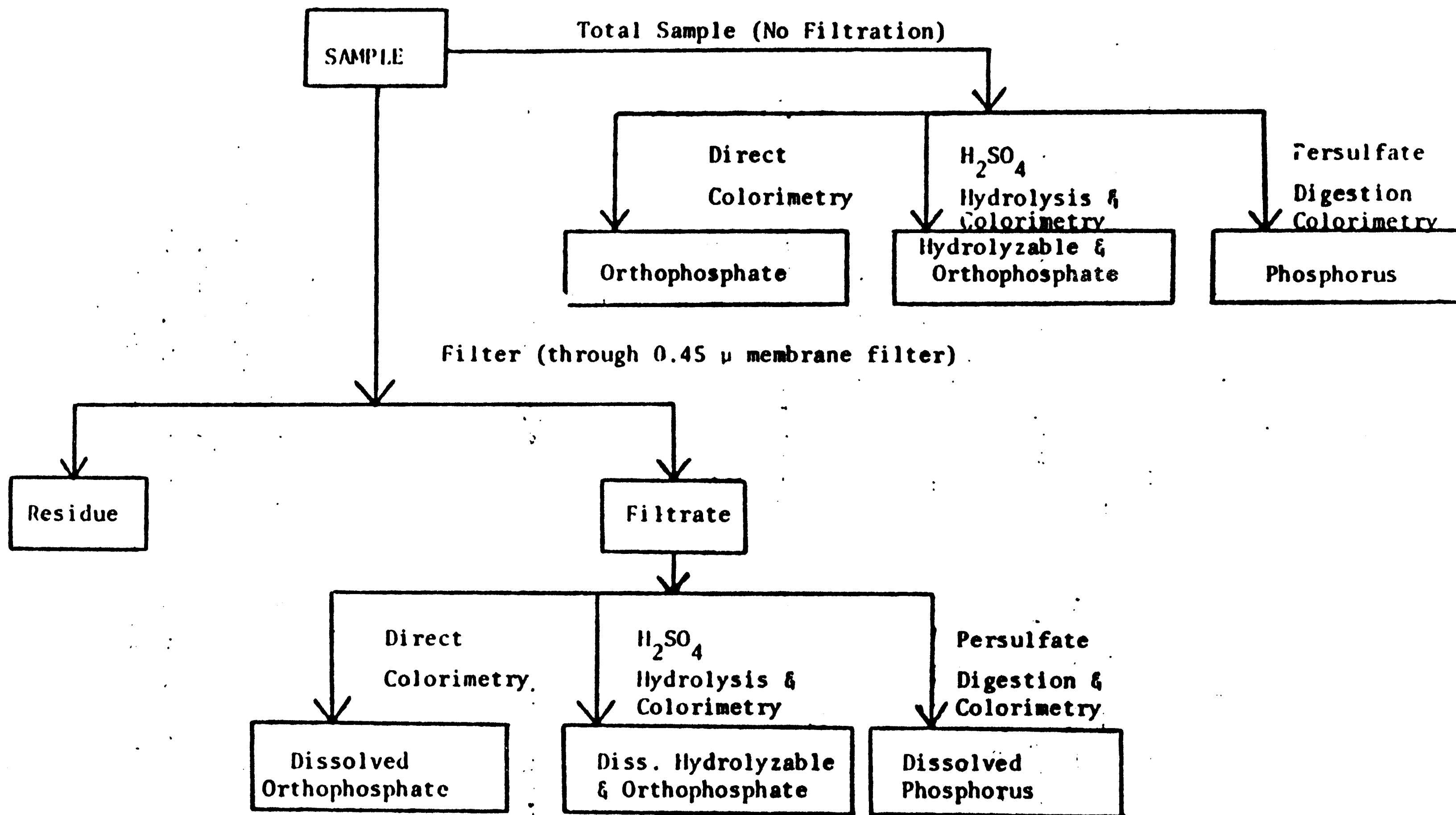

FIGURE 1. ANALYTICAL SCHEME FOR DIFFERENTIATION OF PHOSPHORUS FORMS

4.1.2 Total Hydrolyzable Phosphorus (P, hydro) - phosphorus in the sample as measured by the sulfuric acid hydrolysis procedure, and minus pre-determined orthophosphates. This hydrolyzable phosphorus includes polyphosphorus. [$(P_2O_7)^{-4}$, $(P_3O_{10})^{-5}$, etc.] plus some organic phosphorus. (00669)

4.1.3 Total Organic Phosphorus (P, org)--phosphorus (inorganic plus oxidizable organic) in the sample measured by the persulfate digestion procedure, and minus hydrolyzable phosphorus and orthophosphate. (00670)

4.2 Dissolved Phosphorus (P-D)--all of the phosphorus present in the filtrate of a sample filtered through a phosphorus-free filter of 0.45 micron pore size and measured by the persulfate digestion procedure. (00666)

4.2.1 Dissolved Orthophosphate (P-D, ortho)--as measured by the direct colorimetric analysis procedure. (00671)

4.2.2 Dissolved Hydrolyzable Phosphorus (P-D, hydro)--as measured by the sulfuric acid hydrolysis procedure and minus pre-determined dissolved orthophosphates. (00672)

4.2.3 Dissolved Organic Phosphorus (P-D, org)--as measured by the persulfate digestion procedure, and minus dissolved hydrolyzable phosphorus and orthophosphate. (00673)

4.3 The following forms, when sufficient amounts of phosphorus are present in the sample to warrant such consideration, may be calculated:

4.3.1 Insoluble Phosphorus (P-I) = (P)-(P-D). (00667)

4.3.1.1 Insoluble orthophosphate (P-I, ortho)=(P, ortho)-(P-D, ortho). (00674)

4.3.1.2 Insoluble Hydrolyzable Phosphorus (P-I, hydro)=(P, hydro)-(P-D, hydro). (00675)

4.3.1.3 Insoluble Organic Phosphorus (P-I, org)=(P, org) - (P-D, org). (00676)

4.4 All phosphorus forms shall be reported as P, mg/L, to the third place.

5.0 Interferences

5.1 No interference is caused by copper, iron, or silicate at concentrations many times greater than their reported concentration in sea water. However, high iron concentrations can cause precipitation of and subsequent loss of phosphorus.

5.2 The salt error for samples ranging from 5 to 20% salt content was found to be less than 1%.

5.3 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.

6.0 Apparatus

6.1 Photometer - A spectrophotometer or filter photometer suitable for measurements at 650 or 880 nm with a light path of 1 cm or longer.

6.2 Acid-washed glassware: All glassware used should 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. Preferably, this glassware should be used only for the determination of phosphorus and after use it should be rinsed with distilled water and kept covered until needed again. If this is done, the treatment with 1:1 HCl and reagents is only required occasionally. Commercial detergents should never be used.

7.0 Reagents

7.1 Sulfuric acid solution, 5N: Dilute 70 mL of conc H_2SO_4 with distilled water to 500 mL.

7.2 Antimony potassium tartrate solution: Weigh 1.3715 g $K(SbO)C_4H_4O \bullet 1/2H_2O$ dissolve in 400 mL distilled water in 500 mL volumetric flask, dilute to volume. Store at 4°C in a dark, glass-stoppered bottle.

7.3 Ammonium molybdate solution: Dissolve 20 g $(NH_4)_6Mo_70_{24} \bullet 4H_2O$ in 500 mL of distilled water. Store in a plastic bottle at 4°C.

7.4 Ascorbic acid, 0.1 M: Dissolve 1.76 g of ascorbic acid in 100 mL of distilled water. The solution is stable for about a week if stored at 4°C.

7.5 Combined reagent: Mix the above reagents in the following proportions for 100 mL of the mixed reagent: 50 mL of 5N H_2SO_4, (7.1), 5 mL of antimony potassium tartrate solution (7.2), 15 mL of ammonium molybdate solution (7.3), and 30 mL of ascorbic acid solution (7.4). 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 proceeding. Since the stability of this solution is limited, it must be freshly prepared for each run.

7.6 Sulfuric acid solution, 11 N: Slowly add 310 mL conc. H_2SO_4 to 600 mL distilled water. When cool, dilute to 1 liter.

7.7 Ammonium persulfate.

7.8 Stock phosphorus solution: Dissolve in distilled water 0.2197 g of potassium dihydrogen phosphate, KH2PO4, which has been dried in an oven at 105°C. Dilute the solution to 1000 ml; 1.0 mL = 0.05 mg P.

7.9 Standard phosphorus solution: Dilute 10.0 mL of stock phosphorus solution (7.8) to 1000 mL with distilled water; 1.0 mL = 0.5 μg P.

7.9.1 Using standard solution, prepare the following standards in 50.0 mL volumetric flasks:

mL of Standard Phosphorus Solution (7.9)	Conc., mg/L
0	0.00
1.0	0.01
3.0	0.03
5.0	0.05
10.0	0.10
20.0	0.20
30.0	0.30
40.0	0.40
50.0	0.50

7.10 Sodium hydroxide, 1 N: Dissolve 40 g NaOH in 600 mL distilled water. Cool and dilute to 1 liter.

8.0 Procedure

8.1 Phosphorus

8.1.1 Add 1 mL of H_2SO_4 solution (7.6) to a 50 mL sample in a 125 mL Erlenmeyer flask.

8.1.2 Add 0.4 g of ammonium persulfate.

8.1.3 Boil gently on a pre-heated hot plate for approximately 30 10 minutes or 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).

8.1.4 Cool and dilute the sample to about 30 mL and adjust the pH of the sample to 7.0 ±0.2 with 1 N NaOH (7.10) using a pH meter. If sample is not clear at this point, add 2-3 drops of acid (7.6) and filter. Dilute to 50 mL. Alternatively, if autoclaved see NOTE 1.

8.1.5 Determine phosphorus as outlined in 8.3.2 Orthophosphate.

8.2 Hydrolyzable Phosphorus

8.2.1 Add 1 mL of H_2SO_4 solution (7.6) to a 50 mL sample in a 125 mL Erlenmeyer flask.

8.2.2 Boil gently on a pre-heated hot plate for 30 10 minutes or 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).

8.2.3 Cool and dilute the sample to about 30 mL and adjust the pH of the sample to 7.0 ±0.2 with NaOH (7.10) using a pH meter. If sample is not clear at this point, add 2-3 drops of acid (7.6) and filter. Dilute to 50 mL. Alternatively, if autoclaved see NOTE 1.

8.2.4 The sample is now ready for determination of phosphorus as outlined in 8.3.2 Orthophosphate.

8.3 Orthophosphate

8.3.1 The pH of the sample must be adjusted to 7 ± 0.2 using a pH meter.

8.3.2 Add 8.0 mL of combined reagent (7.5) to sample and mix thoroughly. After a minimum of ten minutes, but no longer than thirty minutes, measure the color absorbance of each sample at 650 or 880 nm with a spectrophotometer, using the reagent blank as the reference solution. NOTE 1: If the same volume of sodium hydroxide solution is not used to adjust the pH of the standards and samples, a volume correction has to be employed.

9.0 Calculation

9.1 Prepare a standard curve by plotting the absorbance values of standards versus the corresponding phosphorus concentrations.

9.1.1 Process standards and blank exactly as the samples. Run at least a blank and two standards with each series of samples. If the standards do not agree within ±2% of the true value, prepare a new calibration curve.

9.2 Obtain concentration value of sample directly from prepared standard curve. Report results as P, mg/L. SEE NOTE 1.

10.0 Precision and Accuracy

10.1 Thirty-three analysts in nineteen laboratories analyzed natural water samples containing exact increments of organic phosphate, with the following results:

Increment as Total Phosphorus mg P/liter	Precision as Standard Deviation mg P/liter	Accuracy as Bias, %	Accuracy as Bias, mg P/liter
0.110	0.033	+3.09	+0.003
0.132	0.051	+11.99	+0.016
0.772	0.130	+2.96	+0.023
0.882	0.128	-0.92	-0.008

(FWPCA Method Study 2, Nutrient Analyses)

10.2 Twenty-six analysts in sixteen laboratories analyzed natural water samples containing exact increments of orthophosphate, with the following results:

Increment as Orthophosphorus mg P/liter	Precision as Standard Deviation mg P/liter	Accuracy as Bias, %	Accuracy as Bias mg P/liter
0.029	0.010	-4.95	-0.001
0.038	0.008	-6.00	-0.002
0.335	0.018	-2.75	-0.009
0.383	0.023	-1.76	-0.007

(FWPCA Method Study 2, Nutrient Analyses)

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APPENDIX G

EPA Method 365.4

Phosphorous, Total (Colorimetric, Automated, Block Digester AA II)

METHOD #: 365.4 Pending Approval for NPDES, CWA (Issued 1974)

TITLE: Phosphorous, Total (Colorimetric, Automated, Block Digester AA II)

ANALYTE: CAS # P Phosphorus 7723-14-0

INSTRUMENTATION: Autoanalyzer

STORET No. 00665

1.0 Scope and Application

1.1 This method covers the determination of total phosphorus in drinking water, surface water and domestic and industrial wastes. The applicable range of this method is 0.01 to 20 mg P/L.

2.0 Summary of Method

2.1 The sample is heated in the presence of sulfuric acid, K_2SO_4 and $HgSO_4$ for two and one half hours. The residue is cooled, diluted to 25 mL and placed on the AutoAnalyzer for phosphorus determination.

3.0 Sample Handling and Preservation

3.1 Sample containers may be of plastic material, such as a cubitainer, or of Pyrex glass.

3.2 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 mL of conc. H_2SO_4 per liter and refrigeration at 4°C.

4.0 Apparatus

4.1 Block Digester BD-40

4.2 Technicon Method No. 327-74W for Phosphorus

5.0 Reagents

5.1 Mercuric sulfate: Dissolve 8 g red mercuric oxide (HgO) in 50 mL of 1:4 sulfuric acid (10 conc. H_2SO_4: 40 mL distilled water) and dilute to 100 mL with distilled water.

5.2 Digestion solution: (Sulfuric acid-mercuric sulfate-potassium sulfate solution): Dissolve 133 g of K_2SO_4in 600 mL of distilled water and 200 mL of conc. H_2SO_4. Add 25 mL of mercuric sulfate solution (5.1) and dilute to 1 liter.

5.3 Sulfuric acid solution (0.72 N): Add 20 mL of conc. sulfuric acid to 800 of distilled water, mix and dilute to 1 liter.

5.4 Molybdate/antimony solution: Dissolve 8 g of ammonium molybdate and 0.2g of antimony potassium tartrate in about 800 mL of distilled water and dilute to

1 liter.

5.5 Ascorbic acid solution: Dissolve 60 g of ascorbic acid in about 600 mL of distilled water. Add 2 mL of acetone and dilute to 1 liter.

5.6 Diluent water: Dissolve 40 g of NaCl in about 600 mL of distilled water and dilute to 1 liter.

5.7 Sulfuric acid solution, 4%: Add 40 mL of conc. sulfuric acid to 800 mL of ammonia-free distilled water, cool and dilute to 1 liter.

6.0 Procedure

Digestion

6.1 To 20 or 25 mL of sample, add 5 mL of digestion solution and mix. (Use a vortex mixer).

6.2 Add 4-8 Teflon boiling chips. Too many boiling chips will cause the sample to boil over.

6.3 With Block Digester in manual mode set low and high temperature at 160°C and preheat unit to 160°C. Place tubes in digester and switch to automatic mode. Set low temperature timer for 1 hour. Reset high temperature to 380°C and set timer for 2 1/2 hours.

6.4 Cool sample and dilute to 25 mL with distilled water. If TKN is determined the sample should be diluted with ammonia-free water.

Colorimetric Analysis

6.4.1 Check the level of all reagent containers to ensure an adequate supply.

6.4.2 Excluding the molybdate/antimony line, place all reagent lines in their respective containers, connect the sample probe to the Sampler IV and start the proportioning pump.

6.4.3 Flush the Sampler IV wash receptacle with about 25 mL of 4% sulfuric acid (5.7).

6.4.4 When reagents have been pumping for at least five minutes, place the molybdate/antimony line in its container and allow the system to equilibrate.

6.4.5 After a stable baseline has been obtained, start the sampler.

7.0 Calculations

7.1 Prepare a standard curve by plotting peak heights of processed standards against concentration values. Compute concentrations by comparing sample peak heights with the standard curve.

8.0 Precision and Accuracy

8.1 In a single laboratory (EMSL) using sewage sample containing total P at levels of 0.23, 1.33, and 2.0, the precision was ± 0.01, ±0.04, and ± 0.06, respectively.

8.2 In a single laboratory (EMSL) using sewage samples of concentration 1.84 and 1.89, the recoveries were 95 and 98%, respectively.

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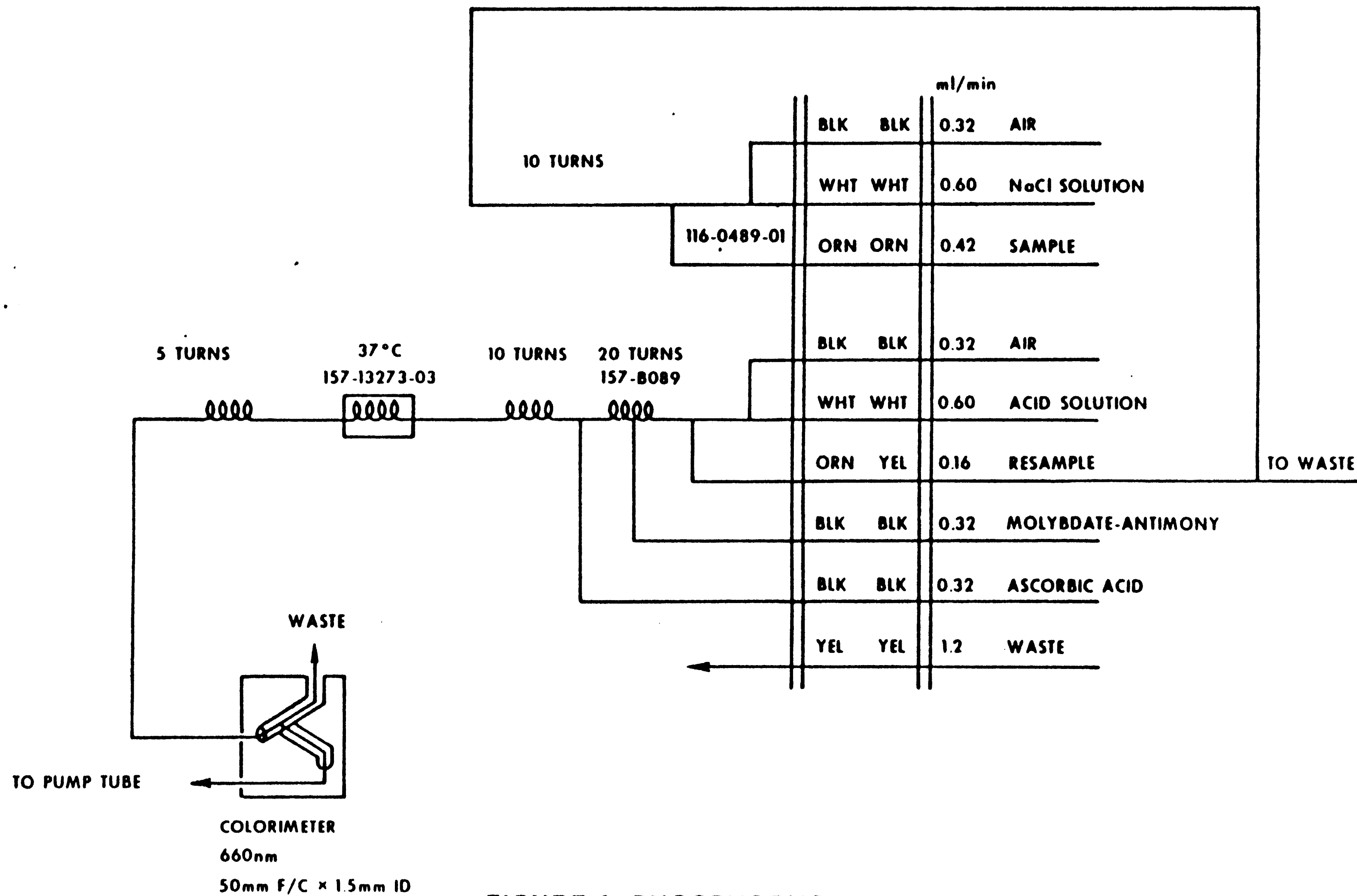

FIGURE 1. PHOSPHORUS MANIFOLD AA11

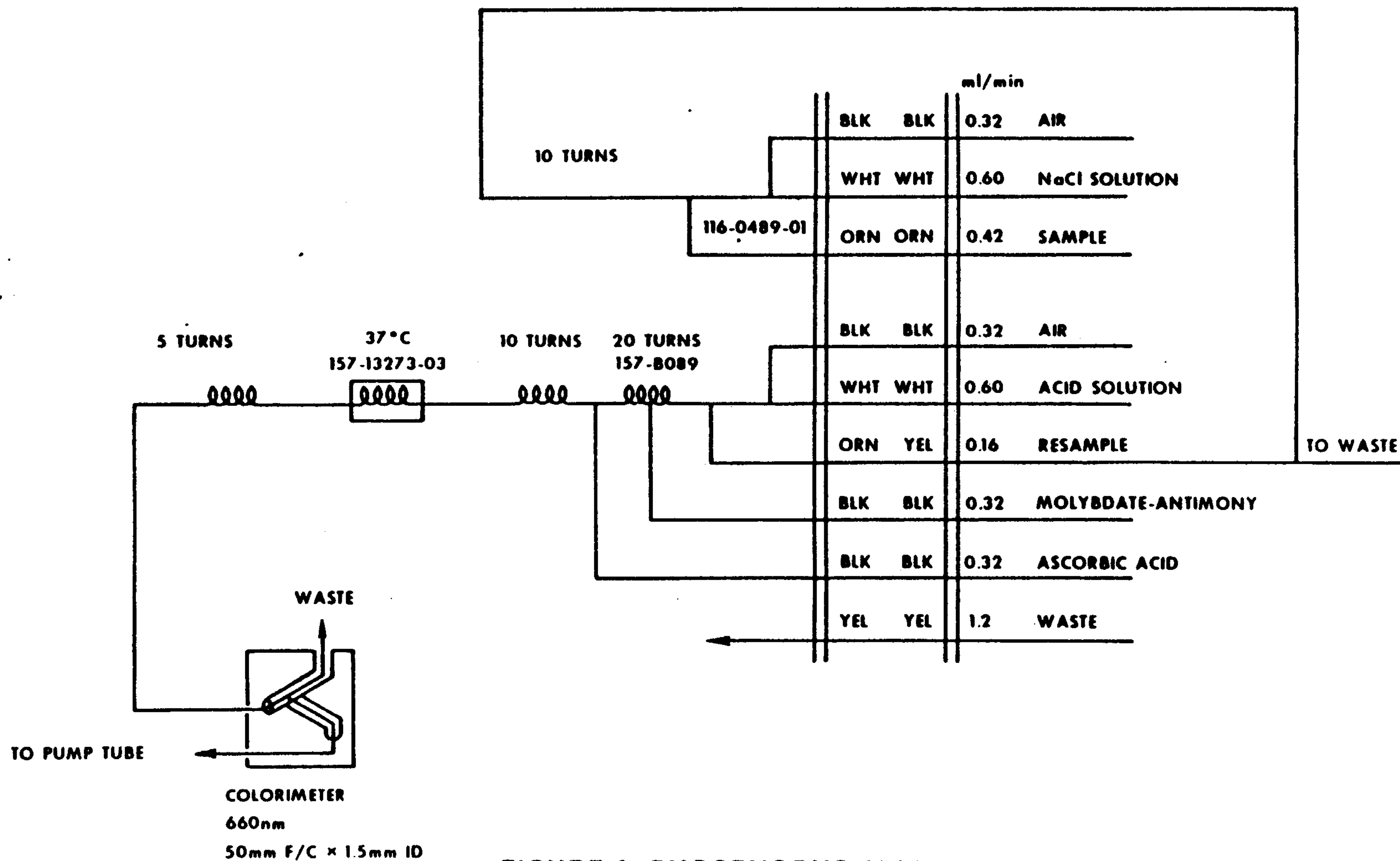

FIGURE 1. PHOSPHORUS MANIFOLD AA11

APPENDIX H

EPA Method 365.3

Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent)

METHOD #: 365.3 Approved for NPDES (Issued 1978)

TITLE: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent)

ANALYTE: CAS # P Phosphorus 7723-14-0

INSTRUMENTATION: Spectrophotometer

STORET No. See Section 4

1.0 Scope and Application

1.1 These methods cover the determination of specified forms of phosphorus in drinking, surface and saline waters, domestic and industrial wastes.

1.2 The methods are based on reactions that are specific for the orthophosphate ion. Thus, depending on the prescribed pretreatment of the sample, the various forms may be determined.

1.2.1 Except for in-depth and detailed studies, the most commonly measured forms are phosphorus and dissolved phosphorus, and orthophosphate and dissolved orthophosphate. Hydrolyzable phosphorus is normally found only in sewage-type samples and insoluble forms of phosphorus are determined by calculation.

1.3 The methods are usable in the 0.01 to 1.2 mg P/L range.

2.0 Summary of Method

2.1 Ammonium molybdate and antimony potassium tartrate react in an acid medium with dilute solutions of phosphorus to form an antimony-phospho-molybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid. The color is proportional to the phosphorus concentration.

2.2 Only orthophosphate forms a blue color in this test. Polyphosphates (and some organic phosphorus compounds) may be converted to the orthophosphate form by sulfuric acid hydrolysis. Organic phosphorus compounds may be converted to the orthophosphate form by persulfate digestion.

3.0 Sample Handling and Preservation

3.1 If benthic deposits are present in the area being sampled, great care should be taken notto include these deposits.

3.2 Sample containers may be of plastic material, such as cubitainers, or of Pyrex glass.

3.3 If the analysis cannot be performed the day of collection, the sample should be preserved by the addition of 2 mL conc. H_2SO_4 pr liter and refrigeration at 4°C.

4.0 Definitions and Storet Numbers

4.1 Total Phosphorus (P)--all of the phosphorus present in the sample, regardless of form, as measured by the persulfate digestion procedure. (00665)

4.1.1 Total Orthophosphate (P, ortho)--inorganic phosphorus $[(PO_4)^{-3}]$ in the sample as measured by the direct colorimetric analysis procedure. (70507)

4.1.2 Total Hydrolyzable Phosphorus (P, hydro) - phosphorus in the sample as measured by the sulfuric acid hydrolysis procedure, and minus pre-determined orthophosphates. This hydrolyzable phosphorus includes polyphosphorus. $[(P_2O_7)^{-4}$, $(P_3O_{10})^{-5}$, etc.] plus some organic phosphorus. (00669)

4.1.3 Total Organic Phosphorus (P, org)--phosphorus (inorganic plus oxidizable organic) in the sample measured by the persulfate digestion procedure, and minus hydrolyzable phosphorus and orthophosphate. (00670)

4.2 Dissolved Phosphorus (P-D)--all of the phosphorus present in the filtrate of a sample filtered through a phosphorus-free filter of 0.45 micron pore size and measured by the persulfate digestion procedure. (00666)

4.2.1 Dissolved Orthophosphate (P-D, ortho)--as measured by the direct colorimetric analysis procedure.(00671)

4.2.2 Dissolved Hydrolyzable Phosphorus (P-D, hydro)--as measured by the sulfuric acid hydrolysis procedure and minus pre-determined dissolved orthophosphates. (00672)

4.2.3 Dissolved Organic Phosphorus (P-D, org)--as measured by the persulfate digestion procedure, and minus dissolved hydrolyzable phosphorus and orthophosphate. (00673)

4.3 The following forms, when sufficient amounts of phosphorus are present in the sample to warrant such consideration, may be calculated:

4.3.1 Insoluble Phosphorus (P-I) = (P)-(P-D). (00667)

4.3.1.1 Insoluble orthophosphate (P-I, ortho)=(P, ortho) - (P-D, ortho). (00674)

4.3.1.2 Insoluble Hydrolyzable Phosphorus (P-I, hydro)=(P, hydro)-(P-D, hydro). (00675)

4.3.1.3 Insoluble Organic Phosphorus (P-I, org)=(P, org) - (P-D, org). (00676)

4.4 All phosphorus forms shall be reported as P, mg/L, to the third place.

5.0 Interferences

5.1 Arsenate is determined similarly to phosphorus and should be considered when present. This interference may be eliminated by reducing the arsenic acid to arsenious acid with sodium bisulfite (7.4).

5.2 When high concentrations of iron are present low recovery of phosphorus will be obtained because it will use some of the reducing agent. The bisulfite treatment will also eliminate this interference.

6.0 Apparatus

6.1 Photometer-A spectrophotometer or filter photometer suitable for measurements at 660 or 880 nm with a light path of 1 cm or longer.

6.2 Acid-washed glassware: All glassware used should 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 absorbed on the glassware. Preferably, this glassware should be used only for the determination of phosphorus and after use it should be rinsed with distilled water and kept covered until needed again. If this is done, the treatment with 1:1 HCl and reagents is only required occasionally. Commercial detergents should never be used.

6.3 Waterbath, 95°C.

7.0 Reagents

7.1 Ammonium molybdate-antimony potassium tartrate solution: Dissolve 8 g of ammonium molybdate and 0.2 g antimony potassium tartrate in 800 mL of distilled water and dilute to 1 liter.

7.2 Ascorbic acid solution: Dissolve 60 g of ascorbic acid in 800 mL of distilled water and dilute to 1 liter. Add 2 mL of acetone. This solution is stable for two weeks.

7.3 Sulfuric acid, 11 N: Slowly add 310 mL of conc. H_2SO_4 to approximately 600 mL distilled water. Cool and dilute to 1000 mL.

7.4 Sodium bisulfite ($NaHSO_3$) solution: Dissolve 5.2 g of $NaHSO_3$ in 100 mL of 1.0 N H_2SO_4.

7.5 Ammonium persulfate.

7.6 Stock phosphorus solution: Dissolve 0.4393 g of predried (105°C for one hour) KH_2PO_4 in distilled water and dilute to 1000 mL. 1.0 mL = 0.1 mg P.

7.7 Standard phosphorus solution: Dilute 100 mL of stock phosphorus solution to 1000 mL with distilled water. 1.0 mL = 0.01 mg P. Prepare an appropriate series of standards by diluting suitable volumes of standard or stock solutions to 100 mL with distilled water.

8.0 Procedure

8.1 Total Phosphorus

8.1.1 Transfer 50 mL of sample or an aliquot diluted to 50 mL into a 125 mL Erlenmeyer flask and add 1 mL of 11 N sulfuric acid (7.3).

8.1.2 Add 0.4 g ammonium persulfate (7.5), mix and boil gently for approximately 30-40 minutes or until a final volume of about 10 mL is reached. Alternatively heat for 30 minutes in an autoclave at 121°C (15-20 psi). Cool, dilute to approximately 40 mL and filter.

8.1.3 For samples containing arsenic or high levels of iron, add 5 mL of sodium bisulfite (7.4), mix and place in a 95°C water bath for 30 minutes (20 minutes after the temperature of the sample reaches 95°C). Cool and dilute to 50 mL.

8.1.4 Determine phosphorus as outlined in (8.3) orthophosphate.

8.2 Hydrolyzable Phosphorus

8.2.1 Add 1 mL of H_2SO_4 solution (8.3) to a 50 mL sample in a 125 mL Erlenmeyer flask.

8.2.2 Boil gently on a pre-heated hot plate for 30-40 minutes or 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). Cool, dilute to approximately 40 mL and filter.

8.2.3 Treat the samples as in 8.1.3.

8.2.4 Determine phosphorus as outlined in (8.3) orthophosphate.

8.3 Orthophosphate

8.3.1 To 50 mL of sample and/or standards, add 1 mL of 11 N sulfuric acid (7.3) and 4 mL of ammonium molybdate-antimony potassium tartrate (7.1) and mix.
NOTE: If sample has been digested for total or hydrolyzable phosphorus do not add acid.

8.3.2 Add 2 mL of ascorbic acid solution (7.2) and mix.

8.3.3 After 5 minutes, measure the absorbance at 650 nm with a spectrophotometer and determine the phosphorus concentration from the standard curve. The color is stable for at least one hour. For concentrations in the range of 0.01 to 0.3 mg P/L, a 5 cm cell should be used. A one cm cell should be used for concentrations in the range of 0.3 to 1.2 mg P/L.

9.0 Calculation

9.1 Prepare a standard curve by plotting the absorbance values of standards versus the corresponding phosphorus concentrations on linear graph paper.

9.2 Obtain concentration value of sample directly from prepared standard curve. Report results as P, mg/L.

10.0 Precision and Accuracy

10.1 Precision data is not available at this time.

10.2 In a single laboratory (EMSL) using industrial waste and sewage samples at concentrations of 7.6 and 0.55 mg P/L, recoveries were 99 and 100%, respectively.

APPENDIX I

EPA Method 350.1

Determination of Ammonia Nitrogen by Semi-Automated Colorimetry

METHOD 350.1

DETERMINATION OF AMMONIA NITROGEN BY SEMI-AUTOMATED COLORIMETRY

Edited by James W. O'Dell
Inorganic Chemistry Branch
Chemistry Research Division

Revision 2.0
August 1993

ENVIRONMENTAL MONITORING SYSTEMS LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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METHOD 350.1

DETERMINATION OF AMMONIA NITROGEN BY SEMI-AUTOMATED COLORIMETRY

1.0 SCOPE AND APPLICATION

1.1 This method covers the determination of ammonia in drinking, ground, surface, and saline waters, domestic and industrial wastes.

1.2 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.

1.3 This method is described for macro glassware; however, micro distillation equipment may also be used.

2.0 SUMMARY OF METHOD

2.1 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.

2.3 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.

2.4 Limited performance-based method modifications may be acceptable provided they are fully documented and meet or exceed requirements expressed in Section 9.0, Quality Control.

3.0 DEFINITIONS

3.1 **Calibration Blank (CB)** -- A volume of reagent water fortified with the same matrix as the calibration standards, but without the analytes, internal standards, or surrogate analytes.

3.2 **Calibration Standard (CAL)** -- A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.3 **Instrument Performance Check Solution (IPC)** -- A solution of one or more method analytes, surrogates, internal standards, or other test substances used to evaluate the performance of the instrument system with respect to a defined set of criteria.

3.4 **Laboratory Fortified Blank (LFB)** -- An aliquot of reagent water or other blank matrices to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

3.5 **Laboratory Fortified Sample Matrix (LFM)** -- An aliquot of an environmental sample to which known quantities of the method analytes are added in the laboratory. The LFM is analyzed exactly like a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and the measured values in the LFM corrected for background concentrations.

3.6 **Laboratory Reagent Blank (LRB)** -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.7 **Linear Calibration Range (LCR)** -- The concentration range over which the instrument response is linear.

3.8 **Material Safety Data Sheet (MSDS)** -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

3.9 **Method Detection Limit (MDL)** -- The minimum concentration of an analyte that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero.

3.10 **Quality Control Sample (QCS)** -- A solution of method analytes of known concentrations that is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.11 **Stock Standard Solution (SSS)** -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

4.0 INTERFERENCES

4.1 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.

4.2 Residual chorine must be removed by pretreatment of the sample with sodium thiosulfate or other reagents before distillation.

4.3 Method interferences may be caused by contaminants in the reagent water, reagents, glassware, and other sample processing apparatus that bias analyte response.

5.0 SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method have 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.

5.2 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.

5.3 The following chemicals have the potential to be highly toxic or hazardous, consult MSDS.

5.3.1 Sulfuric acid (Section 7.6)

5.3.2 Phenol (Section 7.7)

5.3.3 Sodium nitroprusside (Section 7.10)

6.0 EQUIPMENT AND SUPPLIES

6.1 Balance - Analytical, capable of accurately weighing to the nearest 0.0001 g.

6.2 Glassware - Class A volumetric flasks and pipets as required.

6.3 An all-glass distilling apparatus with an 800-1000 mL flask.

6.4 Automated continuous flow analysis equipment designed to deliver and react sample and reagents in the required order and ratios.

6.4.1 Sampling device (sampler)

6.4.2 Multichannel pump

6.4.3 Reaction unit or manifold

6.4.4 Colorimetric detector

6.4.5 Data recording device

7.0 REAGENTS AND STANDARDS

7.1 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.

7.2 Boric acid solution (20 g/L): Dissolve 20 g H_3BO_3 (CASRN 10043-35-3) in reagent water and dilute to 1 L.

7.3 Borate buffer: Add 88 mL of 0.1 N NaOH (CASRN 1310-73-2) solution to 500 mL of 0.025 M sodium tetraborate solution (5.0 g anhydrous $Na_2B_4O_7$ [CASRN 1330-43-4] or 9.5 g $Na_2B_4O_7{\bullet}10H_2O$ [CASRN 1303-96-4] per L) and dilute to 1 L with reagent water.

7.4 Sodium hydroxide, 1 N: Dissolve 40 g NaOH in reagent water and dilute to 1 L.

7.5 Dechlorinating reagents: A number of dechlorinating reagents may be used to remove residual chlorine prior to distillation. These include:

7.5.1 Sodium thiosulfate: Dissolve 3.5 g $Na_2S_2O_3{\bullet}5H_2O$ (CASRN 10102-17-7) 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.

7.5.2 Sodium sulfite: Dissolve 0.9 g $Na2SO_3$ (CASRN 7757-83-7) in reagent water and dilute to 1 L. One mL removes 1 mg/L Cl per 500 mL of sample.

7.6 Sulfuric acid 5 N: Air scrubber solution. Carefully add 139 mL of conc. sulfuric acid (CASRN 7664-93-9) to approximately 500 mL of reagent water. Cool to room temperature and dilute to 1 L with reagent water.

7.7 Sodium phenolate: Using a 1-L Erlenmeyer flask, dissolve 83 g phenol (CASRN 108-95-2) 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.

7.8 Sodium hypochlorite solution: Dilute 250 mL of a bleach solution containing 5.25% NaOCl (CASRN 7681-52-9) (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.

7.9 Disodium ethylenediamine-tetraacetate (EDTA) (5%): Dissolve 50 g of EDTA (disodium salt) (CASRN 6381-92-6) and approximately six pellets of NaOH in 1 L of reagent water.

7.10 Sodium nitroprusside (0.05%): Dissolve 0.5 g of sodium nitroprusside (CASRN 14402-89-2) in 1 L of reagent water.

7.11 Stock solution: Dissolve 3.819 g of anhydrous ammonium chloride, NH_4Cl (CASRN 12125-02-9), dried at 105°C, in reagent water, and dilute to 1 L. 1.0 mL = 1.0 mg NH_3-N.

7.12 Standard Solution A: Dilute 10.0 mL of stock solution (Section 7.11) to 1 L with reagent water. 1.0 mL = 0.01 mg NH_3-N.

7.13 Standard Solution B: Dilute 10.0 mL of standard solution A (Section 7.12) to 100.0 mL with reagent water. 1.0 mL = 0.001 mg NH_3-N.

8.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 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 (if required), and minimize waste disposal.

8.2 Samples must be preserved with H_2SO_4 to a pH <2 and cooled to 4°C at the time of collection.

8.3 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.

9.0 QUALITY CONTROL

9.1 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.

9.2 INITIAL DEMONSTRATION OF PERFORMANCE

9.2.1 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.

9.2.2 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.

9.2.3 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.

9.2.4 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.[9] 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:

$$MDL = (t) \times (S)$$

where,
- t = Student's 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.

9.3 ASSESSING LABORATORY PERFORMANCE

9.3.1 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.

9.3.2 Laboratory Fortified Blank (LFB) -- The laboratory must analyze at least one LFB with each batch of samples. Calculate accuracy as percent recovery (Section 9.4.2). 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.

9.3.3 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 five 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 established 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.

9.3.4 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.

9.4 ASSESSING ANALYTE RECOVERY AND DATA QUALITY

9.4.1 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.

9.4.2 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 calculate using the following equation:

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

where,
- R = percent recovery
- C_s = fortified sample concentration
- C = sample background concentration
- s = concentration equivalent of analyte added to sample

9.4.3 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 (Section 9.3), the recovery problem encountered with the LFM is judged to be either matrix or solution related, not system related.

9.4.4 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.

10.0 CALIBRATION AND STANDARDIZATION

10.1 Prepare a series of at least three standards, covering the desired range, and a blank by diluting suitable volumes of standard solutions (Sections 7.12 and 7.13) to 100 mL with reagent water.

10.2 Process standards and blanks as described in Section 11.0, Procedure.

10.3 Set up manifold as shown in Figure 1.

10.4 Prepare flow system as described in Section 11.0, Procedure.

10.5 Place appropriate standards in the sampler in order of decreasing concentration and perform analysis.

10.6 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.

10.7 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. Periodic reanalysis of the QCS is recommended as a continuing calibration check.

11.0 PROCEDURE

11.1 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.

11.2 Sample preparation: Remove the residual chorine in the sample by adding dechlorinating agent (Section 7.5) equivalent to the chlorine residual. To 400 mL of sample add 1 N NaOH (Section 7.4), until the pH is 9.5, check the pH during addition with a pH meter or by use of a short range pH paper.

11.3 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 (Section 7.3). Distill 300 mL at the rate of 6-10 mL/min. into 50 mL of 2% boric acid (Section 7.2) 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.

11.4 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.

11.5 Allow analysis system to warm up as required. Feed wash water through sample line.

11.6 Arrange ammonia standards in sampler in order of decreasing concentration of nitrogen. Complete loading of sampler tray with unknown samples.

11.7 Switch sample line from reagent water to sampler and begin analysis.

12.0 DATA ANALYSIS AND CALCULATIONS

12.1 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.

12.2 Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.

12.3 Report results in mg NH_3-N/L.

13.0 METHOD PERFORMANCE

13.1 In a single laboratory (EMSL-Cincinnati), using surface water samples at concentrations of 1.41, 0.77, 0.59, and 0.43 mg NH_3-N/L, the standard deviation was ±0.005.

13.2 In a single laboratory (EMSL-Cincinnati), using surface water samples at concentrations of 0.16 and 1.44 mg NH_3-N/L, recoveries were 107% and 99%, respectively.

13.3 The interlaboratory precision and accuracy data in Table 1 were developed using a reagent water matrix. Values are in mg NH_3-N/L.

14.0 POLLUTION PREVENTION

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 The quantity of chemicals purchased should be based on expected usage during its shelf life and disposal cost of unused material. Actual reagent preparation volumes should reflect anticipated usage and reagent stability.

14.3 For information about pollution prevention that may be applicable to laboratories and research institutions, consult "Less is Better: Laboratory Chemical Management for Waste Reduction", available from the American Chemical Society's Department of Government Regulations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036, (202)872-4477.

15.0 WASTE MANAGEMENT

15.1 The U.S. Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. Excess reagents, samples and method process wastes should be characterized and disposed of in an acceptable manner. The Agency urges laboratories to protect the air, water and land by minimizing and controlling all releases from hoods, and bench operations, complying with the letter and spirit of any waste discharge permit and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult the "Waste Management Manual for Laboratory Personnel", available from the American Chemical Society at the address listed in Section 14.3.

16.0 REFERENCES

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17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. INTERLABORATORY PRECISION AND ACCURACY DATA

Number of Values Reported	True Value (T)	Mean (X)	Residual for X	Standard Deviation (S)	Residual for S
134	0.270	0.2670	-0.0011	0.0342	0.0015
157	0.692	0.6972	0.0059	0.0476	-0.0070
136	1.20	1.2008	0.0001	0.0698	-0.0112
195	1.60	1.6095	0.0076	0.1023	0.0006
142	3.00	3.0128	0.0069	0.1677	-0.0067
159	3.50	3.4991	-0.0083	0.2168	0.0165
156	3.60	3.5955	-0.0122	0.1821	-0.0234
200	4.20	4.2271	0.0177	0.2855	0.0488
196	8.76	8.7257	-0.0568	0.4606	-0.0127
156	11.0	11.0747	0.0457	0.5401	-0.0495
142	13.0	12.9883	-0.0465	0.6961	0.0027
199	18.0	17.9727	-0.0765	1.1635	0.2106

REGRESSIONS: $X = 1.003T - 0.003$, $S = 0.052T + 0.019$

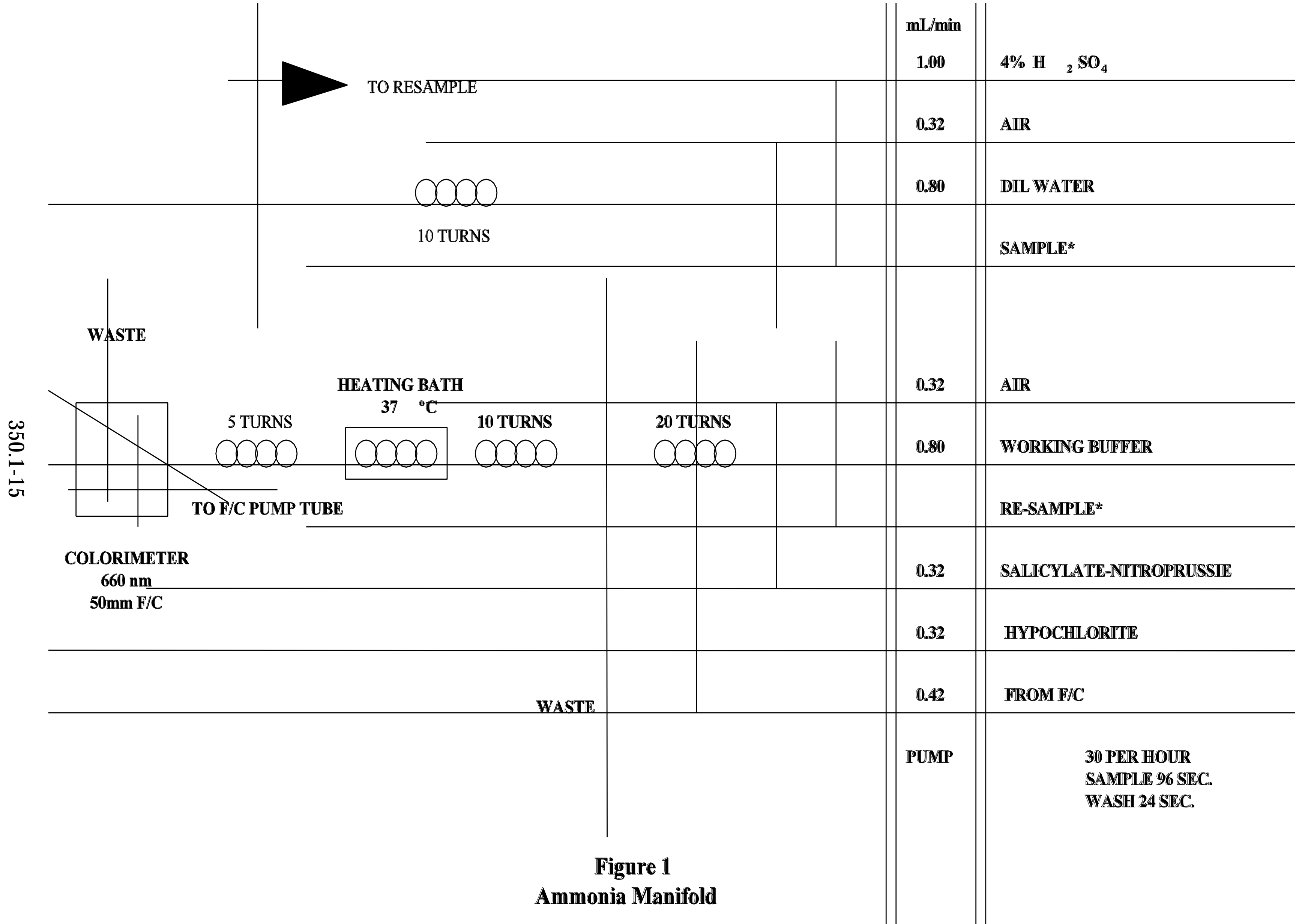


Figure 1
Ammonia Manifold

APPENDIX J

EPA Method 353.2

Nitrogen, Nitrate-Nitrite (Colorimetric, Automated, Cadmium Reduction)

METHOD #: 353.2	Approved for NPDES and SDWA (Ed. Rev. 1974, 1978)
TITLE:	Nitrogen, Nitrate-Nitrite (Colorimetric, Automated, Cadmium Reduction)
ANALYTE:	CAS # N Nitrogen 7727-37-9 NO3 Nitrate NO2 Nitrite
INSTRUMENTATION:	Autoanalyzer
STORET No.	Total 00630

1.0 Scope and Application

1.1 This method pertains to the determination of nitrite singly, or nitrite and nitrate combined in surface and saline waters, and domestic and industrial wastes. The applicable range of this method is 0.05 to 10.0 mg/L nitrate-nitrite nitrogen. The range may be extended with sample dilution.

2.0 Summary of Method

2.1 A filtered sample is passed through a column containing granulated copper-cadmium to reduce nitrate to nitrite. The nitrite (that 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, rather than combined nitrate-nitrite, values are readily obtained by carrying out the procedure first with, and then without, the Cu-Cd reduction step.

3.0 Sample Handling and Preservation

3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with sulfuric acid (2 mL conc. H_2SO_4 per liter) and refrigeration.
CAUTION: Samples for reduction column must not be preserved with mercuric chloride.

4.0 Interferences

4.1 Build up of suspended matter in the reduction column will restrict sample flow. Since nitrate-nitrogen is found in a soluble state, the sample may be pre-filtered.

4.2 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.

4.3 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.

5.0 Apparatus

5.1 Technicon AutoAnalyzer (AAI or AAII) consisting of the following components:

- 5.1.1 Sampler.
- 5.1.2 Manifold (AAI) or analytical cartridge (AAII).
- 5.1.3 Proportioning Pump
- 5.1.4 Colorimeter equipped with a 15 mm or 50 mm tubular flow cell and 540 nm filters.
- 5.1.5 Recorder
- 5.1.6 Digital printer for AAII (Optional).

6.0 Reagents

6.1 Granulated cadmium: 40-60 mesh (MCB Reagents).

6.2 Copper-cadmium: The cadmium granules (new or used) are cleaned with dilute HCl (6. 7) and copperized with 2% solution of copper sulfate (6. 8) in the following manner:

- 6.2.1 Wash the cadmium with HCl (6.7) and rinse with distilled water. The color of the cadmium so treated should be silver.
- 6.2.2 Swirl 10 g cadmium in 100 mL portions of 2% solution of copper sulfate (6.8) for five minutes or until blue color partially fades, decant and repeat with fresh copper sulfate until a brown colloidal precipitate forms.
- 6.2.3 Wash the cadmium-copper with distilled water (at least 10 times) to remove all the precipitated copper. The color of the cadmium so treated should be black.

6.3 Preparation of reduction column AAI: The reduction column is an 8 by 50 mm glass tube with the ends reduced in diameter to permit insertion into the system. Copper-cadmium granules (6.2) are placed in the column between glass wool plugs. The packed reduction column is placed in an up-flow 20° incline to minimize channeling. See Figure 1.

6.4 Preparation of reduction column AAII: The reduction column is a U-shaped, 35 cm length, 2 mm I.D. glass tube (Note 1). Fill the reduction column with distilled water to prevent entrapment of air bubbles during the filling operations. Transfer the copper- cadmium granules (6.2) 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 1: A 0.081 I.D. pump tube (purple) can be used in place of the 2 mm glass tube.

6.5 Distilled 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.

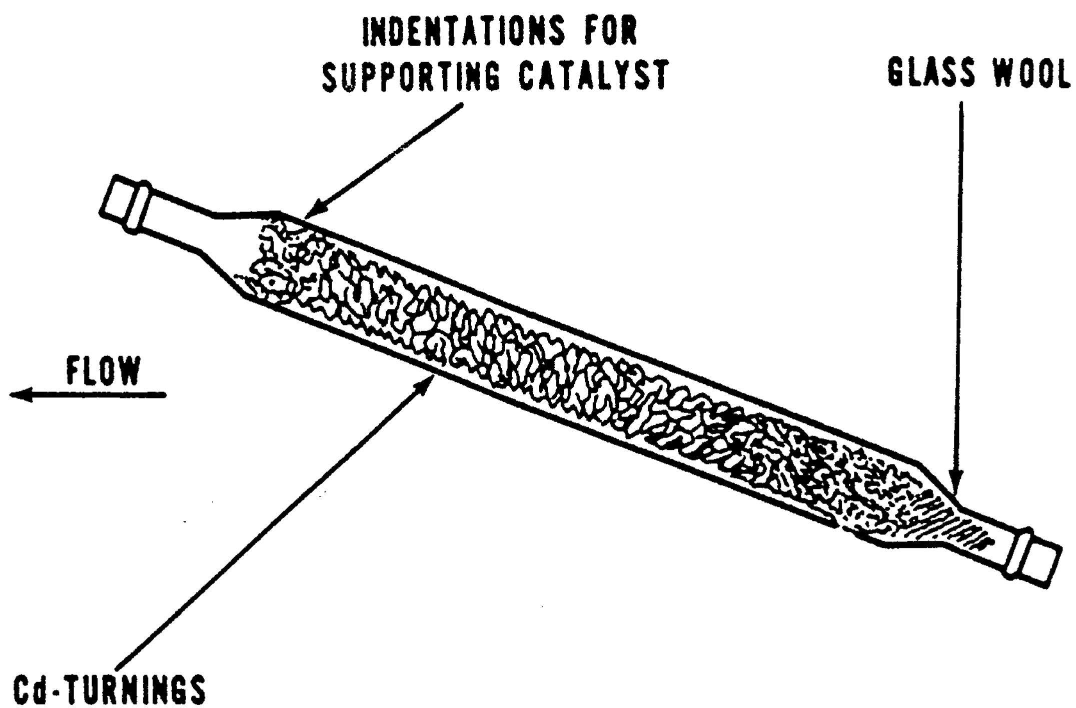
6.6 Color reagent: To approximately 800 mL of distilled water, add, while stirring, 100 mL conc. phosphoric acid, 40 g sulfanilamide, and 2 g N-1-naphthylethylenediamine dihydrochloride. Stir until dissolved and dilute to 1 liter. Store in brown bottle and keep in the dark when not in use. This solution is stable for several months.

6.7 Dilute hydrochloric acid, 6N: Dilute 50 mL of conc. HCl to 100 mL with distilled water.

6.8 Copper sulfate solution, 2%: Dissolve 20 g of $CuSO_4 \cdot 5H_2O$ in 500 mL of distilled water and dilute to 1 liter.

6.9 Wash solution: Use distilled water for unpreserved samples. For samples preserved with H_2SO_4, use 2 mL H_2SO_4 per liter of wash water.

6.10 Ammonium chloride-EDTA solution: Dissolve 85 g of reagent grade ammonium chloride and 0.1 g of disodium ethylenediamine tetraacetate in 900 mL of distilled water. Adjust the pH to 8.5 with conc. ammonium hydroxide and dilute to 1 liter. Add 1/2 mL Brij-35 (available from Technicon Corporation).



TILT COLUMN TO 20° POSTION

FIGURE 1. COPPER CADMIUM REDUCTION COLUMN
(1 1/2 ACTUAL SIZE)

6.11 Stock nitrate solution: Dissolve 7.218 g KNO_3 and dilute to 1 liter in a volumetric flask with distilled water. Preserve with 2 mL of chloroform per liter. Solution is stable for 6 months. 1 mL = 1.0 mg NO_3-N.

6.12 Stock nitrite solution Dissolve 6.072 g KNO_2 in 500 mL of distilled water and dilute to 1 liter in a volumetric flask. Preserve with 2 mL of chloroform and keep under refrigeration. 1.0 mL = 1.0 mg NO_2-N.

6.13 Standard nitrate solution: Dilute 10.0 mL of stock nitrate solution (6.11) to 1000 mL. 1.0 mL = 0.01 mg NO_3-N. Preserve with 2 mL of chloroform per liter. Solution is stable for 6 months.

6.14 Standard nitrite solution: Dilute 10.0 mL of stock nitrite (6.12) solution to 1000 mL. 1.0 mL = 0.01 mg NO_2-N. Solution is unstable; prepare as required.

6.15 Using standard nitrate solution (6.13), prepare the following standards in 100.0 mL volumetric flasks. At least one nitrite standard should be compared to a nitrate standard at the same concentration to verify the efficiency of the reduction column.

Conc., mgNO,-N or NO_3-N/L	mL Standard Solution/100 mL
0.0	0
0.05	0.5
0.10	1.0
0.20	2.0
0.50	5.0
1.00	10.0
2.00	20.0
4.00	40.0
6.00	60.0

NOTE 2: When the samples to be analyzed are saline waters, Substitute Ocean Water (SOW) should be used for preparing the standards; otherwise, distilled water is used. A tabulation of SOW composition follows:

NaCl- 24.53 g/L	$MgCl_2$ - 5.20 g/L	Na_2SO_4 - 4.09 g/L
$CaCl_2$ - 1.16 g/L	KCl - 0.70 g/L	$NaHCO_3$ - 0.20 g/L
Kbr - 0.10 g/L	H_3BO_3 - 0.03 g/L	$SrCl_2$- 0.03 g/L
NaF- 0.003 g/L		

7.0 Procedure

7.1 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.

7.2 Set up the manifold as shown in Figure 2 (AAI) or Figure 3 (AAII). Note that reductant column should be in 20- incline position (AAI). Care should be taken not to introduce air into reduction column on the AAII.

7.3 Allow both colorimeter and recorder to warm up for 30 minutes. Obtain a stable baseline with all reagents, feeding distilled water through the sample line.

NOTE 3: 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.

7.4 Place appropriate nitrate and/or nitrite standards in sampler in order of decreasing concentration of nitrogen. Complete loading of sampler tray with unknown samples.

7.5 For the AAI system, sample at a rate of 30/hr, 1:1. For the AAII, use a 40/hr, 4:1 cam and a common wash.

7.6 Switch sample line to sampler and start analysis.

8.0 Calculations

8.1 Prepare appropriate standard curve or curves derived from processing NO_2 and/or NO_3 standards through manifold. Compute concentration of samples by comparing sample peak heights with standard curve.

9.0 Precision and Accuracy

9.1 Three laboratories participating in an EPA Method Study, analyzed four natural water samples containing exact increments of inorganic nitrate, with the following results:

Increment as Nitrogen, Nitrate mg N/liter	Precision as Standard Deviation mg N/liter	Accuracy as Bias, %	Accuracy as Bias, mg N/liter
0.29	0.012	+ 5.75	+0.017
0.35	0.092	+ 18.10	+0.063
2.31	0.318	+ 4.47	+0.103
2.48	0.176	- 2.69	-0.067

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3. Annual Book of ASTM Standards, Part 31,"Water", Standard D1254, p 366 (1976).
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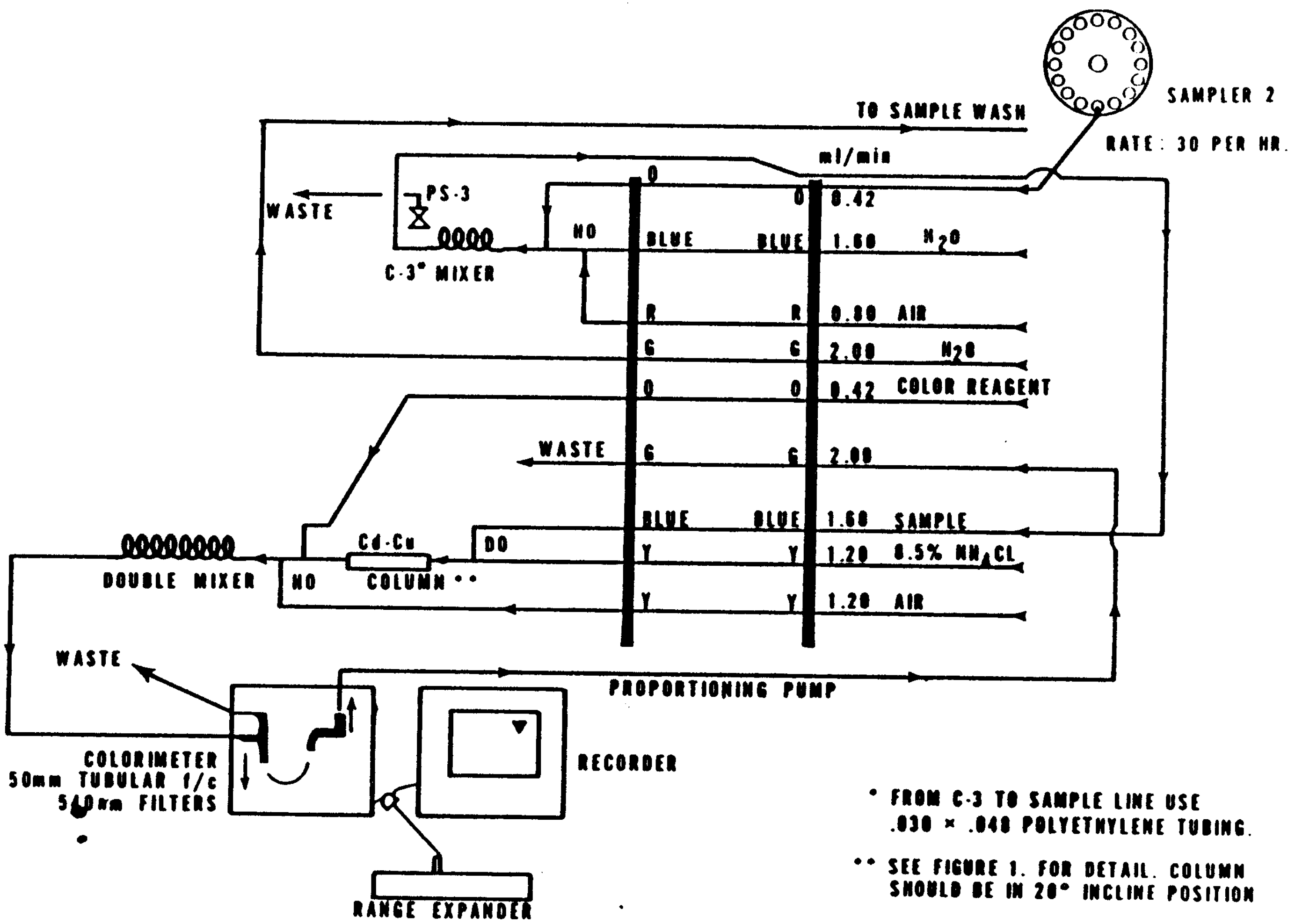

FIGURE 2. NITRATE - NITRITE MANIFOLD AA-I

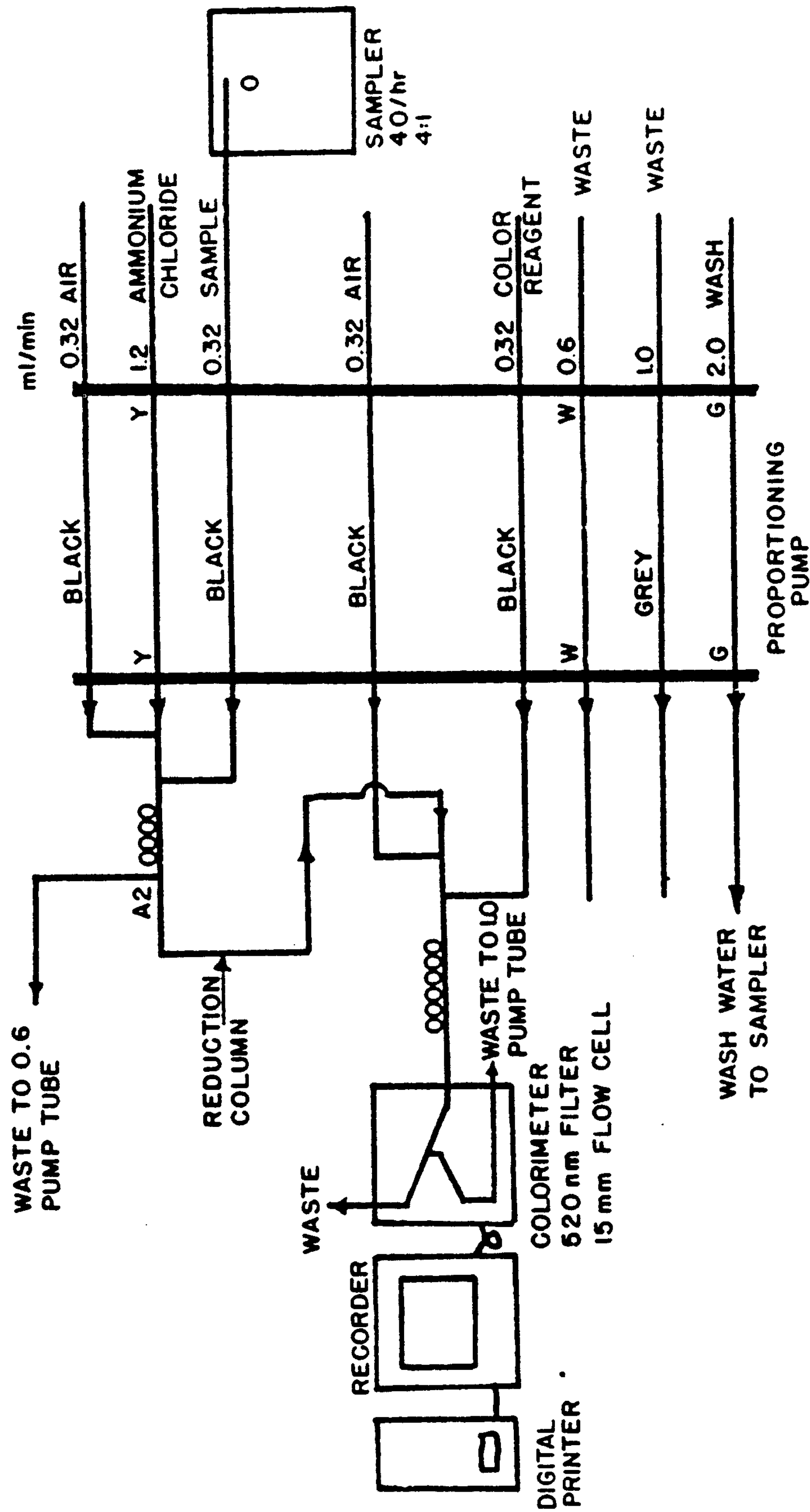

FIGURE 3 NITRATE-NITRITE MANIFOLD AA II

APPENDIX K

EPA Method 445.0

In Vitro Determination of Chlorophyll a and Pheophytin a in Marine and Freshwater Algae by Fluorescence

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

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and

Gary B. Collins

Revision 1.2
September 1997

National Exposure Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

445.0-1

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
NATIONAL EXPOSURE RESEARCH LABORATORY
CINCINNATI, OH 45268

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a*
in Marine and Freshwater Algae by Fluorescence

OFFICE OF
RESEARCH AND DEVELOPMENT

Revision 1.2

ERRATA SHEET

Section1.4 - References numbered 5-8 should be numbered 6-8.

Section 12.2 - Equation for calculating the "corrected" concentration of chlorophyll *a* in the whole water sample is as follows:

$$C_{S,c} = \frac{C_{E,c} \text{ X extract volume (L) X DF}}{\text{Sample volume (L)}}$$

Where, $C_{S,c}$ = corrected chlorophyll *a* concentration (µg/L) in the whole water sample
extract volume = volume (L) of extract prepared before dilution

Section 13.4.1 - The following has been added to the second paragraph.

The reported p-EDLs reflect between-lab variability and extraction variability. There was, however, a major flaw in the study design. Even though the concentrations used could be easily determined by fluorometry after appropriate dilution, we did not specify to the labs the dilution factor to be used. Because of that, each lab diluted at their discretion. Dilution factors ranged from 10-2000. The "observed" concentration by each fluorometer was not the reported concentration used in the multi-lab statistical analysis. Since p-EDLs are based on an estimate of variance (standard deviation) of the reported concentration in the extract, the p-EDL for fluorometry is not reflective of the concentration actually observed by the instrument. Since all the participants used different dilution factors there was no way to correct the determined p-EDLs for the fluorometric techniques. It is safe to say that the statistically determined p-EDLs are at least 1000 times too high. Still, fluorometry yielded the lowest p-EDLs. The p-EDLs for the other methods are valid.

Table 4 - The following has been added to footnote 5.

This is due to a flaw in the study design and not due to any inherent limitations of fluorometry. Please see Section 13.4.1 for a discussion of the determination of p-EDLs. Single-lab EDLs may be 1000 times lower than the p-EDLs reported here.

Tables 4-9 - The following footnote has been added.

Reported concentrations (ppm) are for the 10 mL extraction volume and not the concentrations in the whole water sample. Using the notation of Section 12 of the method, this would be $C_{E,c}$.

Table 9 - Eighth column, last value should be 0.675

Method 445.0

In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence

1.0 Scope and Application

1.1 This method provides a procedure for low level determination of chlorophyll *a* (chl *a*) and its magnesium-free derivative, pheophytin *a* (pheo *a*), in marine and freshwater phytoplankton using fluorescence detection.[1,2] Phaeophorbides present in the sample are determined collectively as pheophytin *a*. For users primarily interested in chl *a* there is currently available a set of very narrow bandpass excitation and emission filters (Turner Designs, Sunnyvale, CA) that nearly eliminates the spectral interference caused by the presence of pheo *a* and chlorophyll *b*. The difference between the modified method and the conventional fluorometric method is that the equations used for the determination of chlor *a* without pheo *a* correction (uncorrected chlor *a*), are used instead of the equations for "corrected chlor *a*". This EPA laboratory has evaluated the modified filters and found the technique to be an acceptable alternative to the conventional fluorometric method using pheo *a* correction.[3]

Analyte	Chemical Abstracts Service Registry Number (CASRN)
Chlorophyll *a*	479-61-8

1.2 Instrumental detection limits (IDL) of 0.05 µg chl *a*/L and 0.06 µg pheo *a*/L in a solution of 90% acetone were determined by this laboratory. Method detection limits (MDL) using mixed assemblages of algae provide little information because the fluorescence of other pigments interferes in the fluorescence of chlorophyll *a* and pheophytin *a*.[4] A single lab estimated detection limit for chlorophyll *a* was determined to be 0.11 µg/L in 10 mL of final extraction solution. The upper limit of the linear dynamic range for the instrumentation used in this method evaluation was 250 µg chl *a*/L.

1.3 This method was multilaboratory validated in 1996.[5] Results from that study may be found in Section 13. Additional QC procedures also have been added as a result of that study.

1.4 This method uses 90% acetone as the extraction solvent because of its efficiency for most types of algae. There is evidence that certain chlorophylls and carotenoids are more thoroughly extracted with methanol[5-8] or dimethyl sulfoxide.[9] Bowles, et al.[8] found that for chlorophyll *a*, however, 90% acetone was an effective extractant when the extraction period was optimized for the dominant species present in the sample.

1.5 Depending on the type of algae under investigation, this method can have uncorrectable interferences (Sect. 4.0). In cases where taxomonic classification is unavailable, a spectrophotometric or high performance liquid chromatographic (HPLC) method may provide more accurate data for chlorophyll *a* and pheophytin *a*.

1.6 This method is for use by analysts experienced in the handling of photosynthetic pigments and in the operation of fluorescence detectors or by analysts under the close supervision of such qualified persons.

2.0 Summary of Method

2.1 Chlorophyll-containing phytoplankton in a measured volume of sample water 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 for a minimum of 2 h, but not to exceed 24 h, to ensure thorough extraction of the chlorophyll *a*. The filter slurry is centrifuged at 675 g for 15 min (or at 1000 g for 5 min) to clarify the solution. An aliquot of the supernatant is transferred to a glass cuvette and fluorescence is measured before and after acidification to 0.003 N HCl with 0.1 N HCl. Sensitivity calibration factors, which have been previously determined on solutions of

pure chlorophyll *a* of known concentration, are used to calculate the concentration of chlorophyll *a* and pheophytin *a* in the sample extract. The concentration in the natural water sample is reported in µg/L.

3.0 Definitions

3.1 Estimated Detection Limit (EDL) -- The minimum concentration of an analyte that yields a fluorescence 3X the fluorescence of blank filters which have been extracted according to this method.

3.2 Linear Dynamic Range (LDR) -- The absolute quantity or concentration range over which the instrument response to an analyte is linear.

3.3 Instrument Detection Limit (IDL) -- The minimum quantity of analyte or the concentration equivalent which gives an analyte signal equal to three times the standard deviation of the background signal at the selected wavelength, mass, retention time, absorbance line, etc. For this method the background is a solution of 90% acetone.

3.4 Stock Standard Solution (SSS) -- A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

3.5 Primary Dilution Standard Solution (PDS) -- A solution of the analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.6 Calibration Standard (CAL) -- A solution prepared from the primary dilution standard solution or stock standard solutions containing the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

3.7 Response Factor (RF) -- The ratio of the response of the instrument to a known amount of analyte.

3.8 Laboratory Reagent Blank (LRB) -- An aliquot of reagent water or other blank matrices that are treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or apparatus.

3.9 Field Duplicates (FD1 and FD2) -- Two separate samples collected at the same time and place under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analyses of FD1 and FD2 give a measure of the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.10 Quality Control Sample (QCS) -- A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. Ideally, the QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

3.11 Material Safety Data Sheet (MSDS) -- Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire, and reactivity data including storage, spill, and handling precautions.

4.0 Interferences

4.1 Any substance extracted from the filter or acquired from laboratory contamination that fluoresces in the red region of the spectrum may interfere in the accurate measurement of both chlorophyll *a* and pheophytin *a*.

4.2 The relative amounts of chlorophyll *a*, *b* and *c* vary with the taxonomic composition of the phytoplankton. Chlorophylls *b* and *c* may significantly interfere with chlorophyll *a* measurements depending on the amount present. Due to the spectral overlap of chlorophyll *b* with pheophytin *a* and chlorophyll *a*, underestimation of chlorophyll *a* occurs accompanied by overestimation of pheophytin *a* when chlorophyll *b* is present in the sample. The degree of interference depends upon the ratio of *a*:*b*. This laboratory found that at a ratio of 5:1, using the acidification procedure to correct for pheophytin *a*, chlorophyll *a* was underestimated by approximately 5%. Loftis and Carpenter[10] reported an underestimation of 16% when the *a*:*b* ratio was 2.5:1. A ratio of 1:1 is the highest ratio likely to occur in nature. They also reported overestimation of chlorophyll *a* in the presence of chlorophyll *c* of as much as 10% when the *a*:*c* ratio was 1:1 (the theoretical maximum likely to occur in nature). The presence of chlorophyll *c* also causes the under-

estimation of pheophytin *a*. The effect of chlorophyll *c* is not as severe as the effect of chlorophyll *b* on the measurement of chlorophyll *a* and pheophytin *a*. Knowledge of the taxonomy of the algae under consideration will aid in determining if the spectrophotometric method using trichromatic equations to determine chlorophyll *a*, *b*, and *c* or an HPLC method would be more appropriate.[11-16] In the presence of chlorophyll *b* or pheopigments, the modified fluorometric method described here is also appropriate.[5]

4.3 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.

4.4 Fluorescence is temperature dependent with higher sensitivity occurring at lower temperatures. Samples, standards, LRBs and QCSs must be at the same temperature to prevent errors and/or low precision. Analyses of samples at ambient temperature is recommended in this method. Ambient temperature should not fluctuate more than ± 3°C between calibrations or recalibration of the fluorometer will be necessary.

4.5 Samples must be clarified by centrifugation prior to analysis.

4.6 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 at -20°C or -70°C to prevent degradation.

5.0 Safety

5.1 The toxicity or carcinogenicity of the chemicals used in this method have not been fully established. Each chemical should be regarded as a potential health hazard and handled with caution and respect. Each laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method.[17-20] A file of MSDS should also be made available to all personnel involved in the chemical analysis.

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

6.0 Apparatus and Equipment

6.1 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). A Turner Designs Model 10 Series fluorometer was used in the evaluation of this method. The modified method requires excitation filter (436FS10) and emission filter (680FS10).

6.2 Centrifuge, capable of 675 g.

6.3 Tissue grinder, Teflon pestle (50 mm X 20 mm) with grooves in the tip with 1/4" stainless steel rod long enough to chuck onto a suitable drive motor and 30-mL capacity glass grinding tube.

6.4 Filters, glass fiber, 47-mm or 25-mm, nominal pore size of 0.7 µm unless otherwise justified by data quality objectives. Whatman GF/F filters were used in this work.

6.5 Petri dishes, plastic, 50 X 9-mm, or some other solid container for transporting and storing sampled filters.

6.6 Aluminum foil.

6.7 Laboratory tissues.

6.8 Tweezers or flat-tipped forceps.

6.9 Vacuum pump or source capable of maintaining a vacuum up to 6 in. Hg.

6.10 Room thermometer.

6.11 Labware -- All reusable labware (glass, polyethylene, Teflon, etc.) that comes in contact with chlorophyll solutions should be clean and acid free. An acceptable cleaning procedure is soaking for 4 h in laboratory grade detergent and water, rinsing with tap water, distilled deionized water and acetone.

6.11.1 Assorted Class A calibrated pipets.

6.11.2 Graduated cylinders, 500-mL and 1-L.

6.11.3 Volumetric flasks, Class A calibrated, 25-mL, 50-mL, 100-mL and 1-L capacity.

6.11.4 Glass rods.

6.11.5 Pasteur type pipets or medicine droppers.

6.11.6 Disposable glass cuvettes for the fluorometer.

6.11.7 Filtration apparatus consisting of 1 or 2-L filtration flask, 47-mm fritted glass disk base and a glass filter tower.

6.11.8 Centrifuge tubes, polypropylene or glass, 15-mL capacity with nonpigmented screw-caps.

6.11.9 Polyethylene squirt bottles.

7.0 Reagents and Standards

7.1 Acetone, HPLC grade, (CASRN 67-64-1).

7.2 Hydrochloric acid (HCl), concentrated (sp. gr. 1.19), (CASRN 7647-01-0).

7.3 Chlorophyll *a* free of chlorophyll *b*. May be obtained from a commercial supplier such as Sigma Chemical (St. Louis, MO). Turner Designs (Sunnyvale, CA) supplies ready-made standards.

7.4 **Water** -- ASTM Type I water (ASTM D1193) is required. Suitable water may be obtained by passing distilled water through a mixed bed of anion and cation exchange resins.

7.5 ***0.1 N HCl Solution*** -- Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 L.

7.6 ***Aqueous Acetone Solution*** -- 90% acetone /10% water. Carefully measure 100 mL of water into the 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.

7.7 ***Chlorophyll Stock Standard Solution*** (SSS) -- Chlorophyll *a* from a commercial supplier will be shipped in an amber glass ampoule which has been flame sealed. This dry standard should be stored at -20 or -70°C in the dark and the SSS prepared just prior to use. Tap the ampoule until all the dried chlorophyll is in the bottom of the ampoule. In subdued light, carefully break the tip off the ampoule. Transfer the entire contents of the ampoule into a 50-mL volumetric flask. Dilute to volume with 90% acetone, label the flask and wrap with aluminum foil to protect from light. The concentration of the solution must be determined spectrophotometrically using a multiwavelength spectrophotometer.[10] When stored in a light and airtight container at freezer temperatures, the SSS is stable for at least six months. The concentration of all dilutions of the SSS must be determined spectrophotometrically each time they are made.

7.8 ***Laboratory Reagent Blank*** (LRB) -- A blank filter which is extracted and analyzed just as a sample filter. The LRB should be the last filter extracted of a sample set. It is used to assess possible contamination of the reagents or apparatus.

7.9 ***Chlorophyll*** *a* ***Primary Dilution Standard Solution*** (PDS) -- Add 1 mL of the SSS (Sect. 7.8) to a clean 100-mL flask and dilute to volume with the aqueous acetone solution (Sect. 7.7). 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.

7.10 ***Quality Control Sample*** (QCS) -- Since there are no commercially available QCSs, dilutions of a stock standard of a different lot number from that used to prepare calibration solutions may be used.

8.0 Sample Collection, Preservation and Storage

8.1 ***Water Sample Collection*** -- Water may be obtained by a pump or grab sampler. Data quality objectives will determine the depth at which samples are taken. Healthy phytoplankton, however, are generally obtained from the photic zone (depth at which the illumination level is 1% of surface illumination). Enough water should be collected to concentrate phytoplankton on at least three filters so that precision can be assessed. Filtration volume size will depend on the particulate load of the water. Four liters may be required for open ocean water where phytoplankton density is usually low, whereas 1 L or less is generally sufficient for lake, bay or estuary water. All apparatus should be clean and acid-free. Filtering should be performed in subdued light as soon as possible after sampling since algal poulations, thus chlorophyll *a* concentration, can change in relatively short periods of time. Aboard ship filtration is highly recommended.

Assemble the filtration apparatus and attach the vacuum source with vacuum gauge and regulator. Vacuum filtration should not exceed 6 in. Hg (20 kPa). Higher

filtration pressures and excessively long filtration times (> 10 min) may damage cells and result in loss of chorophyll.

Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates (stir or invert several times). Pour the subsample into a graduated cylinder and accurately measure the volume. Pour the subsample into the filter tower of the filtration apparatus and apply a vacuum (not to exceed 20 kPa). A sufficient volume has been filtered when a visible green or brown color is apparent on the filter. Do not suck the filter dry with the vacuum; instead slowly release the vacuum as the final volume approaches the level of the filter and completely release the vacuum as the last bit of water is pulled through the filter. Remove the filter from the fritted base with tweezers, fold once with the particulate matter inside, lightly blot the filter with a tissue to remove excess moisture and place it in the petri dish or other suitable container. If the filter will not be immediately extracted, then wrap the container with aluminum foil to protect the phytoplankton from light and store the filter at -20 or -70°C. Short term storage (2 to 4 h) on ice is acceptable, but samples should be stored at -20 or -70°C as soon as possible.

8.2 ***Preservation*** -- Sampled filters should be stored frozen (-20°C or -70°C) in the dark until extraction.

8.3 ***Holding Time*** -- Filters can be stored frozen at -20 or -70°C for as long as 3½ weeks without significant loss of chlorophyll *a*.[21]

9.0 Quality Control

9.1 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 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 thus generated.

9.2 Initial Demonstration of Performance (Mandatory)

9.2.1 The initial demonstration of performance is used to characterize instrument performance (instrumental detection limits, linear dynamic range and MDLs) and laboratory performance (analyses of QCSs) prior to sample analyses.

9.2.2 Linear Dynamic Range (LDR) -- The LDR should be determined by analyzing a minimum of 5 calibration standards ranging in concentration from 0.2 µg/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 setting multiplier. 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 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. Should samples be encountered that have a concentration which is 90% of the upper limit of the LDR, these samples must be diluted and reanalyzed.

9.2.3 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.

9.2.4 Estimated Detection Limit (EDL) -- Several blank filters should be extracted according to the procedure in Sect. 11, using clean glassware and apparatus, and the fluorescence measured. A solution of pure chlorophyll *a* in 90% acetone should be serially diluted until it yields a response which is 3X the average response of the blank filters.

9.2.5 Quality Control Sample (QCS) -- When beginning to use this method, on a quarterly basis or as required to meet data quality needs, verify the calibration standards and acceptable instrument performance with the analysis of a QCS (Sect. 7.10). 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 continuing analyses.

9.2.6 Extraction Proficiency -- Personnel performing this method for the first time should demonstrate proficiency in the extraction of sampled filters (Sect. 11.1).

Twenty to thirty natural samples should be obtained using the procedure outlined in Sect. 8.1 of this method. Sets of 10 or more samples should be extracted and analyzed according to Sect. 11.2. The percent relative standard deviation (%RSD) of uncorrected values of chlorophyll *a* should not exceed 15% for samples that are approximately 10X the IDL. RSD for pheophytin *a* might typically range from 10 to 50%.

9.2.7 Corrected Chl *a* -- Multilaboratory testing of this method revealed that many analysts do not adequately mix the acidified sample when determining corrected chl *a*. The problem manifests itself by highly erratic pheo-*a* results, high %RSDs for corrected chl *a* and poor agreement between corrected and uncorrected chl *a*. To determine if a new analyst is performing the acidification step properly, perform the following QC procedure:

Prepare 100 mL of a 50 ppb chl *a* solution in 90% acetone. The new analyst should analyze 5-10 separate aliquots, using separate cuvettes, according to instructions in Section 11.2. Process the results according to Section 12 and calculate separate means and %RSDs for corrected and uncorrected chl *a*. If the means differ by more than 10%, then the stock chl *a* has probably degraded and fresh stock should be prepared. The %RSD for corrected chl *a* should not exceed 5%. If the %RSD exceeds 5%, repeat the procedure until the %RSD 5%.

9.3 Assessing Laboratory Performance (Mandatory)

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

10.0 Calibration and Standardization

10.1 ***Calibration*** -- Calibration should be performed bimonthly or when there has been an adjustment made to the instrument, such as replacement of lamp, filters or photomultiplier. Prepare 0.2, 2, 5, 20 and 200 µg chl *a*/L calibration standards from the PDS (Sect. 7.11). Allow the instrument to warm up for at least 15 min. 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 chl *a* determination, **DO NOT** acidify. Use the "uncorrected" chl *a* calculation described in Section 12.1.

If pheophytin *a* determinations will be made, it will be necessary to obtain before-to-after acidification response ratios of the chlorophyll *a* calibration standards as follows: (1) measure the fluorescence of the standard, (2) remove the cuvette from the fluorometer, (3) acidify the solution to .003 N HCl[6] with the 0.1 N HCl solution, (4) use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample, (5) wait 90 sec and measure the fluorescence of the standard solution again. Addition of the acid may be made using a medicine dropper. It will be necessary to know how many drops are equal to 1 mL of acid. For a cuvette that holds 5 mL of extraction solution, it will be necessary to add 0.15 mL of 0.1 N HCl to reach a final acid concentration of 0.003N in the 5 mL. Calculate the ratio, r, as follows:

$$r = R_b/R_a$$

where:

R_b = fluorescence of pure chlorophyll *a* standard solution before acidification.

R_a = fluorescence of pure chlorophyll *a* standard solution after acidification.

11.0 Procedure

11.1 Extraction of Filter Samples

11.1.1 If sampled filters have been 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 minimum that is necessary to read instructions and operate instrumentation. Remove a filter from its container and place it in the glass grinding tube. The filter may be torn into smaller pieces to facilitate extraction. Push it to the bottom of the tube with a glass rod. With a volumetric pipet, add 4 mL of the aqueous acetone solution (Sect. 7.6) to the grinding tube. Grind the filter until it has been converted to a slurry. (**NOTE:** Although grinding is required, care must be taken not to overheat the sample. Good judgement and common sense will help you in deciding when the sample has been sufficiently macerated.) Pour the slurry into a 15-mL screw-cap centrifuge tube and, using a 6-mL volumetric pipet, rinse the pestle and the grinding tube with 90% acetone. Add the rinse to the centrifuge tube containing the filter slurry. Cap the tube and shake it vigorously. Place it in the dark before proceeding to the next filter extraction. Before placing another filter in the grinding tube, use the acetone and water squirt bottles to thoroughly rinse the pestle, grinding tube and glass rod. The last rinse should be with acetone. Use a clean tissue to remove any filter residue that adheres to the pestle or to the steel rod of the pestle. Proceed to the next filter and repeat the steps above. The entire extraction with transferring and rinsing steps takes 5 min. Approximately 500 mL of acetone and water waste are generated per 20 samples from the rinsing of glassware and apparatus.

11.1.2 Shake each tube vigorously before placing them to steep in the dark at 4°C. Samples should be allowed to steep for a minimum of 2 h but not to exceed 24 h. The tubes should be shaken at least once during the steeping period.

11.1.3 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. This can be done by placing the tubes in a constant temperature water bath or by letting them stand at room temperature for 30 min. Recalibrate the fluorometer if the room temperature fluctuated ± 3°C from the last calibration date.

11.2 SAMPLE ANALYSIS

11.2.1 After the fluorometer has warmed up for at least 15 min, use the 90% acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.

11.2.2 Pour or pipet the supernatant of the extracted sample into a sample cuvette. The volume of sample required in your instrument's cuvette should be known so that the correct amount of acid can be added in the pheophytin *a* determinative step. For a cuvette that holds 5 mL of extraction solution, 0.15 mL of the 0.1 N HCl solution should be used. Choose a sensitivity setting that yields a midscale reading when possible and avoid the minimum sensitivity setting. If the concentration of chlorophyll *a* in the sample is ≥ 90% of the upper limit of the LDR, then dilute the sample with the 90% acetone solution and reanalyze. Record the fluorescence measurement and sensitivity setting used for the sample. Remove the cuvette from the fluorometer and acidify the extract to a final concentration of 0.003 N HCl using the 0.1 N HCl solution. Use a pasteur type pipet to thoroughly mix the sample by aspirating and dispensing the sample into the cuvette, keeping the pipet tip below the surface of the liquid to avoid aerating the sample. Wait 90 sec before measuring fluorescence again. NOTE: Proper mixing is critical for precise and accurate results. Twenty-five to thirty-five samples can be extracted and analyzed in one 8 hr day.

NOTE: If you are using special narrow bandpass filters for chl *a* determination, **DO NOT** acidify samples. Use the "uncorrected" chl *a* calculations described in Section 12.1.

12.0 Data Analysis and Calculations

12.1 For "uncorrected chlorophyll *a*," calculate the chlorophyll *a* concentration in the extract as:

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

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

R_b = fluoresence response of sample extract before acidification, and

F_s = fluoresence respnse factor for sensitivity setting S.

Calculate the “uncorrected” concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{S,u} = \frac{C_{E,u} \ x \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

where $C_{S,u}$ **=** uncorrected 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.

12.2 For “corrected chlorophyll *a*”, calculate the chlorophyll *a* concentration in the extract as :

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

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 (Sect. 10.1),

R_b = fluorescence of sample extract before acidification, and

R_a = fluorescence of sample extract after acidification.

Calculate the “corrected” concentration of chlorophyll *a* in the whole water sample as follows:

$$C_{s,c} = \frac{C_{E,u} \ x \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

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

extract volume = volume (L) of extract prepared before dilution,

12.3 Calculate the pheophytin *a* concentration as follows:

$P_E = F_s \ (r/r\text{-}1) \ (rR_a - R_b)$

$$P_s = \frac{P_E \ X \ extract \ volume \ (L) \ X \ DF}{sample \ volume \ (L)}$$

where P_E = pheophytin *a* concentration (µg/L) in the sample extract; and

P_s = pheophytin *a* concentration (µg/L) in the whole water sample.

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

13.0 Method Performance

13.1 The single lab EDL for the instrument used in the evaluation of this method was 0.05 µg/L for chlorophyll *a* and 0.06 µg/L pheophytin *a*.

13.2 The precision (%RSD) for chlorophyll *a* in mostly blue-green and green phytoplankton natural samples which were steeped for 2 h vs 24 h is reported in Table 1. Although the means were the same, precision was better for samples which were allowed to steep for 24 h prior to analysis. Since pheophytin *a* was found in the samples, the chlorophyll *a* values are "corrected" (Sect. 12.2). Table 2 contains precision data for pheophytin *a*. A statistical analysis of the pheophytin *a* data indicated a significant difference in the mean values at the 0.05 significance level. The cause of the lower pheophytin *a* values in samples extracted for 24 h is not known.

13.3 Three QCS ampoules obtained from the USEPA were analyzed and compared to the reported confidence limits in Table 3. **NOTE:** The USEPA no longer provides these QCSs.

13.4 **Multilaboratory Testing** - A multilaboratory validation and comparison study of EPA Methods 445.0, 446.0 and 447.0 for chlorophyll *a* was conducted in 1996 by Research Triangle Institute, Research Triangle Park, N.C. (EPA Contract No. 68-C5-0011). There were 21 volunteer participants in the fluorometric methods

component that returned data; 10 that used the modified fluorometric method and 11 that used the conventional method. The primary goals of the study were to determine estimated detection limits and to assess precision (%RSD) and bias (as percent recovery) for select unialgal species, and natural seawater.

13.4.1 The term, pooled estimated detection limit (p-EDL), is used in this method to distinguish it from the EPA defined method detection limit (MDL). An EPA MDL determination is not possible nor practical for a natural water or pure species sample due to known spectral interferences and to the fact that it is impossible to prepare solutions of known concentrations that incorporate all sources of error (sample collection, filtration, processing). The statistical approach used to determine the p-EDL was an adaptation of the Clayton, et.al.[22] method that does not assume constant error variances across concentration and controls for Type II error. The statistical approach used involved calculating an estimated DL for each lab that had the desired Type I and Type II error rates (0.01 and 0.05, respectively). The median DLs over labs was then determined and is reported in Table 4. It is referred to as pooled-EDL (p-EDL).

Solutions of pure chlorophyll *a* in 90% acetone were prepared at three concentrations (0.11, 0.2 and 1.6 ppm) and shipped with blank glass fiber filters to participating laboratories. Analysts were instructed to spike the filters in duplicate with a given volume of solution and to process the spiked filters according to the method. The results from these data were used to determine a p-EDL for each method. Results (in ppm) are given in Table 4. The standard fluorometric and HPLC methods gave the lowest p-EDLs while the spectrophotometric (monochromatic equations) gave the highest p-EDLs. Due to the large dilutions required to analyze these solutions, the fluorometric p-EDLs are unrealistically high compared to what is achievable by a single lab. Typical single lab EDLs can easily be 1000 fold lower than the p-EDL reported in Table 4.

13.4.2 To address precision and bias in chlorophyll *a* determination for different algal species, three pure unialgal cultures (Amphidinium, Dunaliella and Phaeodactylum) were cultured and grown in the laboratory. Four different “concentrations” of each species were prepared by filtering varying volumes of the algae. The filters were frozen and shipped to participant labs. Analysts were instructed to extract and analyze the filters according to the respective methods. The “true” concentration was assigned by taking the average of the HPLC results for the highest concentration algae sample since chlorophyll *a* is separated from other interfering pigments prior to determination. Pooled precision (as determined by %RSD) data are presented in Tables 5-7 and accuracy data (as percent recovery) are presented in Table 8. No significant differences in precision were observed across concentrations for any of the species. It should be noted that there was considerable lab-to-lab variation (as exhibited by the min and max recoveries in Table 8) and in this case the median is a better measure of central tendency than the mean.

In summary, the mean and median concentrations determined for Amphidinium carterae (class dinophyceae) are similar for all methods. No method consistently exhibited high or low values relative to the other methods. The only concentration trend observed was that the spectrophotometric method-trichromatic equations (SP-T) showed a slight percent increase in recovery with increasing algae filtration volume.

For Dunaliella tertiolecti (class chlorophyceae) and Phaeodactylum tricornutum (class bacillariophyceae) there was generally good agreement between the fluorometric and the spectrophotometric methods, however, the HPLC method yielded lower recoveries with increasing algae filtration volume for both species. No definitive explanation can be offered at this time for this phenomenon. A possible explanation for the Phaeodactylum is that it contained significant amounts of chlorophyllide *a* which is determined as chlorophyll *a* in the fluorometric and spectrophotometric methods. The conventional fluorometric method (FL-STD) showed a slight decrease in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The spectrophotometric-trichromatic equations (SP-T) showed a slight increase in chlorophyll *a* recovery with increasing Dunaliella filtration volume. The fluorometric and the spectrophotometric methods both showed a slight decrease in chlorophyll *a* recovery with increasing Phaeodactylum filtration volume.

Results for the natural seawater sample are presented in Table 9. Only one filtration volume (100 mL) was provided in duplicate to participant labs.

14.0 Pollution Prevention

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of

environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation (e.g., Sect. 11.1.1). When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

14.2 For information about pollution prevention that may be applicable to laboratories and research institutions, consult *Less is Better: Laboratory Chemical Management for Waste Reduction*, available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington D.C. 20036, (202)872-4477.

15.0 Waste Management

15.1 The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management consult *The Waste Management Manual for Laboratory Personnel*, available from the American Chemical Society at the address listed in the Sect. 14.2.

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17.0 TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. COMPARISON OF PRECISION OF TWO EXTRACTION PERIODS

CORRECTED CHLOROPHYLL *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	49.6	52.9	78.6	78.8
Standard Deviation (µg/L)	4.89	2.64	6.21	2.77
Relative Standard Deviation (%)	9.9	5.0	7.9	3.5

1 Values reported are the mean measured concentrations (n=6) of chlorophyll *a* in the natural water based on a 100-mL filtration volume.

2 Values reported are the mean measured concentrations (n=9) of the extraction solution. Sample filtration volume was 300 mL.

3 The length of time that the filters steeped after they were macerated.

TABLE 2. COMPARISON OF PRECISION OF TWO EXTRACTIONS PERIODS FOR Pheophytin *a*

Pheophytin *a*

	Sample A[1]		Sample B[2]	
	2 h[3]	24 h[3]	2 h[3]	24 h[3]
Mean Concentration (µg/L)	9.22	8.19	13.1	10.61
Standard Deviation (µg/L)	2.36	3.55	3.86	2.29
Relative Standard Deviation (%)	25.6	43.2	29.5	21.6

[1] Values reported are the mean measured concentrations (n=6) of pheophytin *a* in the natural water based on a 100-mL filtration volume.

[2] Values reported are the mean measured concentrations (n=9) of pheophytin *a* the extraction solution. Sample filtration volume was 300 mL.

[3] The length of time that the filters steeped after they were macerated.

TABLE 3. ANALYSES OF USEPA QC SAMPLES

ANALYTE	REFERENCE VALUE	CONFIDENCE LIMITS
Chlorophyll *a*	2.1 µg/L	0.5 to 3.7 µg/L
Pheophytin *a*	0.3 µg/L	-0.2 to 0.8 µg/L

ANALYTE	MEAN MEASURED VALUE	% Relative Standard Deviation[1]
Chlorophyll *a*	2.8 µg/L	1.5
Pheophytin *a*	0.3 µg/L	33

[1] N = 3

TABLE 4. POOLED ESTIMATED DETECTION LIMITS FOR CHLOROPHYLL *A* METHODS[1]

Method[2]	N[3]	p-EDL[4] (mg/L)
FL -Mod[5]	8	0.096
FL - Std[5]	9	0.082
HPLC	4	0.081
SP-M	15	0.229
SP-T	15	0.104

(1) See Section 13.4.1 for a description of the statistical approach used to determine p-EDLs.

(2) FL-Mod = fluorometric method using special interference filters.

FL-Std = conventional fluorometric method with pheophytin *a* correction.

HPLC = EPA method 447.0

SP-M = EPA method 446.0, monochromatic equation.

SP-T = EPA method 446.0, trichromatic equations.

(3) N = number of labs whose data was used.

(4) The p-EDL was determined with p = 0.01 and q (type II error rate) = 0.05.

(5) Due to the large dilutions required to analyze the solutions by fluorometry, the fluormetric p-EDLs are unrealistically high.

TABLE 5. POOLED PRECISION FOR DUNALIELLA TERTIOLECTI SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.163	0.037	22.8
	10	7	0.298	0.080	26.7
	50	7	1.684	0.385	22.9
	100	7	3.311	0.656	19.8
Fl-Std	5	8	0.185	0.056	30.4
	10	8	0.341	0.083	24.4
	50	8	1.560	0.311	19.9
	100	8	3.171	0.662	20.9

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 6. POOLED PRECISION FOR AMPHIDINIUM CARTERAE SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.066	0.010	14.6
	10	7	0.142	0.045	31.5
	50	7	0.757	0.208	27.5
	100	7	1.381	0.347	25.1
Fl-Std	5	8	0.076	0.018	23.2
	10	8	0.165	0.040	24.3
	50	8	0.796	0.140	17.5
	100	8	1.508	0.324	21.5

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

TABLE 7. POOLED PRECISION FOR PHAEODACTYLUM TRICORNUTUM SAMPLES

Method[1]	mLs of culture filtered	N[2]	Mean (mg chl*a*/L)	Std. Dev.	%RSD
Fl-Mod	5	7	0.221	0.040	18.0
	10	7	0.462	0.094	20.3
	50	7	2.108	0.491	23.3
	100	7	3.568	1.186	33.2
Fl-Std	5	8	0.214	0.053	24.8
	10	8	0.493	0.091	18.4
	50	8	2.251	0.635	28.2
	100	8	4.173	0.929	22.3

(1) Fl-Mod = fluorometric method using special interference filters.

Fl-Std = conventional fluorometric method with pheophytin *a* correction.

(2) N = number of volunteer labs whose data was used.

NOTE: The phaeodactylum extract contained significant amounts of chlorophyll *c* and chlorophyllide *a* which interferes in chlorophyll *a* measurement in the fluorometric method, therefore, the concentration of chlorophyll *a* is overestimated compared to the HPLC method which separates the three pigments. The FL-Mod interference filters minimize this interference more so than the conventional filters.

TABLE 8. MINIMUM, MEDIAN, AND MAXIMUM PERCENT RECOVERIES BY GENERA, METHOD, AND CONCENTRATION LEVEL

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
Amphidinium	Minimum	FL-MOD	70	73	75	76
		FL-STD	66	91	91	90
		HPLC	82	85	87	88
		SP-M	36	48	68	64
		SP-T	21	63	71	70
	Median	FL-MOD	105	112	105	104
		FL-STD	109	107	111	109
		HPLC	102	106	112	105
		SP-M	99	101	101	101
		SP-T	95	96	106	107
	Maximum	FL-MOD	121	126	143	146
		FL-STD	156	154	148	148
		HPLC	284	210	131	116
		SP-M	141	133	126	125
		SP-T	115	116	119	117
Dunaliella	Minimum	FL-MOD	162	159	157	156
		FL-STD	179	171	165	164
		HPLC	165	109	64	41
		SP-M	120	188	167	164
		SP-T	167	169	166	165
	Median	FL-MOD	206	246	227	223
		FL-STD	250	228	224	210
		HPLC	252	177	89	80

Table 8 cont'd

Species	Statistic	Method	Percent Recovery			
			Conc. Level 1	Conc. Level 2	Conc. Level 3	Conc. Level 4
		SP-M	240	247	247	243
		SP-T	225	244	256	256
Dunaliella	Maximum	FL-MOD	295	277	287	288
		FL-STD	439	385	276	261
		HPLC	392	273	172	154
		SP-M	342	316	296	293
		SP-T	291	283	283	283
Phaeodactylum	Minimum	FL-MOD	216	183	157	154
		FL-STD	189	220	223	219
		HPLC	150	119	84	75
		SP-M	161	138	156	160
		SP-T	203	195	216	244
	Median	FL-MOD	292	285	250	245
		FL-STD	296	263	254	254
		HPLC	225	203	114	90
		SP-M	287	274	254	253
		SP-T	286	281	277	274
	Maximum	FL-MOD	357	337	320	318
		FL-STD	371	415	415	334
		HPLC	394	289	182	139
		SP-M	446	344	330	328
		SP-T	357	316	318	299

TABLE 9. CHLOROPHYLL *A* CONCENTRATIONS IN MG/L DETERMINED IN FILTERED SEAWATER SAMPLES

Method	Con.[1]	No. Obs.	No. Labs	Mean	Std. Dev.	RSD(%)	Minimum	Median	Maxium
FL-MOD	100	14	7	1.418	0.425	30.0	0.675	1.455	2.060
FL-STD	100	15	8	1.576	0.237	15.0	1.151	1.541	1.977
HPLC	100	10	5	1.384	0.213	15.4	1.080	1.410	1.680
SP-M	100	38	19	1.499	0.219	14.6	0.945	1.533	1.922
SP-T	100	36	18	1.636	0.160	9.8	1.250	1.650	1.948
All Methods	100	113	57	1.533	0.251	16.4	0.657	1.579	2.060

(1) Con = mLs of seawater filtered.

APPENDIX L

Van Heukelem and Thomas, 2001

Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments

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Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments

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Abstract

We used chromatography modeling software to assist in HPLC method development, with the goal of enhancing separations through the exclusive use of gradient time and column temperature. We surveyed nine stationary phases for their utility in pigment purification and natural sample analysis. For purification, a complex algal matrix was separated on an efficient monomeric column, from which partially purified fractions were collected and purified on polymeric columns that exaggerated resolution between pigments of interest. Additionally, we feature an HPLC method that is simple, fast, demonstrates excellent transferability and is ideal for quantitative analysis of pigments in dilute natural water samples.
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Keywords: Chromatography modeling software; Stationary phases, LC; Phytoplankton pigments; Carotenoids; Chlorophylls

1. Introduction

HPLC methods for the analysis of phytoplankton pigments are used to help assign algal species to phylogenetic divisions, assess phytoplankton abundance and diversity in natural samples [1] and provide information on photosynthetic and photoprotective physiology [2]. However, no one method can resolve all pigments important to these purposes as there are many pigments to separate and as pigment separations are improved, new pigments are often identified. Several approaches have been used to improve pigment separations as recently reviewed by Jeffrey et al. [1]. These approaches have included the use of ternary solvent systems (using three pumps) [3,4], C_{18} columns [3–16] and C_8 columns [17–20] and manipulations to column temperature (T) [9,13–16,20]. The C_{30} stationary phase has been useful in the analysis of pigments in food and tissues as recently reviewed [21] and has had limited use thus far in the analysis of phytoplankton pigments [22,23]. A very promising new method which utilizes a pyridine-containing mobile phase and a C_8 column separates many pigments not previously resolved and has been used to identify the elution position of many new pigments [20]. We used an alternative approach to improve separations: the combined use of column T and gradient time (t_G), in conjunction with a simple binary solvent system. Our approach was enabled by method development software.

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The software selected (DryLab, from LC Resources, Walnut Creek, CA, USA) has been successfully used [24] to optimize separations of 14 different sample types, including the separation of 29 algal pigments. To use this software, retention times (t_R) are recorded for a suite of compounds analyzed on the same HPLC column under four chromatographic conditions, including two values each of t_G and T. From the observed t_R values (referred to as input data), the software predicts the t_R values which would result from hypothetical chromatographic conditions selected by the user (including such things as t_G, gradient shape, column T, column dimensions and flow-rate (F)). The conditions required for optimal separations can then be easily identified.

Our ultimate goals in using this software were to identify methods which would enhance our abilities to isolate pigments as standards from natural sources and to identify a method that would simultaneously separate as many important pigments as possible and be suitable for the routine quantitative analysis of pigments in natural water samples. Additional requirements of this latter method were that it be based on a simple methanol-based binary solvent system, use linear gradients only, have a short analysis time and excellent detectability and be easily transferred to other instruments. We collected pigment t_R data from nine different columns for use with the simulation software. We used changes to values of T and t_G for optimizing conditions as we wanted to see how effectively we could improve separations based exclusively on these two variables.

We identify columns and methods well suited to the isolation of pigments from algal monocultures for use as pigment standards and we introduce a new method where we describe the elution position of 57 algal pigments.

2. Experimental

2.1. Equipment and software

A Hewlett-Packard (HP, Waldbronn, Germany) series 1100 HPLC system with autoinjector (900 μl syringe head), refrigerated autosampler compartment, thermostatted column compartment, quaternary pump with in-line vacuum degasser, and photo-diode array detector set to monitor 450 and 665 nm (both with 20 nm bandwidths) was used for primary studies. In-line photo-diode array spectra (from 350 to 700 nm) was collected for each pigment (Table 1) and compared with published values [20,25] for pigment identifications. Dwell volume was 3 ml. A Beckman (Beckman Coulter, Fullerton, CA, USA) System Gold HPLC was equipped with a dual pump (125 solvent module), photo-diode array detector set to monitor 450 nm, manual injector (200 μl loop), and an external column heating device (Eldex Laboratories, Napa, CA, USA). Dwell volume was 2.2 ml.

Chromatography modeling software (DryLab, version 2.05, LC Resources, Walnut Creek, CA, USA) was donated for use. This software requires (for predictions based on simultaneous variation of column T and t_G) retention times gathered from four sets of conditions, referred to as the input data (Table 2, further details in Section 3.1). We used t_R values for pigments in various algal culture and mutant corn leaf extracts for input data. After developing suitable methods for pigment isolation, we isolated pigments for use as standards and then recombined them into one standard test mixture containing 33 pigments. This mixture was used in subsequent testing.

After the chromatography modeling software was used to generate hypothetical conditions for the best separation, those conditions were implemented on the HPLC. For these analyses, an injector program was used which mixed sample with buffer (28 m*M* tetrabutyl ammonium acetate (TBAA), pH 6.5) in the sample loop before injection. With the Beckman Gold HPLC and when using large injection volumes (900 μl) on the HP HPLC (during pigment isolations), sample was premixed by hand with buffer and then allowed to equilibrate for 5 min before injecting. All samples were equilibrated to the autosampler compartment temperature (5°C) of the HP HPLC before injecting.

2.2. Materials

2.2.1. Reagents and solvents

All reagents except ethanol (JT Baker, Phillipsburg, NJ, USA) were HPLC grade: methanol (EM Science, Gibbstown, NJ, USA or JT Baker), acetone

Table 1
Pigments, SCOR abbreviations used in the text, code (number or letter) used to identify elution position of pigments in Figs. 2–9 and the pigment sources used to document retention time (t_R) values are shown[a]

Code		Pigment name	SCOR [26] WG 78 abbreviation	Source[b]	t_R (min)	R_s (peak code)	Visible absorbance spectra (nm)
1	*	Chlorophyll *c*3	chl *c*3	C J L N S T	3.88		456, 588 (625)
2		Monovinyl chl *c*3	MV chl *c*3	J	4.14		448, 585 (626)
3	*	Chlorophyll *c*2	chl *c*2	A–E H J L–O R–T	5.70	NR 3/4	446, 584, 634
4	*	Mg 3,8-divinyl pheoporphyrin a_5 monomethyl ester	Mg DVP	G P	5.81	NR 4/a	440, 576, 632
a		Unknown	–	K	5.92	NR a/5	–
5	*	Chlorophyll *c*1	chl *c*1	B D E R	6.05	NR 5/6	442, 580, 634, 668
6		Chlorophyllide *a*	chlide *a*	F H N O	6.06		(390), 434, 620, 668
7	**	Peridinin	perid	A B M	9.32		476
8		Peridinin isomer	–	A B M	9.58		478
b		Unknown	–	P	11.37		456, 476
9	**	19′-Butanoyloxy fucoxanthin	but-fuco	VKI[c] C N S T	12.31		448, 464
c		Unknown	–	G	12.68	NR c/10	458
10	**	Fucoxanthin	fuco	C D E L N R S T	12.63		454
11	**	Neoxanthin	neo	F G P U	13.29	NR 11/12	414, 438, 466
12		4-Keto-19′-hexanoyloxyfucoxanthin	4 k-hex-fuco	J	13.31		448, 470
d		Unknown	–	L	13.73	NR d/13	446, 468
13	**	Prasinoxanthin	pras	VKI[c] G P	13.74		462
14	**	Violaxanthin	viola	VKI[c] F G P Q U	13.99	R_s = 1.3, 14/15	418, 442, 470
15	**	19′-Hexanoyloxy fucoxanthin	hex-fuco	VKI[c] C J L	14.16		(430), 452, 480
16	*	Astaxanthin	asta	shrimp carapace G	14.53		480
e		Unknown	–	P	14.78		466
17		Diadinochrome	diadchr	M	15.02	NR 17/f	(410), 428, 456
f		Unknown	–	J	15.09	NR f/18	448, 470
18		Unknown (myxo-like spectra)	–	K	15.13	NR 18/19	452, 474, 506
19	*	Diadinoxanthin	diadino	A–E J L–N R–T	15.23	R_s = 1.4, 19/20	(428), 446, 476
20		Dinoxanthin	dino	A M	15.49		416, 440, 470
21	*	Antheraxanthin	anth	F	15.99		(425), 446, 474
22	**	Alloxanthin	allo	H O	16.53		(430), 452, 480
23	*	Diatoxanthin	diato	C D E M R T	17.12	NR 23/24	(430), 454, 480
24		Monadoxanthin	monado	H O	17.22		(422), 444, 472
25	**	Zeaxanthin	zea	C F G I K P Q V	17.79		(430), 452, 478
26	**	Lutein	lut	F G T V	17.98		424, 446, 474
g		Unknown	–	Q	18.24	NR g/h	422, 444, 472
h		Unknown	–	G	18.32		(408), 428, 454
i		Unknown	–	L N S T	18.84		(424), 448, 472
27	**	Canthaxanthin	cantha	W	19.07		480
j		Unknown	–	Q	19.23		422, 444, 472
28	*	Gyroxanthin diester-like 1 [27]	–	C	19.94		(426), 444, 472
29	*	Gyroxanthin diester-like 2 [27]	–	C	21.00		(426), 444, 472
30	*	Divinyl chlorophyll *b*	DV chl *b*	U	21.92	R_s = 0.8, 30/31	478, 608, 654
31	**	Monovinyl chlorophyll *b*	chl *b*	Fluka[d] F G P	22.03		468, 602, 652
32		Divinyl chlorophyll *b*′	DV chl *b*′	U	22.29		480, 608, 658
33		Crocoxanthin	croco	H O	22.42	NR 33/34	(428), 446, 476
34		Monovinyl chlorophyll *b*′	chl *b*′	Fluka[d] F G P	22.50		470, 602, 652
35		Chlorophyll *a* allomer 1	chl *a* allom	Fluka[d] A–T	23.30		(390), 432, 620, 666
36		Chlorophyll *a* allomer 2	chl *a* allom	Fluka[d] A–T	23.43	NR 36/k	(390), 432, 620, 666
k		Unknown	–	R	23.52	NR k/L	(464–474)
L		Unknown	–	R	23.52	NR L/37	454

Table 1. Continued

Code		Pigment name	SCOR [26] WG 78 abbreviation	Source[b]	t_R (min)	R_s (peak code)	Visible absorbance spectra (nm)
37		Phytylated chlorophyll *c*-like	phytyl-chl *c*	J R	23.53		460, 588, 636
38	**	Divinyl chlorophyll *a*	DV chl *a*	U	23.76		(390), 442, 622, 666
m		Unknown	–	L	23.91	NR m/39	458, 588, 638
39	**	Monovinyl chlorophyll *a*	chl *a*	Fluka[d] A–T	23.96		(390), 432, 620, 666
40		Divinyl chlorophyll *a′*	DV chl *a′*	U	24.13		(386), 440, 622, 666
41		Monovinyl chlorophyll *a′*	chl *a′*	Fluka[d] A–T	24.33		(388), 432, 618, 666
n		Unknown	–	P	25.58		(422), 442, 470
42	*	β,ϵ-Carotene (α-carotene)[e]	β,ϵ-car	Sigma[f] G H J L O P S	26.65	NR 42/43	(422), 446, 474
43	*	β,β-Carotene (β-carotene)[d]	β,β-car	Fluka[d], A–G, I–N P–U	26.71		(430), 452, 476

[a] Pigments with * or ** were used with all columns (t_R and R_s data given are specific to the method described in Section 3.6). R_s is ≥ 1.5 unless otherwise noted (NR $= R_s < 1.0$). Visible absorbance spectra was obtained from the in-line HPLC photo-diode array detector (350–700 nm) (solvent A, 70:30 methanol, 28 m*M* TBAA, pH 6.5; solvent B, methanol). Parentheses indicate spectral shoulder.

[b] Sources: Horn Point batch cultures, (A) *Prorocentrum minimum*, (B) *Gyrodinium uncatenum*, (C) *Gyrodinium galatheanum*, (D) *Thalassiosira pseudonana*, (E) *Isochrysis* sp. (TISO strain, CCMP 1324), (F) *Dunaliella tertiolecta* (CCMP 1320), (G) *Pycnococcus provasolii* (CCMP 1203), (H) *Pyrenomonas salina*, (I) *Synecoccocus sp.*; CCMP individual cultures, (J) *Emiliania huxleyi* (CCMP 373), (K) *Synechococcus* cf. *elongatus* (CCMP 1629), (L) *Chrysochromulina polyepsis* (CCMP 1757), (M) *Amphidinium carterae* (CCMP 1314), (N) *Pelagococcus subviridis* (CCMP 1429), (O) *Guillardia theta* (CCMP 327), (P) *Micromonas pusilla* (CCMP 1545), (Q) *Nannochoropsis* sp. 1 (CCMP 531), (R) *Isochrysis galbana* (CCMP 1323); all other sources, (S) *Pelagamonas calceolata*, (T) *Aureococcus anophagefferens*, (U) mutant corn, (V) marigold petals, (W) gift from Perdue, Salisbury, MD, USA.

[c] VKI Water Quality Institute, Denmark.

[d] Fluka (Milwaukee, WI, USA).

[e] Trivial name.

[f] Sigma (St. Louis, MO, USA).

(EM Science), ammonium acetate (JT Baker) and 0.4 *M* tetrabutyl ammonium hydroxide titrant (JT Baker). Water was deionized and filtered. Solvent A was 70:30 (v/v) methanol, 28 m*M* aqueous TBAA, pH 6.5. Solvent B was methanol or ethanol (Table 2).

Table 2
Chromatographic conditions used for generating pigment t_R databases for use with DryLab chromatography modeling software[a]

Column	Column dimensions (mm)	Column temperatures (°C)	Gradient times (min)	Initial %B	Flow rate (ml/min)
C_8 columns					
Hypersil mos-2	100×4.6	40, 60	15, 45	5	1.0
Luna C_8 (2)	100×4.6	40, 60	15, 45	5	0.8
Eclipse XDB	150×4.6	45, 60	20, 60	0	1.0
C_{18} columns					
Supelcosil LC 318	250×4.6	45, 60	20, 60	5	1.0
Supelcosil LC PAH	100×4.6	45, 60	15, 45	5	1.0
Vydac 201TP	250×3.2	45, 60	20, 60	0	0.6
YMC ODS-AL	150×4.6	40, 60	20, 60	5	1.0
Zorbax Bonus-RP C_{14}	250×4.6	45, 60	20, 60	0	1.2
YMC C_{30}	250×4.6	40, 60	20, 60	20	1.0

[a] Pigments were analyzed on each column at two values of *T* and two values of t_G. All gradients were linear from the specified initial percent solvent B to 100% solvent B. Solvent A, 70:30 methanol, 28 m*M* TBAA, pH 6.5; solvent B, methanol, except for the YMC C_{30} column where it was ethanol. Flow-rates were adjusted to keep backpressure below 180 bar.

2.2.2. Columns

Ten different reversed-phase, silica-based columns were used in this study. Aliphatic chain length and physical characteristics varied (Table 3). The Supelcosil LC318 C_{18} was used in two dimensions: 250×4.6 mm for computer modeling work and 100×4.6 mm for pigment isolations.

2.2.3. Algal cultures

Algal cultures with well characterized pigment content were used for the isolation of pigment standards and for collecting input data. They were (their clonal designation is indicated when known): (1) *Dunaliella tertiolecta* (CCMP 1320), (2) *Pycnococcus provasolii* (CCMP 1203), (3) *Synechococcus* sp., (4) *Isochrysis* sp. (T.ISO strain, CCMP 1324), (5) *Gyrodinium uncatenum*, (6) *Prorocentrum minimum*, (7) *Thalassiosira pseudonana*, (8) *Pyrenomonas salina* and (9) *Gyrodinium galatheanum*. These cultures were grown in large batches at Horn Point Laboratory, harvested onto glass fiber filters and frozen at −75°C until used. Additional cultures used with the new method on the Eclipse XDB C_8 column were grown at Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), harvested onto GF/F filters, frozen immediately in liquid nitrogen and shipped to Horn Point Laboratory.

2.2.4. Pigment standards

The Scientific Committee on Oceanic Research (SCOR) abbreviations will be used throughout when referring to pigments (Table 1). We purchased β,ϵ-car (discontinued, Sigma), β, β-car (Fluka), chl *a* (Fluka) and chl *b* (Fluka). Lut (isolated from marigold petals) and cantha were donated by Perdue. Other pigments were either isolated from algal monocultures, mutant maize leaves grown in our laboratory [28] or shrimp carapace.

Standard concentrations (after transfer to the solvent required for use with the appropriate extinction coefficients) were determined using a dual beam spectrophotometer (model U-3110, Hitachi, Tokyo, Japan), bandwidth 2 nm, corrected for absorbance at 750 nm [29]. Spectrophotometer accuracy was assessed using NIST traceable neutral density filters (Starna Cells, Atascadero, CA, USA). Standards were stored at −20°C in darkness in amber bottles with PTFE-lined lids or PTFE bottles.

2.2.5. Sample extraction

Algal monocultures and field samples were collected on glass fiber filters and extracted in acetone (90 or 100%) or ethanol (as with some cultures used for pigment isolations). Samples were chilled while disrupted with an ultrasonic probe (model 450, Branson Ultrasonics, Danbury, CT, USA). Extracts

Table 3
Physical characteristics of HPLC columns evaluated[a]

Column name	Dimensions (mm)	Particle size (μm)	Surface area (m^2/g)	Pore size (Å)	Endcapped	% Carbon load, bonding chemistry	pH tolerance
Eclipse® XDB C_8 [b]	150×4.6	3.5	180	80	Yes	7.6, monomeric	2–9
Hypersil® C_8 mos-2[c]	100×4.6	3	170	120	Yes	7, monomeric	2–7
Luna™ C_8 (2)[c]	100×4.6	3	400	100	Yes	13.5, monomeric	1.5–10
Supelcosil™ LC318 C_{18} [d]	250×4.6	5	75	300	Yes	6, proprietary	2–7
Supelcosil™ LCPAH C_{18} [d]	100×4.6	3	170	120	Proprietary	proprietary	2–7.5
ODS-AL C_{18} [e]	150×4.6	5	335	120	No	17, monomeric	2–6
Vydac® 201TP™ C_{18} [f]	250×3.2	5	73	300	No	8 to 9, polymeric	2–7
Zorbax® Bonus-RP C_{14} [b]	250×4.6	5	180	80	Yes	9.3, monomeric	1–9
The Carotenoid Column™ C_{30} [e]	250×4.6	5	175	Proprietary	No	20, polymeric	2–6

[a] All stationary phase particles are spherical silica, except Vydac® 201TP™ C_{18} which is irregular silica. Data provided by vendors.
[b] Aglient Technologies (formerly Hewlett-Packard), Waldbronn, Germany.
[c] Phenomenex®, Torrance, CA, USA.
[d] Supelco, Bellefonte, PA, USA.
[e] YMC, Wilmington, NC, USA.
[f] Separations Group, Hesperia, CA, USA.

were clarified using 0.45-μm, PTFE, HPLC syringe cartridge filters fitted with glass fiber prefilters (Scientific Resources Inc., Eatontown, NJ, USA).

3. Results and discussion

3.1. Criteria for conditions used for collecting input data

The conditions used for collecting input data on each column are detailed in Table 2. We selected values of T between 40 and 60°C because these above-ambient temperatures are easily attainable by most column thermostatting devices and because our previous work [9,23,24] had indicated that these values of T provided resolution (R_s) between a great number of pigments. We felt that differences in T not greater than 20°C would be more likely to ensure accurate predictions as Sander and Wise [30] had observed that separation selectivity (α) does not necessarily change in a linear fashion over a wide range of T (−20 to 100°C). Temperatures we used do not necessarily result in the best R_s between all pigments. In fact, others have seen [9,13,14,16] that lower T values often improved R_s between selected pigments.

We selected values for t_G of 15 and 45 min or 20 and 60 min (Table 2) depending on column retentiveness. Some pigments eluted after the gradient and an isocratic hold on solvent B was necessary in these cases.

We used the same mobile phase with all columns as this study did not extend to the effects of mobile phase on separations. Thus we used methanol as solvent B even when a solvent of stronger elutropic strength may have been more practical, except with the C_{30} column. Because it was extremely retentive, it was necessary to change to a stronger solvent (ethanol).

Historically, many pigment separation methods have used a gradient system where solvent A is based on that of Mantoura and Llewellyn [5]: (80:20) methanol:0.5 *M* aqueous ammonium acetate and 0.025 *M* TBAA, pH 7.1. Many pigment analysts have subsequently deleted TBAA from solvent A [3,4,7–14,16–20,31] as reviewed in Jeffrey et al. [1]. During initial data collection we observed abnormal chl *a* retention (as with excessive chl *a* allomerization) when highly concentrated algal extracts and chl *a* standards were injected while ammonium acetate was used in solvent A without TBAA. This abnormal retention was eliminated by adding butylated hydroxy toluene (BHT) to the mobile phase (as suggested by an anonymous reviewer) or as we did, by replacing ammonium acetate with TBAA. We have avoided the costs ordinarily associated with the purchase of TBAA ion-pairing reagent by formulating it ourselves from the acidification of tetrabutyl ammonium hydroxide with acetic acid. TBAA was also advantageous in positioning the early eluting chlorophylls in front of the early eluting xanthophylls on the C_{30} column. In contrast, when ammonium acetate was used in solvent A, the early eluting chlorophylls were retained longer, eluted among the early eluting xanthophylls and caused many pigments to co-elute.

3.2. Column characteristics

Physical characteristics of the columns used were diverse (Table 3). Small particle sizes and long column dimensions generally increase column efficiency, N, while other stationary phase characteristics have a greater effect on α. Of the nine stationary phases used, two were listed by the vendors as polymeric and five as monomeric. The remaining two, the Supelcosil LC PAH and the LC 318, for which the bonding chemistry was listed as proprietary, were characterized as polymeric and monomeric, respectively, by Epler et al. [32].

Plate number (N) calculations for gradient data are complex but are conveniently calculated by the simulation software. Hence we easily compared N among columns. With DryLab one can enter the value of N observed during data collection or, as we did, enter various values of N until the accurate value is found (at which point the software predictions of peak width and R_s match those observed during data collection). Specifically, we used the peak widths and R_s of pairs eluting midway in the gradient from input data derived from the cooler T and longer t_G (Table 2) to identify the accurate value of N for each column. Next, we used the software to determine N for each column after normalizing the variables which affect N. Specifically, we entered the same

column dimension (250×4.6 mm), particle size (5 μm) and flow-rate (F=1 ml/min), as these varied among columns in the experimental procedures. In all cases, the monomeric columns were more efficient (with higher N values) than the polymeric C_{18} columns (Table 4). The columns with the highest efficiencies were the Luna C_8 and the Eclipse XDB C_8. We show no data for the C_{30} column as we had used a different mobile phase and thus the observed N (8500) cannot be compared exactly with values of N for the other columns.

We used the simulation software to compare the resolving power of each column free from the effects of their differences in N and column dimensions. Inspection of the R_s equation for gradient elution, $R_s = 1/4N^{1/2}(\alpha - 1)[k^*/(1 + k^*)]$ [33], reveals the interaction among N, α and k^*. In the computer simulation software, we normalized N and factors affecting k^* among columns. With these variables normalized, changes in R_s attained from manipulations to T or t_G reflect their effects on separation selectivity (α). The quantity k^* is defined by $(t_G F)/(V_m \Delta\phi S)$, where t_G is gradient time in min, $\Delta\phi$ the percent change in solvent B during the gradient divided by 100, F the flow-rate (ml/min), V_m the column volume (ml) and S is a constant that is a function of the molecular structure of each compound. We adjusted variables affecting k^* so that it was equal for each column by using a gradient range of 20–100% solvent B, F of 1 ml/min and column dimensions of 250×4.6 mm. Values of S can be assumed to be approximately equal for a given solute on different columns [34]. After normalizing k^*, we entered the same N value (10 000) for each column. Next we set t_G to 20 min (T=50°C) and used the simulation software to predict the R_s between adjacent pigments. The R_s predicted from the 20 min t_G was compared to the R_s predicted between the same pigment pairs when t_G was 60 min (T=50°C). We then used the simulation software to predict the R_s between adjacent pigments at 45°C (t_G=45 min) and calculated the change in R_s seen between the same pairs when T was set to 60°C (t_G=45 min.). We used the same pigments with each column and included only those that eluted during the gradient with all sets of conditions used. As a result, chlorophylls were excluded.

The effects of variations to t_G and T on the resolving power of each column are shown in Fig. 1. We illustrate the results with columns sorted first by bonding chemistry (either monomeric or polymeric) and second by aliphatic chain length (C_8 to C_{30}) (Fig. 1, x axis). (Note that data from the C_{30} column is not relevant to the other columns as previously stated.) The change in R_s associated with the 3-fold change in t_G (●) and the 15° difference in T (○) are shown (y axis). There was tremendous variability (as indicated by the distribution of data along the y axis) for both t_G and T for all columns. Changes in R_s resulting from T (○) were generally greater on the polymeric columns relative to the monomeric columns. In fact, the average median value of changes in R_s associated with T on the two C_{18} polymeric columns (1.17±0.12) was 3.7 times the average

Table 4
Observed values of N (derived from input data and experimental conditions given) and normalized values of N[a]

Column	Experimental conditions			N	
	Column dimensions (mm)	Particle size (μm)	Flow rate (ml/min)	Observed	Normalized
Eclipse XDB C_8	150×4.6	3.5	1.0	17 000	22 000
Luna C_8 (2)	100×4.6	3	0.8	10 500	21 850
Hypersil mos-2 C_8	100×4.6	3	1.0	8000	12 550
Bonus-RP C_{14}	250×4.6	5	1.2	10 500	10 250
LC318 C_{18}	250×4.6	5	1.0	15 000	15 000
ODS AL C_{18}	150×4.6	5	1.0	8800	14 800
201TP C_{18}	250×3.2	5	0.6	5250	4370
LC PAH C_{18}	100×4.6	3	1.0	2200	4400

[a] When normalizing N: column dimensions, 250×4.6 mm; particle size, 5 μm; and flow-rate, 1 ml/min.

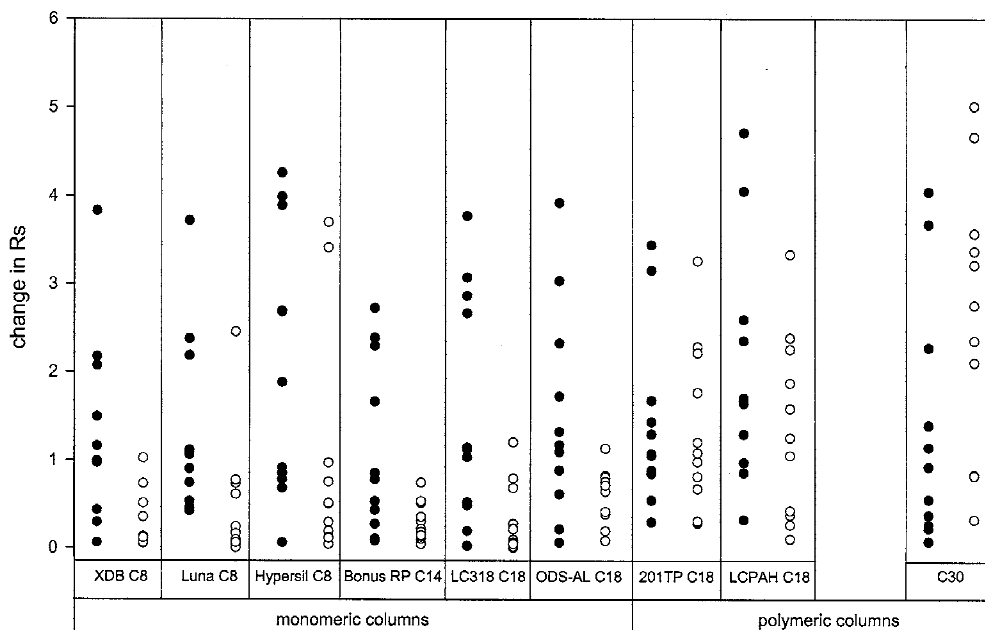

Fig. 1. Changes in R_s as a function of t_G or T. All data derived from computer simulations using the input data from each column. The theoretical change in R_s (y axis) resulting from two values of t_G (●), 20 and 60 min (where $T=50$°C) and the change in R_s resulting from two values of T (○), 45 and 60°C (where $t_G=45$ min) is shown for each column. Columns were normalized for factors that affect R_s (N and k^*; details in Section 3.2) prior to calculating R_s. Mobile phase: solvent A, 70:30 (v:v) methanol, 28 mM aqueous TBAA, pH 6.5; solvent B methanol for all but the C_{30} column (ethanol). Data from this column are therefore not directly comparable to the others.

median value associated with T on the monomeric columns (0.32±0.23). Others have also demonstrated that phytoplankton pigment separations on polymeric columns are highly sensitive to changes in T [9,13–16]. The average median value of changes in R_s associated with t_G for the two C_{18} polymeric columns (1.37±0.42) and the six monomeric columns (1.17±0.38) were more similar relative to the differences seen with T. While data for the C_{30} column is not directly comparable to others, it was sensitive to both changes of T and t_G with somewhat greater sensitivity to T.

These data (Fig. 1) explore separation characteristics among columns independent of differences in N. However, factors affecting separations are only useful if peak widths are sufficiently narrow so that adequate R_s (ideally $\not<1.5$) is attained. Highly efficient columns are therefore advantageous. In fact, the relatively large changes in R_s shown for the LC PAH C_{18} column (Fig. 1, where N was normalized to 10 000) were in reality unattainable as peak widths were abnormally broad during experimental conditions (observed $N=2200$). The low efficiency seen with this column may have resulted from an incompatibility with the conditions used. We show no subsequent data for this column.

While the independent effects of T and t_G are of interest, their combined effects are more important. The resolution map of the DryLab simulation software is most useful with its visual display of the R_s attainable at all possible combinations of t_G and T, as Dolan et al. [24] illustrated with 14 different samples (including algal pigments).

3.3. Using computer simulations to develop methods for pigment isolation

Our strategy for isolating pigment standards was to isolate as many as possible from the same source using methods which were fast and resulted in a final product of high chromatographic and peak purity. Here we demonstrate how we used computer simulations to develop methods for isolating pigments from

extracts of *G. galatheanum* and *T. pseudonana* combined.

For a column to be selected for pigment isolation, we required that it: (1) exhibit high sample capacity, (2) low back pressure, (3) high efficiency, (4) not excessively retain the latest eluting pigment in the extract (β,β-car) and (5) provide exaggerated R_s (>3.0) between pigments to be isolated. The LC 318 C_{18} stationary phase met our requirements and computer simulations indicated that the desired R_s could be achieved between a number of the 10 pigments to be isolated from this algal mixture. The simulation software also indicated that a shorter column (100 mm) would fulfil our requirements and shorten run times.

In Fig. 2A, we show the chromatogram produced on the LC 318 C_{18} column (100×4.6 mm) from the injection of the combined extract of *G. galatheanum* and *T. pseudonana*. The chl *c*3 (1), hex-fuco (15), diato (23), and the gyroxanthin diester-like (28 and 29) pigment fractions were collected free of contamination from this injection. Three impure fractions, chl *c*1, chl *c*2 and chlide *a* (5, 3, 6), but-fuco and fuco (9, 10) and diadino plus an unknown degradant (19+?) were collected and subsequently purified using other methods.

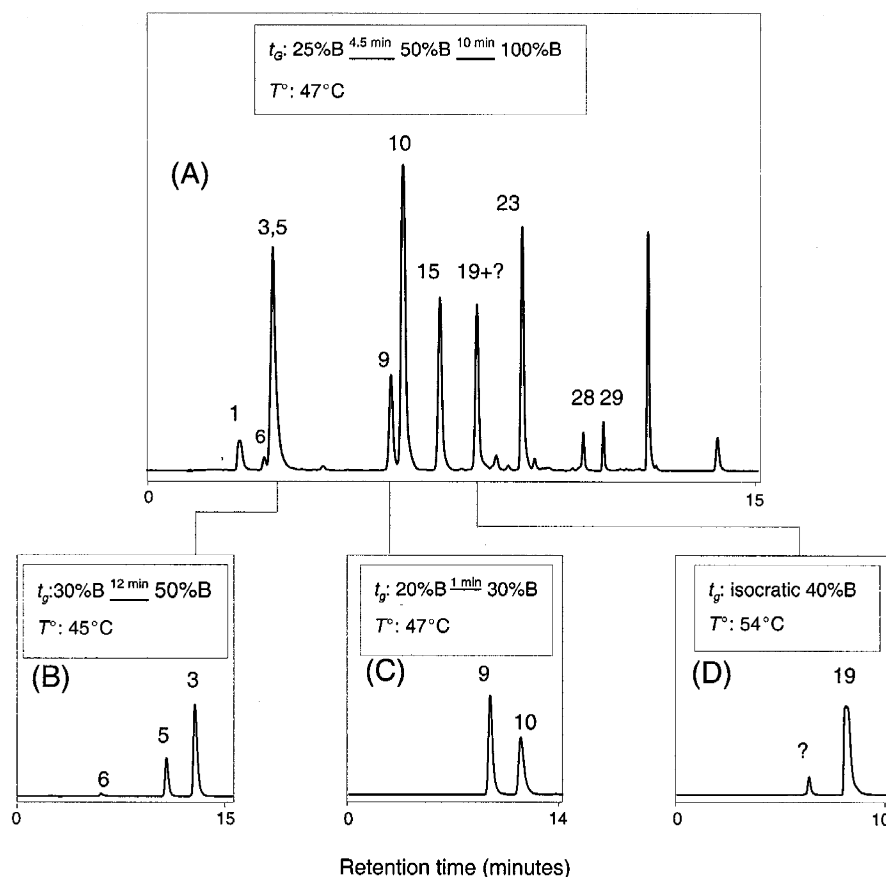

Fig. 2. Chromatograms from methods developed with the simulation software for the isolation of pigment standards from *T. pseudonana* and *G. galatheanum*. Peaks 1, 15, 23, 28 and 29 (identities in Table 1) were collected as pure fractions from the initial injection (A). Impure fractions were collected and subsequently purified on other methods (B–D). Mobile phase: solvent A, 70:30 (v:v) methanol, 28 m*M* aqueous TBAA, pH 6.5; solvent B, methanol. For the segmented gradient in (A) 50%B is reached at 4.5 min and 100%B at 10 min. Flow was individually adjusted to shorten run time. Columns used (details in Table 3): (A) LC 318 C_{18}, 100×4.6 mm; (B) C_{30}; (C,D) 201TP C_{18}. Inject volume 900 μl (sample premixed with buffer before injection).

For final purification of the three impure fractions, we developed methods using polymeric columns capable of achieving exaggerated R_s in short analysis times (Fig. 2B–D). The chl *c*1 (5) and chl *c*2 (3) fractions were each collected free of contamination from chlide *a* (6) with a method using the C_{30} column (Fig. 2B). We used the 201TP C_{18} column for isolating but-fuco (9) from fuco (10) (Fig. 2C) and diadino (19) free of contamination by the unknown pigment (Fig. 2D). After isolation, we transferred the pigments from the mobile phase in which they had been collected to the solvent required for use with the corresponding extinction coefficient. Chl *c*1 and *c*2 were transferred to acetone using solid-phase extraction [35]. The other pigments were transferred to ethanol by individually injecting each onto the C_{30} column with ethanol as the isocratic mobile phase and then collecting the pigment as it eluted from the column. We varied values of T to optimize the retention of each pigment such that the peak to be isolated was sufficiently removed from the injection peak and the run time was not excessively long.

Finally, the isolated standards were checked for chromatographic purity on each of two analytical methods using different columns (methods not shown). Chromatographic purity was >95% in all cases. Peak purity was also demonstrated by comparing the absorbance spectra on the peak upslope, apex and downslope attained from the HPLC in-line photo-diode array detector.

3.4. Optimizing pigment separations through changes to column temperature and gradient time

We used computer simulations to identify chromatographic conditions capable of resolving the greatest number of very important pigments in our test mixture. Some pigments in this mixture provide more meaningful information than others with regard to algal class diversity and abundance so we ranked them by their degree of importance as we anticipated that not all could be resolved in one analysis. Fourteen pigments classified as very important to resolve (those marked with **, Table 1) and 13 pigments considered less important to resolve (those marked with *, Table 1) were included in the test mixture. Pigments with no such notation (Table 1) and in our test mixture (peak codes 8, 32–34, 40 and 41, Table 1) were considered as interference peaks only. We sought fast methods, rejected analysis times greater than 60 min as impractical even if the longer analysis times improved separations and avoided segmented gradients in favor of simple linear gradients. We emphasize that the values of T and t_G available for use were constrained by the conditions used while collecting input data and are not necessarily optimal for all pigment pairs.

Analyses were conducted on each of eight columns using the chromatographic conditions identified in DryLab. The resulting chromatograms and conditions used are illustrated (Figs. 3–6). No column was able to resolve all pigments ($R_s \geq 1.0$) in the same analysis. The co-eluting pigments seen with each method are summarized (Table 5).

Only the C_{30} column was capable of separating all pigments (with ** or *, Table 1) in our test mixture, albeit not in one run (Fig. 3A,B). With simple manipulations to t_G and T, this column resolved all chlorophylls (Fig. 3A) and all carotenoids and xanthophylls (Fig. 3B). We used a segmented gradient in Fig. 3B simply to shorten run time. The other polymeric column, the 201TP C_{18}, achieved separation between the maximum number of carotenoids at 60°C. Others have shown that at sub-ambient [9,14,16] and ambient [13] temperatures, this column can resolve the chlorophyll pigments not resolved here.

The co-elution of some pigment pairs was related to the aliphatic chain length of the stationary phase. For example, the separation of monovinyl chl species (chl *c*1 and chl *a*) from their divinyl counterparts (chl *c*2 and DV chl *a*) was achieved easily on the C_{30} and C_8 columns but not on others (DV chl *b* and chl *b* were resolved only on the C_{30} column). β,ϵ-car and β,β-car were resolved on all but the C_8 columns. The co-elution of these carotene pigments is of little importance in describing algal class diversity in natural samples; however, the identification of DV chl *a* in a natural sample uniquely indicates the presence of prochlorophytes and therefore this separation is very important in the analysis of oceanic samples.

The co-elution of other pigments did not seem to correlate with stationary phase aliphatic chain length. The Hypersil C_8 and the LC 318 C_{18} (Fig. 4A,B) did

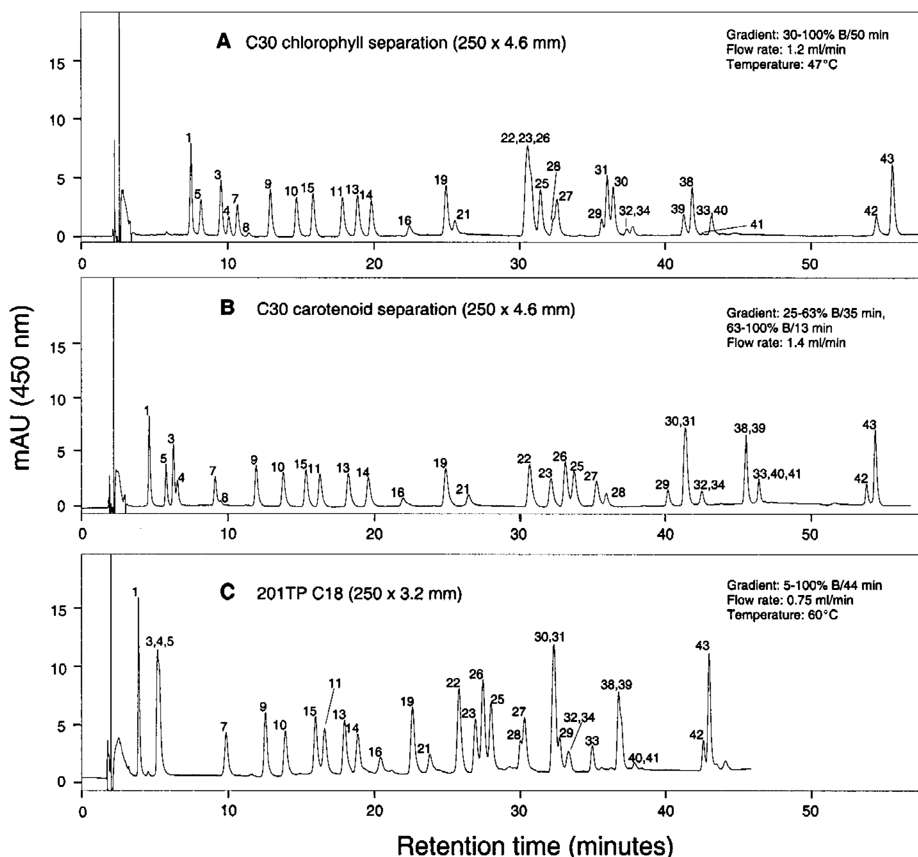

Fig. 3. Separations of pigments in a test mixture from methods developed with the simulation software for use with two polymeric columns. Separations were developed for the C_{30} column to separate all chlorophylls (Fig. 3A) and to separate all carotenoids and xanthophylls (Fig. 3B). The separation shown in Fig. 3C was developed for the 201TP C_{18} column to separate the maximum number of very important pigments in the shortest analysis time. Experimental conditions: mobile phase-solvent A, 70:30 (v:v) methanol, 28 m*M* aqueous TBAA, pH 6.5; solvent B, methanol (except (A,B) when it was ethanol); column details in Table 3; peak identities in Table 1.

not resolve neo, pras, and viola (11, 13, 14). Resolution between lut and zea (26, 25) was the most limiting ($R_s = 0.6$) with the Bonus-RP C_{14} and the ODS-AL C_{18} (Fig. 5A,B); somewhat limiting (R_s 1.0≥1.2) on the other C_{18} columns (Figs. 3C and 4B) and the C_{30} column (Fig. 3B); and the best ($R_s \geq 1.5$) with the C_8 columns (Figs. 4A, 6A and 6B). Resolution between lut and zea was easily improved on the two polymeric columns (the C_{30} and the 201TP C_{18}) with changes to T at the expense of decreases in R_s between other pigments. With the ODS-AL C_{18}, trends seen on the resolution map (not shown) for this column hinted that temperatures <40°C (not available for use with simulations) may have been advantageous for resolving lut from zea. Thus, it is unfortunate that we had not evaluated this column at near-ambient temperatures. The Luna C_8 and the Eclipse XDB C_8 columns provided separations of the maximum number of very important pigments in one analysis (Fig. 6A,B and Table 5).

3.5. Accuracy of computer simulations

We found, as have others [33,36], the computer simulations to be highly accurate when comparing observed data with predicted data. Ghrist et al. [37] found that prediction accuracy was best when input data and modeled data were collected close together in time. We observed an average deviation between predicted and observed t_R values of 0.06 and 0.18

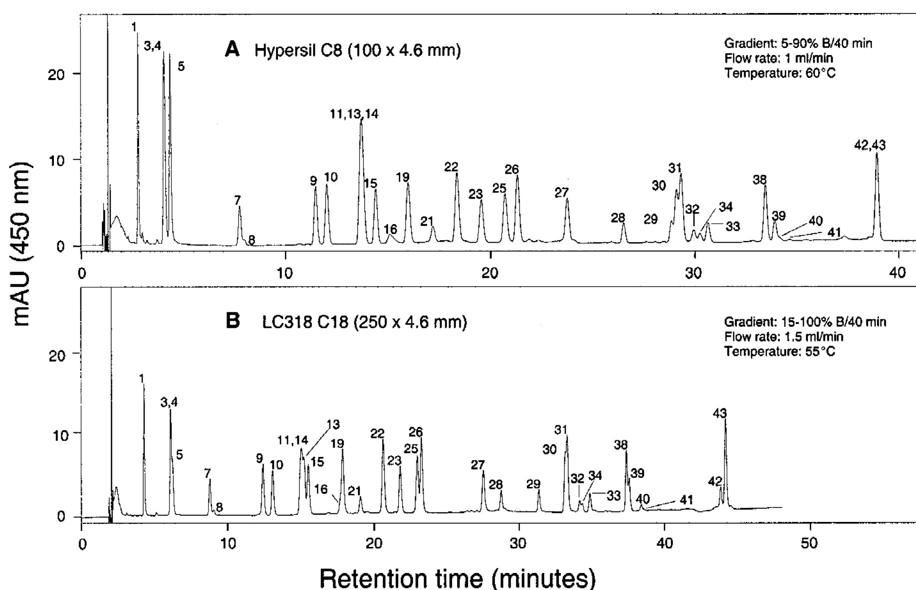

Fig. 4. Separation of pigments in the test mixture on two columns that were unable to resolve neo, pras and viola. Methods were developed for the (A) Hypersil C_8 and (B) LC 318 C_{18} columns for the purpose of separating the maximum number of very important pigments in the shortest analysis time (experimental details in Fig. 3 legend).

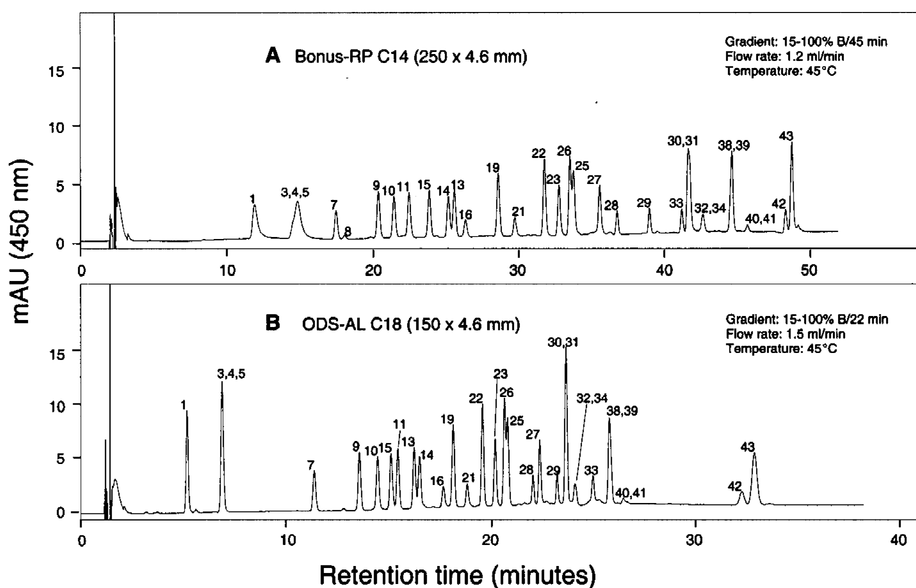

Fig. 5. Separation of pigments in the test mixture on two columns that had difficulty resolving lut and zea. Methods were developed for the (A) Bonus-RP C_{14} and (B) ODS-AL C_{18} columns for the purpose of separating the maximum number of very important pigments in the shortest analysis time (experimental details in Fig. 3 legend).

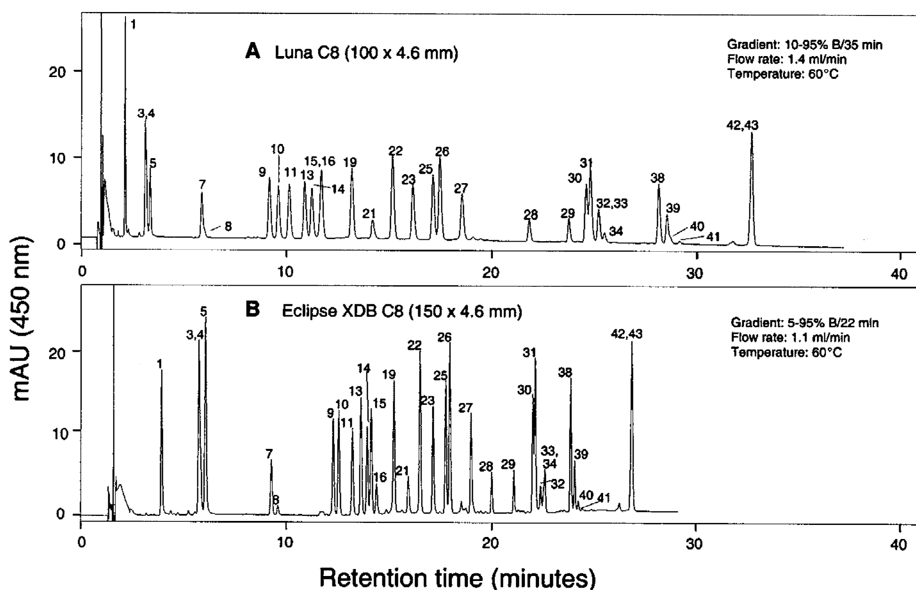

Fig. 6. Separation of pigments in the test mixture on two columns that, relative to the columns in Fig. 3, 4 and 5 separated the most pigments in one run. Methods were developed for the (A) Luna C_8 and (B) the Eclipse XDB C_8 columns for the purpose of separating the maximum number of very important pigments in the shortest analysis time (experimental details in Fig. 3 legend).

min (using all pigments but chl *c*3) when input data and experimental data were collected either within 2 days or up to 21 days, respectively. When all early eluting chlorophylls were excluded from the average, the deviation dropped by 50% in both cases. Injector programming and large injection volumes (especially

Table 5
Pigments in the column test mixture that co-eluted, as shown in Figs. 3–6 (columns and conditions therein described)[a]

Co-eluting pigments (pigment codes)	Columns featured in Figs. 3 through 6								
	Fig. 3A C_{30}	Fig. 3B C_{30}	Fig. 3C 201TP C_{18}	Fig. 4A Hypersil C_8	Fig. 4B LC318 C_{18}	Fig. 5A Bonus-RP C_{14}	Fig. 5B ODS-AL C_{18}	Fig. 6A Luna C_8	Fig. 6B XDB C_8
Chl *c*2, Mg DVP, chl *c*1 (3, 4, 5)			x		x	x	x		
Chl *c*2, Mg DVP (3, 4)			x	x	x	x	x	x	x
Neo, pras, viola (11, 13, 14)				x	x				
Hex-fuco, asta (15, 16)								x	
Asta, diadino (16, 19)					x				
Allo, diato, lut (22, 23, 26)	x								
Zea, Lut (25, 26)						x	x		
Cantha, gyroxanthin-like (27, 28)	x		x						
Gyroxanthin-like, chl *b* (29, 31)	x								
Gyroxanthin-like, DV chl *b*, chl *b* (29, 30, 31)			x	x					
Chl *b*, DV chl *b* (30, 31)		x	x	x	x	x	x	x	x
DV chl *a*, chl *a* (38, 39)		x	x		x	x	x		
β,ϵ-car, β,β-car (42, 43)				x				x	x
No. of very important pigments resolved	10	11	10	10	8	9	9	12	13

[a] Co-elution of interference peaks (8, 32–34, 40 and 41) are not indicated here as they were resolved from important pigments (except for occasional co-elution of DV chl *a′* with chl *a*). Pigments are considered unresolved when $R_s < 1.0$. Pigment codes and their relative importance are shown in Table 1.

when injection solvent is different from the initial mobile phase) create a micro-environment at the head of the column, which has the potential to alter t_R values and peak widths of early eluting compounds, independent of effects resulting from the mobile phase gradient and *T*. In fact, the simulation software often flagged early eluting chlorophylls as exhibiting abnormal chromatographic behavior.

3.6. A new method for the analysis of phytoplankton pigments in natural samples

We selected the method developed for the Eclipse XDB C_8 column for further testing for suitability in the routine quantitative analysis of phytoplankton pigments in natural samples. We chose this method because it provided the fastest analysis time, highest efficiency, maximum number of pigments resolved in one analysis and low solvent use relative to other methods tested. We identified the elution position of additional pigments, evaluated features important to quantitative analysis and demonstrated transferability.

As one is never certain what pigments may be present in natural samples, we analyzed extracts of additional algal monocultures (Fig. 7) containing pigments not in our test mixture to increase the library of pigments whose t_R values were documented by this method. We note several pigments that we could not identify (peak 18 and those listed with letters above the peaks, Fig. 7 and Table 1) but had the potential for co-elution with important pigments. For example, a pigment (18) with myxo-like spectra (Fig. 7E) co-eluted with diadino. Furthermore, extracts of *C. polylepsis*, *E. huxleyi* and *I. galbana* were analyzed on the XDB C_8 column and then again on the C_{30} column to examine the elution position of certain pigments. Data from the C_{30} column (not shown) enabled us to see that in *I. galbana* there were two additional pigments unknown to us (k and L in Fig. 7L and Table 1) imbedded in phytyl-chl *c* (37). Also, in *C. polylepsis* there were two pigments of identical spectra (m in Fig. 7I and Table 1) eluting as a shoulder on the front of chl *a* (39). Phytyl-chl *c* (37) from *E. huxleyi* (Fig. 7K) and *I. galbana* (Fig. 7L) each contained two identical fractions when analyzed on the C_{30} column.

Some additional pigments which we could identify co-eluted with others already identified. These include 4k-hex-fuco (12) shown in Fig. 7K (separated in [20]) that co-eluted with neo; phytyl-chl *c* (37) shown in Fig. 7K,L that co-eluted with chl *a* allom 2 (36) shown in Fig. 9A; and chlide *a* (6) shown in Fig. 7A, F and H that co-eluted with chl *c*1. As the quantitation of chlide *a* is important when reporting equivalent total chl *a* values, we suggest quantitation of chlide *a* (in the presence of chl *c*1) by using a dichromatic equation as in Latasa et al. [38] based on simultaneous monitoring of two different wavelengths (665 and 450 nm). (Note: we have subsequently determined that chlide *a* and chl *c*1 can be separated by increasing the molarity of TBAA.)

We evaluated factors important to quantitative analysis including such things as peak area and t_R reproducibility and minimum detection limits (MDL) [29]. We used data from seven replicate injections of a mixed standard containing 17 pigments ranging in concentration from 1.5 to 5.6 ng/injection (S/N varied from 20 to 70) for this evaluation. Peak area and t_R reproducibility averaged 0.75 %RSD and 0.04 %RSD, respectively. The average MDL of all 17 pigments evaluated was 0.06±0.03 ng per injection. Additionally we tested 14 chl *a* calibration curves on six different XDB C_8 columns and found the mean slope to be 3.436±0.046 (1.325 %RSD). Linear dynamic range was observed from 0.5 to >700 ng of pigment per injection (where 0.5 ng chl $a = S/N$ of 10 at 665±10 nm). We demonstrate the suitability of this method for the analysis of dilute oceanic samples where total chl *a* abundance (DV chl *a* plus chl *a*) was 0.08 μg/l of seawater (Fig. 8).

We successfully transferred this method to a different HPLC (Beckman Gold) and laboratory (data courtesy of Ivy Collins and Alan Lewitus, University of South Carolina, Charleston, SC). We also tested the transfer to an HPLC with a high dwell volume by imposing an isocratic hold on initial conditions on our existing HPLC such that it mimicked an HPLC with a 10 ml dwell volume. This simulation also yielded results which were comparable to the original HPLC (Fig. 9).

Initial attempts to transfer this method to the Beckman Gold HPLC were unsatisfactory, as R_s between several peaks was poor. The column heater used with the Beckman Gold HPLC was a stand-

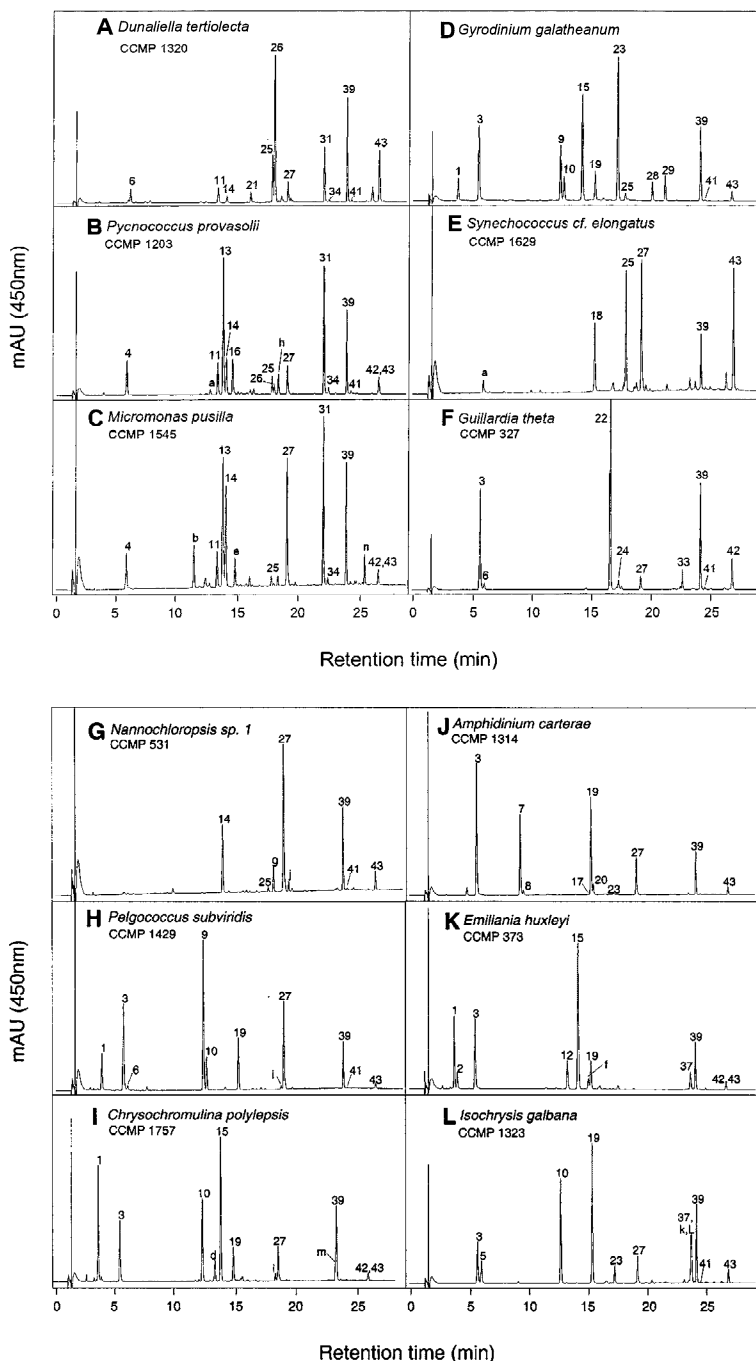

Fig. 7. Chromatograms of algal monocultures from various algal classes analyzed on the Eclipse XDB C_8 column showing the elution position of additional pigments not previously shown with the same method (Fig. 6B). Column details in Table 3; peak identities in Table 1. All cultures except *Gyrodinium galatheanum* (D), which was grown at Horn Point Laboratory, were from the Provasoli-Guillard Culture Collection (CCMP). Canthaxanthin (27) was added as an internal standard to all cultures except those depicted in Fig. 7D and K. Data courtesy of Ajit Subramaniam (Department of Meteorology, UMD, College Park, MD, USA) and Robert Vaillancourt and Robert Guillard (Bigelow Laboratory for Ocean Science, West Boothbay Harbor, ME, USA).

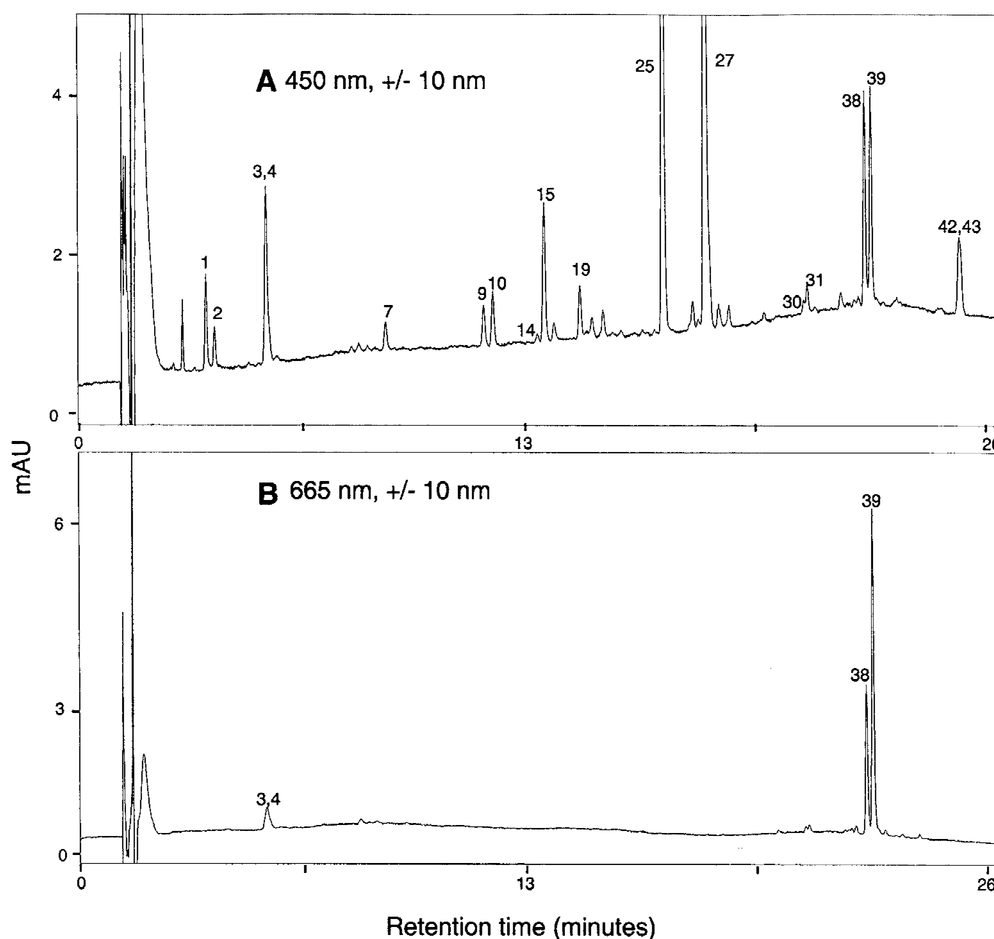

Fig. 8. Dilute natural sample analyzed on the Eclipse XDB C_8 column. DV chl *a* (38) is approximately 2 ng per injection and chl *a* (39) 6 ng per injection Column details in Table 3; pigment identities in Table 1. The sample was collected at 37°29.41′N, 73°23.00′W, which is 250 km ENE of the mouth of Chesapeake Bay (data courtesy of Lawrence Harding, Jr., Horn Point Laboratory, UMCES, Cambridge, MD, USA). Analyses using this method are also shown in Figs. 6B and 7.

alone unit which suspended the column in air inside. The solvent inlet line was not preheated and *T* was monitored by a glass mercury thermometer inserted into the column heater box.

Wolcott et al. [39] noted several reasons for the ineffective transfer of methods between instruments when elevated column temperatures (>40°C) are used. Of these reasons, the most likely to have adversely affected our ability to initially transfer this method to the Beckman HPLC were column thermostat inaccuracy and lack of mobile phase preheating prior to the column inlet (the latter of which causes temperature gradients within the column and a column *T* lower than expected). Column performance equivalent to that seen with our HP HPLC was achieved by increasing the column heater setpoint from 60 to 61.5°C and by inserting a piece of stainless steel tubing (100 cm long×0.007′ I.D.) between the injector and the column with approximately 82 cm coiled within the column heating box so that the mobile phase was pre-heated to the column *T*.

4. Conclusions

The present study shows that method development software (DryLab) can be used to identify methods

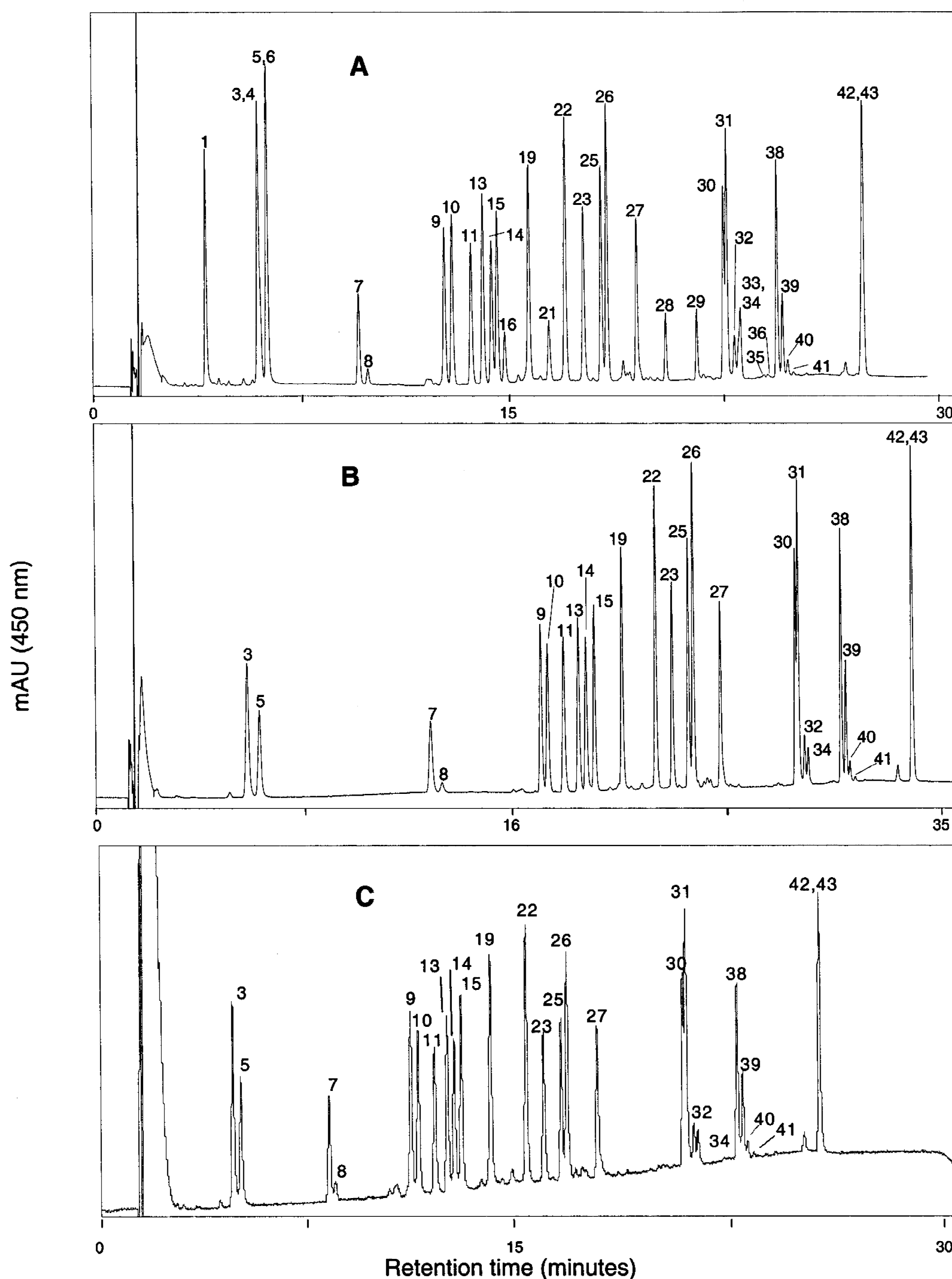

Fig. 9. Chromatograms showing transferability of the method developed for the Eclipse XDB C_8 column. Column details in Table 3; peak identities in Table 1. Some pigments were present in (A) but not in (B,C). (A) HP 1100 HPLC, dwell vol=3 ml; (B) HP 1100 HPLC, dwell vol=10 ml (simulated); (C) Beckman Gold HPLC, dwell vol=2.2 ml. Mobile phase: solvent A, 70:30 (v:v) methanol, 28 m*M* aqueous TBAA, pH 6.5; solvent B, methanol. F=1.1 ml/min. T (A,B)=60°C; T (C)=61.5°C. Gradient conditions in (A,C): linear gradient of 5–95% B in 22 min followed by an isocratic hold for 7 min and return to initial conditions in 2 min; in (B), initial conditions (5% solvent B) were held isocratically for 6.4 min to simulate a 10 ml dwell volume before starting the gradient. Methods in (A,B) used an injector program; in (C) the sample was premixed with buffer (50:50) and manually injected (200 μl).

useful for the isolation and analysis of phytoplankton pigments on columns varying greatly in stationary phase physical characteristics and that changes to column T and t_G alone effectively enhance separations. It is unlikely, however, that the separations we identified are the best attainable for each column as we did not explore the use of other mobile phases and, as we limited our choice of T to what is conveniently attained with most column heating devices, we did not evaluate the effects of ambient or sub-ambient T on these columns.

Changes in R_s seen with adjustments to T were profound on the polymeric columns (relative to the monomeric columns) where changes of T as little as 5°C caused otherwise well-resolved peaks to co-elute. The modeling software was especially useful with the polymeric columns, as many pigments pairs were greatly affected by changes in T and optimal conditions were confined to a fairly narrow range of T and t_G in many cases. This sensitivity to T is advantageous when collecting pigments to be isolated as standards (where exaggerated R_s is required between selected pigments), or when attempting to gain information about peaks that are unresolved on the primary method used (as we did with pigments found in the extracts of *I. galbana*, *E. huxleyi* and *C. polylepsis* analyzed first on our primary method with the XDB C_8 column and secondly on the C_{30} column). Extreme sensitivity to T can be disadvantageous, however, when attempting to reproduce methods between instruments, as inaccuracies in column thermostatting devices can result in unexpected changes to α. Strict adherence to the guidelines set forth by Wolcott et al. [39] is mandatory for success in transferring methods when using elevated column T. We do not advise using polymeric columns without controlling T.

We found that a two-step procedure for the isolation of pigments for use as standards from a complex matrix was very time efficient and produced products of high quality. A highly efficient monomeric column with low back-pressure and high sample capacity (such as the Supelcosil LC 318 C_{18} column) was useful for the isolation of crudely purified fractions when pigments of interest co-eluted, or for the isolation of well-resolved pigments. Partially purified fractions were subsequently re-injected and the constituent pigments isolated as pure fractions from columns (primarily polymeric ones) with selectivities that differed from the LC 318 C_{18} column. We isolated 20 different pigments using methods based on these principles.

From our survey of columns (Figs. 3–6), we found the XDB C_8 column combined high efficiency (important to good detectability) and desirable α. We regret that the additional pigments identified from the analysis of culture extracts (Fig. 7) were not included in the input data from which our T and t_G conditions were derived, as we were unable to determine if other conditions would have been more optimal for resolving the pigment pairs observed to co-elute (Table 1) with conditions used. The method described for this column is well suited to the analysis of pigments in dilute or highly concentrated natural samples as the method has excellent detectability and the linear dynamic range extends to the point of detector saturation. The analysis time is convenient (27 min for elution of the most retained pigment). The method uses a simple mobile phase (composed primarily of an inexpensive organic solvent, methanol) and a linear gradient (which contributes to baseline stability). Because the peak shape, and therefore R_s, of early eluting chlorophylls is affected by injection conditions and injector capabilities, analysts may need to individualize injector programs, ratios of solvent to buffer and injection volumes to achieve similar results for these pigments. This method has been used extensively in our laboratory for the analysis of natural samples and has exhibited excellent column to column reproducibility with regard to α, R_s and response factor stability. This method was recently evaluated in an inter-calibration exercise [40] and compared favorably with other methods tested.

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APPENDIX M

Mekebri et al., 2009

Method validation of microcystins in water and tissue by enhanced liquid chromatography tandem mass spectrometry

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Method validation of microcystins in water and tissue by enhanced liquid chromatography tandem mass spectrometry

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ABSTRACT

A liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) method has been developed and validated to identify and quantify trace levels of cyanotoxins or microcystins (MC) in water, bivalves and fish tissue with enhanced sensitivity and specificity. The method enables confirmation and quantification of six MCs (MC-LA, LF, LR, LW, RR and YR) with a single chromatographic run. The applied chromatography also allows determination of certain MC metabolites (Desmethyl-LR and -RR). By using LC-ESI–MS/MS in multiple reaction monitoring (MRM) mode, the limit of detection and quantitation for the microcystins studied, were determined to be between 0.2 and 1 pg on column (5:1 S/N ratio). These values are below the 2 pg detection limits found in the available literature.

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1. Introduction

1.1. Background and literature review

Cyanobacteria, also known as blue-green algae, are a small group of photosynthetic-planktonic bacteria whose evolution dates back more than 3.5 billion years. Cyanobacteria are widely distributed in eutrophic aquatic environments worldwide. Many of the common cyanobacterial species produce toxic metabolites which can be lethal to wildlife, domestic livestock and humans [1]. Contaminants associated with cyanobacteria are called cyanotoxins. The cyanotoxins are divided into three classes based on chemical structure: cyclic peptides, alkaloids and lipopolysaccharides.

Nodularins (NDLN) and microcystins (MC) are cyclic peptides containing five and seven amino acids, respectively. Microcystins, the most common and important cyanobacterial toxins, are cyclic heptapeptide hepatotoxins. More than 70 structural variants of microcystins, isolated primarily from the freshwater genera *Microcystis*, *Planktothrix* (*Oscillatoria*), *Anabaena* and *Nostoc*, have been described in the scientific literature [2]. Where as nodularins (less than 10 known variants) are cyclic pentapeptides and are produced mainly by Nodularia, found in brackish waters [3]. Toxic cyanobacterial blooms (microcystis) are an emerging issue in the United States and worldwide because of increasing amounts of nutrient pollutants (nitrogen and phosphorous) in surface waters and warmer weather patterns which favor the growth of cyanobacteria and lead to more microcystin outbreaks [4]. The increasing number of cyanobacteria infested surface waters used for drinking, irrigation and recreation water constitute a potential risk to public health, domestic animals and wildlife. These health hazards have led the World Health Organization (WHO) to establish a provisional guideline value of MC-LR of 1 μg/L for drinking water [5]. Health Canada calculated a tolerable daily intake (TDI) of 0.013 μg of MC-LR (kg of body weight)$^{-1}$ day^{-1} (defined as a 60-kg adult consuming 1.5 L of water per day, with an MC-LR content of 0.5 ng/mL water) [6]. MCs are extremely stable in water because of their chemical structure, surviving in both warm and cold water and can tolerate radical changes in water chemistry, including pH. MCs can remain toxic even after being boiled [7].

1.2. Microcystin chemical structures

The general structure of microcystins is cyclo(D-Ala-L-X-D-erythro-methylAsp (iso-linkage)-L-Z-Adda-D-Glu(iso-linkage)-N-methyldehy-droAla), where X and Z are variable L-amino acids (Fig. 1). By using amino acid single letter code classification, each microcystin is designated a name depending on the variable amino acids which complete their structure. For example, one of the most common toxins found in water supplies around the world, microcystin-LR contains the amino acids Leucine (L) and Arginine (R) in these variable positions.

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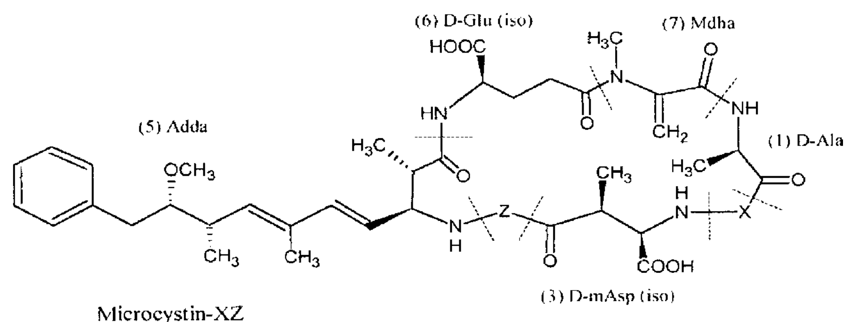

Microcystin congeners variable amino acids		
Microcystin	X	Z
LR	*Leucine*	*Arginine*
RR	*Arginine*	*Arginine*
YR	*Tyrosine*	*Arginine*
LA	*Leucine*	*Alanine*
LF	*Leucine*	*Phenylalanine*
LW	*Leucine*	*Tryptophan*

Fig. 1. Microcystins general structure.

The structures of NDLNs are similar to MCs but with a five-member amino acid ring instead of a seven-member one. The structure of nodularin-R (NDLN-R) is cyclo(D-erythro-methylAsp (iso-linkage)-L-Arg-Adda-D-Glu(iso-linkage)-2-(methyl-amino)-2-(Z)-dehydrobutyric acid). Adda stands for the amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [2,8].

The characteristic feature of both microcystins and nodularins is the presence of ADDA (4*E*, 6*E*-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid). Structural variants of microcystins commonly contain other L-amino acids at two nonconserved sites in the peptide ring. Other structural variants arise from the presence or absence (desmethyl variants) of methyl groups at the β-Me-Asp (dm-MC-RR and dmMC-LR) and *N*-methyldehydroalanine (Mdha) (Nodularin) residues, but such changes have little effect on the toxicity of the molecules [9]. Isomerization of the ADDA moiety to form 6*Z*-ADDA microcystin analogues renders the molecule essentially non-toxic. Thus, 6*E* geometry in the ADDA moiety is considered a prerequisite for toxicity in microcystin congeners [10].

1.3. *Microcystin toxicity*

MCs are associated with freshwater environments and their bioaccumulation by aquatic animals, including zooplankton, fish and water filter feeders such as bivalves, has been reported by several authors [11–14]. Liver, followed by kidney and intestine accumulate most of the MCs in exposed fish. Because these organisms are an important food source, not only for birds and fish but also for mammals, MCs can be transferred to higher trophic levels through the food chain leading to human toxicity.

Microcystins are known liver toxins. Carmichael suggests that "the extraordinarily high rates of liver cancer in humans in parts of China may be tied to the cyanobacterial toxins in water [15]." At a hemodialysis clinic in the town of Caruaru in north-east Brazil, an outbreak of severe hepatitis occurred where dialysis water contaminated with blue-green algal toxins caused the death of 50 patients from acute liver failure [16,17]. Severe cases of gastroenteritis in North and South America [18] and Australia [19] have been linked to the consumption of drinking water contaminated by cyanobacteria. Toxic and non-toxic strains of the same cyanobacterial species show no predictable difference in appearance. Therefore, it is necessary to analyze the toxin content by physico-chemical, biochemical or biological methods [20,21].

1.4. *Analytical method history*

Various analytical techniques have been used to analyze these toxins, such as enzyme-linked immunosorbent assay (ELISA) [22], phosphatase inhibition assay [23], gas chromatography–mass spectrometry (GC–MS) [24], liquid chromatography with UV detection (HPLC-UV) [25], capillary electrophoresis (CE) [26] and, more recently, liquid chromatography–mass spectrometry (LC–MS) [27–29]. LC combined with different detectors, such as UV detection or mass spectrometry (MS), can identify and quantify MCs in freshwater, cyanobacterial blooms, fish, shellfish and other biological samples [30–32]. LC–MS offers the advantage of providing specificity and good sensitivity. For this reason LC–MS has increased in popularity [33–35]. HPLC is a powerful tool to separate specific toxins; however the typical detection technique (UV) does not get near the sensitivity and the selectivity of LC–MS without extensive sample preparation or enrichment prior to analysis. ELISA methods offer a fast screening tool but can suffer from false positives depending upon the matrix. In addition, ELISA can confirm the presence of microcystins but does not identify which specific toxin is present. While chromatographic methods are capable of detecting and identifying single congeners, routine quantification of all known congeners is almost impossible because new analogues, especially of microcystins, continue to be discovered [36–38]. The quantitative analysis by LC–MS of microcystins has usually involved separation on C18 sorbents followed by electrospray ionization and detection by MS instruments [39–44]. Typical detection limits in recent work have been in the low pg range per injection. LC–MS has also been shown to provide valuable molecular weight information.

Because of the low provisional limits set by the WHO and Health Canada, effective consumer protection requires the sensitive and efficient detection of the whole spectrum of cyanobacterial cyclic peptide toxin congeners, many of which are as toxic as MC-LR, and regulation should not be restricted to MC-LR alone [45–46]. This, however, requires that the present methods for cyclic peptide toxin analysis be able to quantify the individual congeners with similar sensitivities and at concentrations well below the proposed limits

(because the toxic effects of the various congeners are expected to be additive).

1.5. Project objectives

The work presented in this article focused on the development of a simple, sensitive and selective LC–MS method to analyze as many of the target toxins as possible. Fig. 1 shows the list for the various toxins studied. One of the biggest challenges to monitoring these toxins is that very few of them are available as analytical standards. For this reason only six of the toxins, the only ones commercially available at the time, were analyzed. Four of the toxins (MC-LR, RR, YR and LA) are listed by the US EPA as the most important algal toxins in the United States, with MC-LR also listed by World Health Organization (WHO) as the most common toxin found.

2. Materials and methods

2.1. Chemicals and reagents

Certified MC standards (LR, RR, LF, LW and NDLN-R) were purchased from Calbiochem (EMD Chemicals, La Jolla, CA) and LR, RR, YR, LA were purchased from Sigma–Aldrich (Allentown, PA). Burdick and Jackson HPLC grade solvents (acetonitrile, methanol, water), glass fiber filters (Type A/E, 90 mm, 1 μm) and Gelman Acrodisc® CR PTFE syringe filters (13 mm, 0.45 μm) were obtained from Pall Corp., Ann Arbor, MI, USA. Mobile phase additives, ACS grade formic acid (98%) and trifluoroacetic acid (99%) were purchased from Sigma–Aldrich, Milwaukee, WI, USA). For method validation purposes, Sacramento River water and Rainbow Trout were obtained from the Nimbus Fish Hatchery, Rancho Cordova, CA. Mussels were purchased from a local fish market. A combined intermediate working solution of MCs was made in methanol from the purchased standards. A matrix spiking solution (20 μg/L) was prepared to fortify samples.

2.2. Sample storage

Tissue samples were kept frozen until time of extraction. Water samples for cyanotoxin analysis were refrigerated in the dark to prevent toxin degradation. Storage time was kept to a minimum (preferably less than 72 h). Where prolonged storage was required, samples were frozen. When dissolved toxin concentrations were required, samples were filtered as soon as possible and, if necessary, the filters and filtered water were frozen.

2.3. Sample preparation

2.3.1. Liquid sample extraction

MCs in water bodies at the time of a bloom are present in both the water (free, dissolved or extra cellular toxins) and the cyanobacterial cells (intracellular toxins). In order to determine total MC in the water, the cell wall must be ruptured or lysed by repeated freeze–thawing and sonication. An aliquot of sample (100 mL) was filtered under vacuum through a glass fiber filter (1 μm). The water and filters were extracted separately, as follows: (1) pre-filtered water samples were acidified with 0.1% FA and 0.05% TFA to obtain pH ~2 and extracted by SPE using J.T. BakerBond C18, 6 cc, 500 mg solid phase cartridges (Mallinckrodt Baker, Phillipsburg, NJ) mounted on a Resprep™ vacuum manifold (Restek Corp., Bellefonte, PA). The cartridges were first pre-conditioned with 10 mL methanol followed by 10 mL acidified water. The samples were loaded through the cartridges at a rate not to exceed 5 mL/min. The cartridges were then dried for 5 min with vacuum and finally, eluded with 2 × 1 mL mixture methanol:water (90:10) acidified with 0.1% TFA, vortexed and filtered through 0.45 μm filters to extend the life of the analytical column. (2) Filters with planktonic material or lyophilized biomass shellfish were extracted twice with 15 mL of methanol:acidified water (90:10, v/v) by homogenizing for 1–2 min using a Polytron, followed by 10 min sonication in an ultrasonic bath. The extracts were centrifuged and the supernatant was evaporated at 35 °C to 5 mL with rotary-evaporator. The concentrated extract was diluted to 100 mL in order to decrease the methanol concentration, acidified and cleaned-up using the SPE procedure.

2.3.2. Sample extraction

Tissue (mussel, liver, fish tissue) samples were homogenized using a Bucchi B-400 mixer equipped with a titanium knife assembly. A 2–5 g sample was transferred to conical centrifuge tubes with 10 mL methanol:acidified water (90:10, v/v) and finely-ground with an Arrow 850 tissue grinder (Arrow Engineering Co., Inc., Hillside, NJ) equipped with a glass pestle for 5 min, followed by sonication with a Branson® 3510 Ultrasonic for 1 h. The extracts were then centrifuged at 3500 rpm for 30 min using a HN-S centrifuge (Damon-IEC Division, Needham Heights, MA). The extract was reduced to minimum volume and diluted with water (not to exceed 5% methanol), acidified and cleaned-up using SPE, as described above. For samples with lipid >1%, an extra step is recommended by washing the SPE cartridge with 5 mL of 10% methanol:water solution before eluting the target analytes.

2.4. Analysis parameters and set up

The LC–MS used was an Agilent 1200 liquid chromatograph connected to an Agilent 6410 triplequadrupole (QqQ) mass spectrometer. The LC–MS was equipped with a vacuum degasser, binary pump, autosampler and a thermostatted column compartment maintained at 40 °C. The LC–MS was purchased from Agilent Technologies, Santa Clara, CA. Agilent Mass Hunter® software was used for identification and quantitation of target analytes.

2.4.1. Chromatography

The mobile phase consisted of HPLC water (A) and acetonitrile (B) (both contained 0.1% formic acid (v/v)). The gradient elution program started with 5% B and held for 2 min. The first linear gradient from 5% B to 50% B over 8 min, a second linear gradient from 50% B to 75% B over 3 min and held for 5 min before returning to initial mobile phase ratio at 19 min and held for 1 min. The run time was 20 min. The flow rate was set at 0.25 mL/min. The injection volume was 20 μL. After each run, the column was equilibrated for 5 min at the initial conditions before the next injection. A Guard column C18, 3.5 μm, 2.1 mm × 30 mm was used to protect the analytical column; dC18, 3 μm and 2.1 mm × 100 mm (Waters Atlantis). The effluent from the LC column was directed from the waste to the mass spectrometer source after the first 5 min of the run.

2.4.2. MS/MS-MRM parameters

Nitrogen (less than 1 ppm oxygen, Praxair, Rancho Cordova, CA) was used as the nebulizing and drying gas. The QqQ was operated using an electrospray ionization (ESI) interface in positive mode as follows: 350 °C drying gas temperature, 13.0 L/min drying gas flow, 40 psi nebulizer pressure, 110 fragment voltage and 4.0 kV electrospray capillary voltage.

Initial experiments were carried out using direct injection of high concentrations of individual toxin standards with Flow Injection Analysis (FIA). The full scan spectra obtained showed the exclusive presence of protonated molecular ions $[M+H]^+$ for all microcystins (MC) except MC-RR and dm-MC-RR, which had a doubly charged ion resulting from the presence of two arginine residues whose side chains are capable of retaining external protons and producing stabilized $[M+2H]^{2+}$.

Table 1
Time segments and MRM parameters.

Time segment #	Time (min)	Target compound	Scan type	Precursor ion	Product ions	Fragment (V)	Collision energy (V)
1	0	na[a]	MS2 Scan (to waste)				
2	5	MC-RR	MRM	520	213, 135.2	110	50
		dm-MC-RR	MRM	512.8	213, 135.2	110	50
3	12.9	MC-LR	MRM	995.7	213, 135.2	110	70
		dm-MC-LR	MRM	981.7	213, 135.2	110	70
		MC-YR	MRM	1045.6	213, 135.2	110	70
4	14.5	MC-LA	MRM	910.6	213, 135.2	110	70
5	15.5	MC-LF	MRM	987.6	213, 135.2	110	70
		MC-LW	MRM	1026.6	213, 135.2	110	70

[a] na: Not applicable.

Detection was accomplished using multiple reaction monitoring (MRM) mode. The MRM parameters are as follows: ultra-pure nitrogen gas was used for collision induced dissociation (CID). The protonated fragment ions served as the precursor ions for MRM mode. The transition from the precursor to the product ion was optimized by varying the voltage of collision induced dissociation (CID) gas from 0 to 50 eV. The voltages resulting in the most intense product ions were selected. The collision energy (CE) was set at 50 V for MC-RR/dm-RR and 70 V for the remaining MCs. The MRM windows were established for MCs using the product ions. Table 1 shows the optimum CE setting for all MCs and time segments of the MRM method. Typical MRM and reconstructed ion chromatograms are shown in Fig. 2. The fragment ions, m/z 135.2 and m/z 213 were chosen as quantifier and qualifier ions, respectively. Fragment of Adda at m/z 135.2 and fragment ion at m/z 227.1 correspond to $[\text{Glu-Mdhb}+\text{H}]^+$ were obtained for the internal standard, NDLN.

2.5. Calibration curve

To evaluate the linearity of the system various concentrations of certified MC mixture standard solution were prepared in methanol–water (90:10) (v/v) to obtain a seven level calibration curve ranging from 2 to 2000 pg on column. The instrument was calibrated for all the toxins except the desmethylated variants. A linear response was found between concentration and area for MCs. The linearity was very good for all MCs with correlation coefficient (r^2) greater than 0.998. The limit of detection in MRM mode was calculated using USEPA procedures found in Title 40 Code of Federal Regulations Part 136 (40CFR 136, Appendix B, revision 1.11) and were below 0.1 μg/L or lower for all the MCs.

Using the above listed MRM parameters, 1 μg/L microcystin standard mixture was easily detected and separated (Fig. 3). The limit of detection and quantitation for all microcystins were determine to be between 0.2 and 1 pg on column, with 5:1 S/N ratio. These values are below the 2 pg found in the available literature.

3. Results and discussion

3.1. Liquid samples method validation

3.1.1. Fresh water solid phase extraction (SPE)

The method was first tested with several types of SPE cartridges (Waters Oasis® HLB {n-vinylpyrrolidone-divinylbenzene copolymer}, J.T. Baker C18 {octadecysilane} and Phenomenex Strata X {surface modified styrene divinylbenzene}). Acidified water samples (100 mL), fortified with MCs mixture at 5 μg/L, were extracted using the SPE procedure detailed earlier in Section 2.3.1. The study demonstrated that J.T. Baker C18 cartridges extract all the tested microcystins and Nodularin from the water with acceptable recoveries as shown in Table 2.

Table 2
Microcystin solid phase extraction results (triplicate analysis).

MC congeners	C18-J.T. Baker % Recovery	C18-HLB Oasis % Recovery	Strata X % Recovery
MC-LR	109	58.8	60.3
MC-RR	143	95.4	114
MC-YR	118	60.5	60.0
MC-LA	48.1	18.6	13.2
MC-LF	157	13.2	ND
MC-LW	139	10.8	8.08
NDLN	82.8	56.3	50.0

The selected SPE C18 cartridges (J.T. Baker) were used to validate the method. River water samples ($n = 3$) and glass fiber filters ($n = 3$) were fortified with MCs and NDLN at 2 μg/L level then extracted following the procedure listed in Section 2.3.1. Average recoveries from triplicate analysis of all tested MCs ranged from 74.0 to 125% and from 73.8 to 110% for water and filter extracts, respectively. Nodularin recoveries ranged from 89.7 to 113% for both. MC-LW showed lower recoveries in water and filter extracts. The recovery of more hydrophobic microcystins such as MC-LW can be very poor [45]. Recoveries for all individual MC and the method detection limit (MDL) calculated from students t times standard deviation are listed in Table 3.

3.1.2. Fresh water direct injection

The triplequadrupole LC–MS operated in MRM can achieve extremely low detection of MCs (1 pg on column). For this reason a direct injection method was validated on the most common microcystins (MC-RR, LR and YR). A set of nine fortified river water samples (0.2 μg/L) were diluted with methanol to obtain (9:1) water–methanol (v/v). A portion of the sample was filtered through 0.45 μm Gelman filters then 20 μL was directly injected

Table 3
MC water (LCS) and filters (FS) method validation result.

Microcystin congeners	LCS Ave, %Recovery, $n = 3$	SD	RSD (%)	MDL = $t \times$ SD (μg/L)
MC-RR	101	1.45	1.43	0.01
MC-LR	106	4.51	4.25	0.01
MC-YR	118	6.08	5.15	0.02
MC-LA	94.3	5.90	6.25	0.01
MC-LF	81.5	6.48	7.95	0.02
MC-LW	73.6	7.69	10.4	0.02
Microcystin congeners	**FS Ave, %Recovery, $n = 3$**	**SD**	**RSD (%)**	**MDL = $t \times$ SD (μg/L)**
MC-RR	76.3	2.72	3.56	0.01
MC-LR	87.9	1.51	1.72	0.02
MC-YR	108	4.36	4.04	0.02
MC-LA	81.0	3.61	4.45	0.02
MC-LF	85.9	5.68	6.61	0.02
MC-LW	45.4	6.97	15.3	0.03

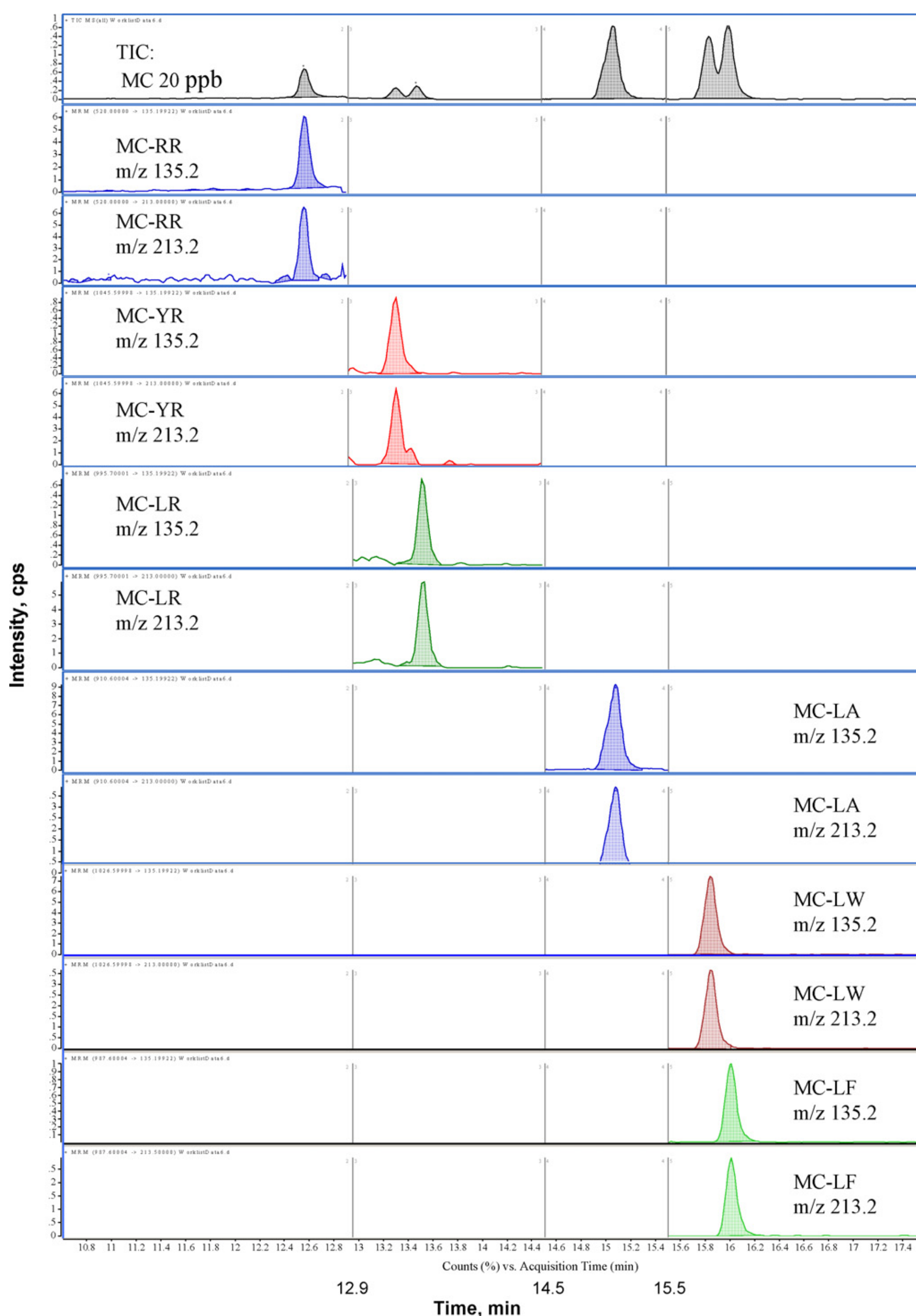

Fig. 2. MRM constructed ion chromatogram for microcystin standard.

into LC–MS/MS. The MDL for water using direct injection was determined to be 0.2 μg/L based on signal-to-noise equivalent to 5:1. The MRM results obtained in Table 4 shows the mean recoveries were 104, 97.0 and 95.4% for MC-RR, LR and YR, respectively, with RSD <11%.

3.1.3. Round Robin study

This validated method was tested by analyzing split contaminated water samples received as part of a Round Robin study organized by the Florida Department of Environmental Protection. Twelve laboratories throughout the United States participated in

Table 4
Percent recovery (%R) from direct injection results from 0.2 μg/L fortified water samples.

Toxins	LCS1 (%R)	LCS2 (%R)	LCS3 (%R)	LCS4 (%R)	LCS5 (%R)	LCS6 (%R)	LCS7 (%R)	LCS8 (%R)	LCS9 (%R)	Avg (%R)	SD
MC-RR	106	100	102	98.8	104	110	110	110	96.8	104	5.09
MC-LR	88.0	85.1	90.2	87.2	93.9	107	104	101	116	97.0	10.6
MC-YR	99.8	88.4	96.5	94.0	103	106	95.0	83.5	92.4	95.4	7.00

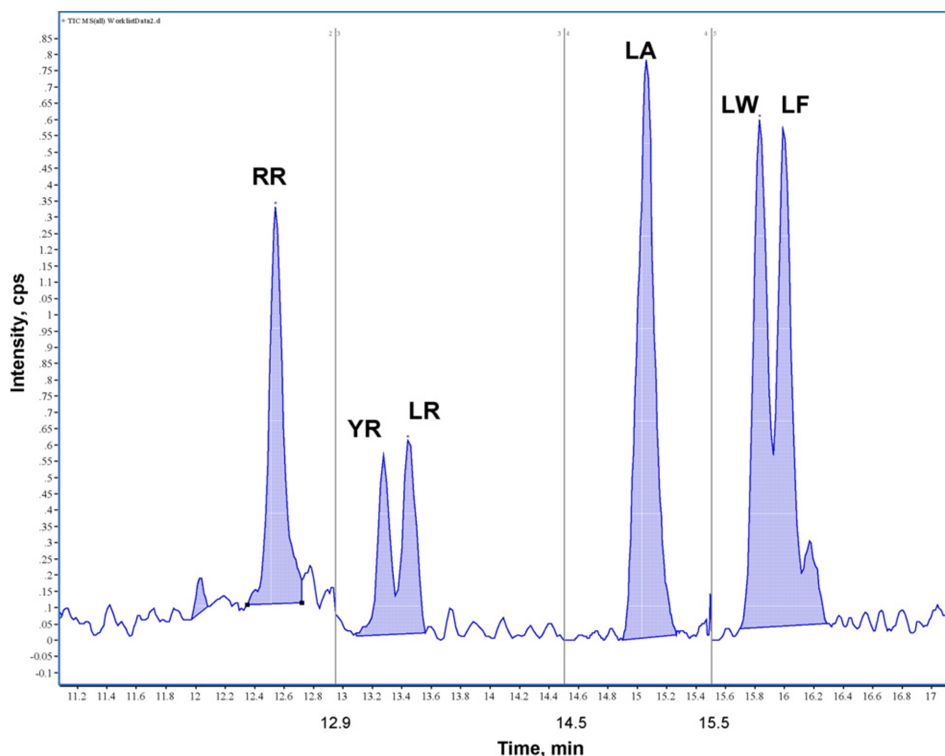

Fig. 3. Total ion chromatogram of a microcystin standard at 1 pg on column.

this exercise. Three types of water samples were received: (1) water from natural bloom (2007) occurred in Lake Munson (M) which was caused by cyanobacteria, *Microcystis aeruginosa*, (2) water sample containing toxin produced by cultures from University of Texas laboratory (T) and (3) a microcystin standard (S) diluted in water. Each laboratory was provided with 10 blind water samples. Either three or four replicates were provided to each laboratory for each sample type. Laboratories were required to hold the samples in the dark at 4 °C for no more than one week before analysis. Two different extractions were performed on the water samples for comparison:

(a) An aliquot of round robin water sample (200 mL) was filtered through 0.45 μm glass fibers filters then extracted with SPE cartridge. The filters were sonicated and both SPE and filters were extracted according to the procedure outlined in Section 2.3.1.
(b) The second extraction consisted of 1 mL of methanol added to 9 mL of the round robin water sample sonicated for 45 min, centrifuged for 30 min and a portion of the methalonic solution was filtered thru Gelman filters and directly injected into LC–MS/MS. Tables 5–7 lists the results from both types of extractions.

Results in Table 5, obtained from the analysis of round robin sample (S), show the presence of low levels of MC-LR (~2 ppb). The combined results obtained from SPE + filters correlate well with the direct injection results. The average MC-LR value obtained from sonication was 2.90 ppb compare to 1.71 ppb obtained with SPE and filters, the difference could be contributed to losses from sample preparation steps.

Table 6 shows the results of a comparison of direct injection vs. SPE + filters for the Texas culture samples. With an average of 62.9 ppb for the direct injection compare to 48.1 ppb obtained by SPE extraction for total MC. The RSD values were below 5% for all replicates. Most of the reported concentrations were from MC-LR but desmethylated microcystins (dmMC-RR and dmMC-LR) were also found in these samples with an average value of 5.82 and 17.13 ppb, respectively.

MC-RR, MC-LR and MC-YR were found in Munson Lake samples with an average of 68.2, 76.5 and 1.68 ppb, respectively. The concentrations of total microcystins obtained from direct injection of the four replicates were nearly the same as those obtained from SPE + filters and were 151 and 173 ppb, respectively. The desmethylated microcystins (dmMC-RR and dmMC-LR) were also found in the samples with an average of 70.2 and 66.5 ppb, respectively (Table 7).

In summary, the results obtained from direct injection showed better precision than those from the combined SPE and filters extractions. This LC–MS/MS technique was also able to detect

Table 5
Comparison of direct injection vs. SPE and filters from Round Robin MC-LR standard (S).

	S-1	S-2	S-3	Avg (μg/L)	SD	RSD (%)
Direct injection	2.80	2.96	2.96	2.91	0.09	3.18
SPE	0.37	0.49	0.55	0.47	0.09	19.4
Filters	1.28	1.27	1.17	1.24	0.06	5.12
Total						
Direct injection	2.80	2.96	2.96	2.91	0.09	3.18
SPE + filters	1.65	1.76	1.72	1.71	0.06	3.26

Table 6
Comparison of direct injection vs. SPE and filters from University of Texas culture (T).

	T-1	T-2	T-3	Avg (μg/L)	SD	RSD (%)
Total MC						
Direct injection	65.0	60.6	63.1	62.9	2.21	3.51
SPE + filters	49.7	47.5	45.9	47.7	1.91	4.00
Microcystins						
dm-MC-RR[a]	6.20	5.65	7.61	6.49	1.01	15.6
dm-MC-LR[a]	16.9	16.5	18.0	17.1	0.78	4.53

[a] Desmethyl analog quantified as parent compound.

Table 7
Comparison of direct injection vs. SPE and filters from Lake Munson (M) natural bloom.

	M-1	M-2	M-3	M-4	Avg (μg/L)	SD	RSD (%)
Direct injection							
MC-RR	57.3	71.9	72.7	70.7	68.2	7.28	10.7
MC-LR	63.6	84.2	79.4	78.8	76.5	8.93	11.7
MC-YR	1.80	1.50	1.50	1.90	1.68	0.21	12.3
Total MCs	123	158	154	151	147	15.9	10.9
SPE							
MC-RR	60.3	67.3	60.3	72.1	65.0	5.77	8.88
MC-LR	58.5	81.5	70.0	80.4	72.6	10.8	14.8
MC-YR	0.19	0.79	1.25	0.65	0.72	0.44	60.6
Total MCs	119	150	132	153	138	16.0	11.6
Filters							
MC-RR	9.88	11.4	16.7	10.1	12.0	3.21	26.7
MC-LR	8.73	7.08	8.96	9.08	8.46	0.93	11.0
MC-YR	0.35	0.54	0.74	0.54	0.54	0.16	29.4
Total MCs	19.0	19.0	26.4	19.7	21.0	3.62	17.2
Total MC							
Direct injection	123	158	154	151	146	15.9	10.9
SPE + filters	138	169	158	173	160	15.7	9.83
Microcystins							
dm-MC-RR[a]	60.0	74.2	74.2	72.6	70.2	6.87	9.79
dm-MC-LR[a]	56.5	72.5	68.1	68.8	66.5	6.92	10.4

[a] Desmethyl analog quantified as parent compound.

the presence of desmethylated variants. This laboratory was the only participating laboratory reporting the desmethylated variants. Since desmethylated standards were not available, the dmMC values were estimated using the methylated congeners' response factors.

3.2. Biota samples (fish and mussels)

California coastal mussels, oysters, Rainbow Trout fillets and livers were used for method development and validation. Samples (2–5 g, fresh weight) were homogenized using a Polytron, fortified with 5 ng/g microcystins mixture standard and extracted with methanol–water (90:10) using the sonication procedure listed in Section 2.3.2. Recovery experiments were performed using replicate samples. The results show that all tested MCs were efficiently extracted. The recoveries obtained from fortified mussels, ranged from 79.9 to 104% with percent RSD <15 ($n = 8$).

The method detection limit (MDL) calculated from Student's t times standard deviation for mussels ($n = 8$) was determined to be $\leq$1 ng/g using MRM.

The average microcystin recovery for oysters ($n = 4$) was 102% with average standard deviation of $\pm$14.9. The mean recoveries were 106% for fish fillet ($n = 4$) and 85.7% for fish liver ($n = 3$). The % RSD was less than 11% for both.

4. Method application

4.1. Lake Success incident

In the middle of July 2007, US Army Corps of Engineers reported a major fish kill at Lake Success, Tulare County, California. Ten western grebes were also found dead around the lake. The number of bird and fish deaths increased to 5000 by August 3. *Flavobacterium columnar* (columnaris disease) was observed on the fish gills. It was unclear if the bacterial infection or cyanobacterial toxin was the direct cause of the deaths. A mixture of dead fish livers and fish guts were received, extracted and analyzed using the validated method. The results obtained show the presence of microcystins with MC-LR as the predominant variant, which most likely is the cause of death. Table 8 summarizes the results obtained from these samples. This was the fourth incident that involved the presence of algal blooms concurrently with a fish die-off in this lake within the past ten years.

4.2. Klamath River

In August 2007, analysis of water taken from Copco and Iron Gate reservoirs located on Klamath River near the Oregon border revealed high levels of MC-LR produced by *M. aeruginosa*. This toxin was found as far away as 125 miles downstream of the reservoirs. Mussels and fish (fillet and liver) were collected from the river and reservoirs. The results of sample extract analysis (Table 9) shows that the mussels collected from Klamath river (filter feeders) had high concentrations of MC-LR (396 μg/g) and five times higher concentrations of MC-LA. Low levels of MC-RR and demethyl-LR were also found in the mussel extracts. The fish (yellow perch) fillet extracts showed mainly the presence of MC-YR and demethyl-LR. The fish liver extracts showed the presence of MC-LA, demethyl-LR and demethyl-RR. As a result of these findings, signs were posted at the sites to alert the public of the danger associated with the consumption of mussels and fish from those areas. The Klamath River is rich in nutrients that supports the growth of blue-green algae. Warm and calm surface water in Iron Gate and Copco reservoirs provide an ideal environment for the growth of large algal blooms. The levels of algae and associated toxins measured are high enough

Table 8
Algal toxin concentrations from one dead black crappie collected at Lake Success, Tulare County, California, July–August 2007.

Microcystin analytes	Fish liver and stomach content ng/g (ppb) Wet wt.	Fish liver and stomach content ng/g (ppb) Wet wt.	Mean ng/g (ppb) Wet wt.
MC-RR	<1.0	<1.0	
dm-MC-RR[a]	<1.0	<1.0	
MC-LR	172	225	198
dm-MC-LR[a]	<1.0	<1.0	
MC-YR	51.7	60.1	55.9
MC-LA	79.0	96.8	87.9
MC-LF	46.4	68.5	57.5
MC-LW	<1.0	<1.0	
Total Microcystins	349	450	399

[a] Desmethyl analog quantified as parent compound.

Table 9
Klamath River toxin results (ng/g) from mussels and yellow perch (fish fillet and liver), July 2007.

Microcystin analytes	Klamath River	Irongate Reservoir		Copco Reservoir		
	Mussel	Fish	Liver	Fish	Liver	Mussel
MC-RR	ND-136		ND-15			
dm-MC-RR[a]			ND-42		25–62	
MC-LR	58–396					
dm-MC-LR[a]	5–37	ND-227		58–422	138–426	
MC-YR		ND-4.2		ND-3.2		
MC-LA	138–2,200		ND-13		8–15	32–57
MC-LF						
MC-LW	ND-14					

ND: non-detect.
[a] Desmethyl analog quantified as parent compound.

Table 10
Pinto Lake toxin results from water samples collected in October 2007.

Microcystin analytes	Pinto Lake 1′ deep water @ end of boat ramp (μg/L)	Pinto Lake surface scum @ beginning of ramp (μg/L)
MC-RR		33.3
dm-MC-RR[a]		
MC-LR	33.3	760,000
dm-MC-LR[a]	1.67	33,000
MC-YR		44.4
MC-LA	61.1	2,100,000
MC-LF		
MC-LW		7.29

[a] Desmethyl analog quantified as parent compound.

to pose health risks to anyone drinking or bathing in the water, particularly children and animals [5,45–46].

4.3. *Pinto Lake*

Pinto Lake is located on the central coast of California. In October 2007, surface scum and water samples were collected from this lake, extracted and analyzed for microcystins using this method. Sample extracts showed extremely high levels of MC-LA (2,100,000 μg/L), MC-LR (760,000 μg/L) and desmethyl-MC-LR (33,000 μg/L). As a consequence of these findings the County of Santa Cruz Environmental Health Services issued an advisory to warn the public to avoid contact with the water. Table 10 summarizes the water and scum results obtained from the analysis.

5. Conclusion

The method was developed and validated for measuring trace concentrations of microcystin toxins (MCs) including desmethyl microcystins (dm-MCs), in different matrices (water, mussels, fish fillet and liver) using LC–MS/MS. The limit of detection and quantitation for all microcystins were determined to be between 0.2 and 1 pg on column. These concentrations are below the 2 pg detection limits found in the available literature. This method enables the quantification of nodularin and microcystins in a single 30 min chromatographic analysis using a simple gradient elution. Analysis of selected microcystins using this method allowed for easy quantification of the target compounds below 1 μg/L levels with no sample preparation required other than filtering the water samples. Since there are no available microcystin certified reference materials (CRMs), accuracy and precision of the method were determined using fortified and unfortified samples. The method was used in a round robin and gave excellent precision and results that were similar to other participating laboratory's LC–MS results. SPE has shown that it can be used for 10–100-fold target analyte enrichment.

The validated method proved to be a powerful tool for monitoring trace levels of harmful algal bloom toxins in different matrices.

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APPENDIX N

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Characterization and deployment of Solid Phase Adsorption Toxin Tracking (SPATT) resin for monitoring of microcystins in fresh and saltwater

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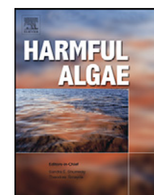

Characterization and deployment of Solid Phase Adsorption Toxin Tracking (SPATT) resin for monitoring of microcystins in fresh and saltwater

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ABSTRACT

Fresh and brackish water cyanobacterial blooms and their associated toxins appear to be increasing globally. Current sampling methodologies for cyanotoxins typically involve point (grab) samples, and are subject to variability due to spatial and temporal heterogeneity, hydrological conditions, and the presence or absence of surface accumulations (scums) of algae. To overcome some of these issues, passive samplers including Solid Phase Adsorption Toxin Tracking (SPATT) and Polar Organic Compound Integrative Samplers (POCIS) have been used for primarily marine phycotoxins with more limited application to freshwater toxins. In this study SPATT was evaluated in both the lab and the field for use as an integrative sampler for microcystins, deployed in freshwater using DIAION HP20 resin. HP20 exhibited excellent adsorption and recovery characteristics for microcystin-LR, -YR, -LA, and -RR. Approximately weekly deployments of SPATT in Pinto Lake, CA were conducted for 16 months and compared to traditional (grab) samples. SPATT proved to be robust, detecting microcystins during every deployment in contrast to the grab samples, 42% of which were below the limit of detection using liquid chromatography–mass spectrometry for microcystin-LR. A simple canonical correlation model was built to determine if toxin concentrations co-varied with environmental parameters such as water temperature, nutrient concentrations, chlorophyll *a*, rainfall, or other easily obtained variables. The best individual correlate to toxin concentration was total biomass (chlorophyll *a*), while the first principal axis of the canonical correlation included chlorophyll *a* and total dissolved nitrogen as statistically significant variables. Overall, SPATT proved to be a useful adjunct or replacement for traditional grab samples.

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1. Introduction

Cyanobacterial blooms and their associated toxins have become increasingly problematic globally (Chen et al., 1993; Dawson, 1998; Amorim and Vasconcelos, 1999; Domingos et al., 1999; Lehman et al., 2005; Guo, 2007; Paerl and Huisman, 2008). *Microcystis aeruginosa* in particular is considered a cyanobacterial harmful algal bloom (CHAB) organism because it can impede recreational use of waterbodies, reduce aesthetics, lower dissolved oxygen concentration, and cause taste and odor problems in drinking water, as well as produce microcystins, powerful hepatotoxins associated with liver cancer and tumors in humans and wildlife (Carmichael, 2001). Extensive *Microcystis* blooms with toxin production occur during summer and fall in impaired waterways in Washington, Oregon and California (Gilroy et al., 2000; Johnston and Jacoby, 2003) and *Microcystis* contamination has been documented at the marine outflows of the Klamath and San Francisco estuaries (Lehman et al., 2005; Fetcho, 2007) as well as from river inputs to Monterey Bay (Miller et al., 2010). The recently documented direct impact to the threatened California Sea Otter (*Enhydra lutris*) has also promoted these blooms and toxins from predominantly a freshwater issue to potentially a land–sea problem, with concomitant risk because of the lack of monitoring in brackish and marine waters (Miller et al., 2010).

Microcystis growth and toxin production has been linked to high nutrient concentrations, increased salinity, warm temperatures, increased vertical stratification of lakes, summer droughts and increased light intensity; all of these factors can be exacerbated by global climate change (Zehnder and Gorham, 1960; Welker and Steinberg, 2000; Guo, 2007; Paerl and Huisman, 2008; Davis et al., 2009). Cyanobacteria such as *Microcystis* can exploit these conditions by accumulating in dense surface blooms that “shade out” nontoxic phytoplankton, thereby increasing local water temperatures through light absorption and creating a positive feedback loop leading to more blooms (Paerl and Huisman, 2008).

Microcystin and other biotoxins can exert their effects in regions that are remote from sources of toxin production and can bio-accumulate in invertebrates and fish, suggesting efficient

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means for exposure to freshwater-derived toxins downstream as well as at the land–sea interface (Garcia et al., 2010; Lehman et al., 2010; Miller et al., 2010). Given the severe and ubiquitous nature of this problem in freshwater habitats and potentially coastal marine systems, surveillance and monitoring is critical. Traditional monitoring programs for phycotoxins typically rely on discrete sampling ("grab" samples) from a particular site or sites, sometimes augmented with automated sampling systems. Such methods are inherently biased if the sampling does not capture the spatial and temporal variability of the system due to (e.g.) behavioral adaptations of the algae such as vertical migration, hydrologic or circulation effects, and ephemeral or episodic events. Furthermore, grab sampling may underestimate the presence of low levels of toxins if the sampling protocol does not include pre-concentration and/or if the toxin concentrations are below the analytical limit of detection.

To overcome some of these issues, various types of passive integrated samplers have been developed and deployed for environmental contaminants and toxins (see reviews in Górecki and Namiésnik, 2002; Kot-Wasik et al., 2007; Seethapathy et al., 2008; MacKenzie, 2010). Solid Phase Adsorption Toxin Tracking (SPATT; MacKenzie et al., 2004) in particular has been widely adopted for the detection of marine phycotoxins (MacKenzie et al., 2004; Lane et al., 2010; MacKenzie, 2010). For freshwater and brackish systems, both SPATT and Polar Organic Compound Integrative Samplers (POCIS) have been deployed successfully to detect or monitor microcystins (Kohoutek et al., 2008, 2010; Miller et al., 2010) and anatoxins (Wood et al., 2011), but characterization and adoption of SPATT for these systems has been much more limited.

Prior to this study, SPATT deployments with DIAON HP20 have been used extensively in marine waters, primarily targeting lipophilic algal toxins and the water-soluble compound domoic acid (cf. MacKenzie et al., 2004; MacKenzie, 2010; Lane et al., 2010). A limited analysis in marine waters using both field deployments and tank experiments (Miller et al., 2010) also documented the potential for detection of microcystins from HP20 SPATT, but the efficacy of SPATT was not fully evaluated. This study demonstrates the potential to use SPATT with a single resin (DIAON HP20) to detect multiple phycotoxins from various environments.

The aims of this study were to characterize SPATT using the resin DIAION HP20 for passive sampling of microcystins in freshwater, and to demonstrate the applicability of SPATT sampling compared to traditional grab sampling using a known CHAB "hotspot", Pinto Lake, California (Miller et al., 2010). Secondarily, the data collected from this study were used to determine whether there are any easily measured environmental correlates that could be used to predict toxin loads for this system.

2. Materials and methods

2.1. Study area

Pinto Lake is a shallow natural lake located 8.3 km inland from Monterey Bay (Fig. 1). It is connected to the Pacific Ocean through an overflow drainage system into Corralitos Creek, which in turn

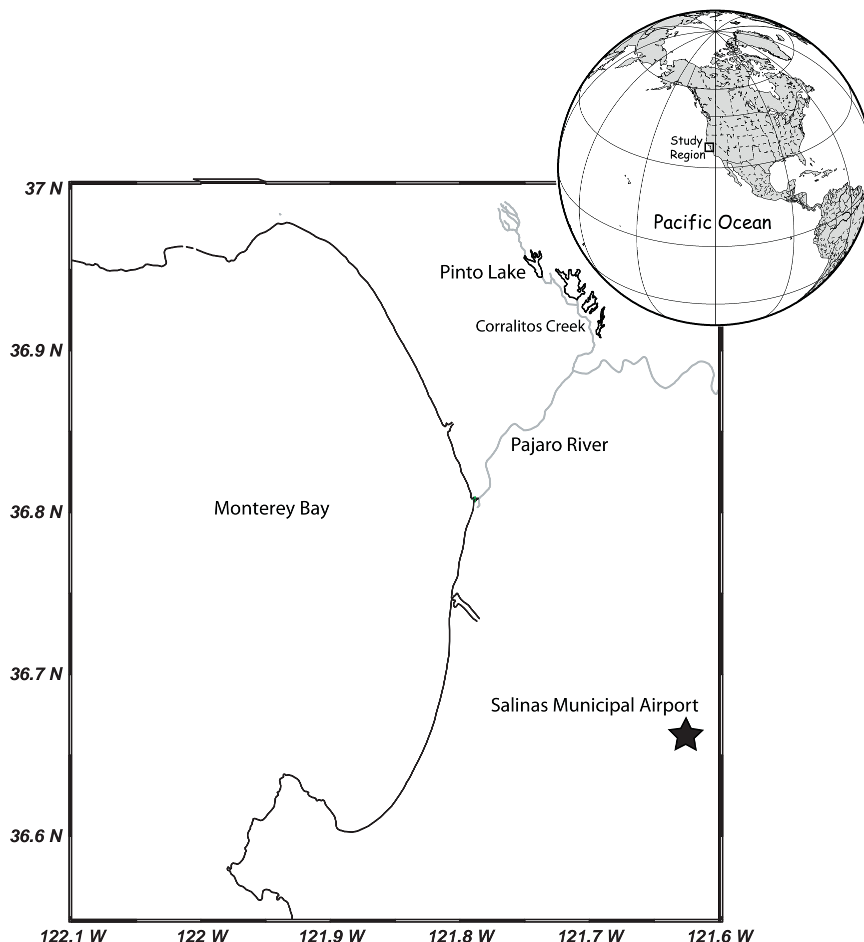

Fig. 1. Map of Monterey Bay, California showing the location of Pinto Lake, Corralitos Creek, and the Pajaro River. The Salinas Municipal Airport (star) is also indicated.

drains into the Pajaro River. This spring-fed lake has a maximum depth of ~10 m and covers 370,000 m^2 (37 surface hectares). Pinto Lake includes parks operated by the City of Watsonville and Santa Cruz County, and is regularly stocked for recreational fishing by the California Department of Fish and Game.

2.2. SPATT construction and toxin analysis

DIAION HP20 was purchased from either Sorbent Technologies, USA, or Sigma–Aldrich. SPATT construction and activation followed the methods described by Lane et al. (2010). Toxin analysis was conducted using an Agilent 6130 liquid chromatography–mass spectrometry (LC–MS) system with an Agilent Zorbax Rapid Resolution column and Selected Ion Monitoring (SIM) of microcystin-LA, -LR, -RR, and -YR generally following the method of Mekebri et al. (2009) but adapted from an LC–MS-MS system to LC–MS as described in Miller et al. (2010). Sample concentrations were determined by calibration with certified standards obtained from various sources (Mekebri et al., 2009; Miller et al., 2010). The Method Detection Limit (MDL) was determined to be <1 ppb ($\mu g\ L^{-1}$) on-column for all toxin congeners. For a subset of samples, toxin extracts were split and run using both LC–MS and commercially available ELISA kits from Abraxis LLC (PN 520011) and Envirologix (PN EP 022) following the manufacturer's guidelines but with additional dilution using the manufacturer-supplied buffer solutions. Grab sample toxins are reported as total MC-LR (dissolved plus particulate) analyzed from whole water with sonication to disrupt cell membranes.

2.3. SPATT extraction protocol

For both laboratory and field samples, SPATT bags were rinsed with deionized water (Milli-Q) and processed using methods described by Lane et al. (2010) with the following modification: SPATT bags were cut open, and the resin was extracted out-of-bag (as free resin) without vortexing; following the first 10 mL 50% methanol (MeOH) extraction, two more sequential extractions with 20 mL 50% MeOH were performed, and the three extracts were analyzed separately. All extracts were analyzed immediately using LC/MS; re-analysis of extracts stored at either room temperature or −20 °C for up to 12 months showed no signs of degradation. Similarly, whole SPATT bags stored at −20 °C exhibited no loss of microcystins for up to 3 months (longer time periods were not assessed).

2.4. Adsorption and extraction efficiency

The adsorption profile for SPATT bags was determined by laboratory trial. SPATT resin bags were incubated, in triplicate, in aliquots of microcystin-fortified filtered Pinto Lake water or seawater in 125 mL glass flasks with silicone stoppers with target initial concentrations of 100 $ng\ L^{-1}$ for each congener (actual concentrations varied from 61 to 184 $ng\ L^{-1}$). Pinto Lake water was obtained from the near surface at the Pinto Lake boat dock, filtered through a 0.2 μm filter, and stored in the dark at 4 °C prior to use. Saltwater was obtained from Moss Landing Marine Laboratories from a sand-filtered flowing seawater system. The seawater was 0.2 μm filtered and stored as for the Pinto Lake water. Basic characteristics of these water samples was salinity = 1.4 and 35.1 (Pinto Lake and seawater), pH = 8.22 and 7.92. The incubations were maintained at a controlled temperature (15 °C) with constant agitation (70 rpm) with 12:12 illumination using "cool white" lamps at approximately 125 $\mu mol\ photons\ m^{-2}\ s^{-1}$. The sample water was regularly assayed for toxin to monitor adsorption by the resin. Three trials were conducted, two for Pinto Lake water spanning 1 and 12 h, and one trial with filtered seawater spanning 1 h. The SPATT bags used for the determination of adsorption efficiencies were subsequently extracted to determine recovery efficiency. To determine SPATT desorption characteristics, the extraction protocol described in the previous section was used but with 10- (first trial) and 50-mL (second trial) sequential 50% MeOH extractions to determine optimal recovery volumes. To determine resin saturation, small (ca. 100 mg) concentrations of free resin were exposed to Pinto Lake water fortified with an excess of MC-RR, MC-YR, and MC-LR (using toxin standards) and kept at room temperature in the dark for 7 days. Total adsorption was determined by the difference between control (no resin) and final free toxin concentrations at the end of the 7-day period; samples were re-tested at 14 days to ensure equilibrium had been reached. The saturation test was conducted in triplicate (fortified resin) with a single control (no resin present).

2.5. Field deployment and environmental variables

SPATT was deployed approximately weekly (3–19 day deployments) from 30 August 2009 to 2 January 2011 at the southern end of Pinto Lake. The majority of deployments (51 out of 64) were 7 ± 1 days; shorter deployments occurred during the peak bloom period during Autumn 2010 to coincide with more frequent grab samples, while longer deployments typically occurred because of logistical issues (e.g. holidays, lack of access to the boat dock during flooding). For deployment, SPATT bags were clamped into plastic embroidery hoops (Susan Bates® HOOP-La, 7.6 cm diameter), and secured to a weighted rope with a plastic zip-tie at approximately 0.5 m depth from the public boat dock in approximately 3 m water depth. Toxin values are reported normalized to 1 g of resin and per days of exposure (e.g. ng toxin per gram resin per day) and were not corrected for estimated extraction efficiency.

Environmental data included simultaneous collection of the macronutrients nitrate + nitrite (hereafter referred to as nitrate), ammonium, urea, phosphate, silicate; total dissolved nitrogen (TDN), and by subtraction, dissolved organic nitrogen (DON), total dissolved phosphorus (TDP) and by subtraction, (DOP), biomass as chlorophyll *a*, non-purgeable organic C (NPOC), and water temperature. Samples were collected in conjunction with the grab samples at the beginning/end of each SPATT deployment in a glass collection bottle from the near surface (<0.5 m), transported to the laboratory and immediately processed for analysis or storage. Nutrients and biomass were determined following standard protocols (Solórzano and Sharp, 1980; Mulvenna and Savidge, 1992; Welschmeyer, 1994; Lachat, 2010); NPOC and TDN were measured following manufacturer's guidelines on a Shimadzu TOC/VCPH TOC/TN analyzer. Additional data were obtained from the Salinas Municipal Airport for daily average air temperature and precipitation. Dominant phytoplankton groups were determined (to genus) by visual inspection of whole water collected from the near surface (0–0.5 m) using a Leica MZ125 stereomicroscope, but were not enumerated. Not all ancillary data were collected for all SPATT deployments as noted in Table 1. Sampling for toxins were initiated on 30 August 2009; ancillary sampling was added approximately 1 month later, after determining that environmental data from other monitoring programs were not available for this analysis. The first two chlorophyll samples were lost during storage, while urea measurements were not added until 24 January 2010 after determining that urea concentrations were not negligible in Pinto Lake.

2.6. Statistics

Relationships between environmental variables and toxins were determined using two statistical packages after applying a log transform, $\ln(X + 1)$, to all data. Correlations and regressions were

Table 1
Toxin data and environmental variables collected for the Pinto Lake time series and used in the statistical models.

Variable	Dates collected (n)	Mean (S.D.)	Range
Microcystin-LR grab samples (ppb)	30-Aug-09/2-Jan-11 (66)	33.25 (183.65)	0.00–1424.9
Microcystin-LR SPATT (ng/g resin/day)	30-Aug-09/2-Jan-11 (62)	137.66 (194.22)	1.01–895.4
Precipitation (in.)	30-Aug-09/2-Jan-11 (66)	0.05 (0.28)	0.0–0.47
Air temperature (°C)	30-Aug-09/2-Jan-11 (66)	14.31 (3.28)	5.0–20.56
Water temperature (°C)	30-Aug-09/2-Jan-11 (66)	17.43 (5.35)	3.64–26.23
Chlorophyll a ($\mu g L^{-1}$)	22-Nov-09/2-Jan-11 (52)	197.78 (565.25)	3.09–2975
Nitrate + nitrite (μM)	8-Nov-09/2-Jan-11 (54)	15.29 (18,24)	0.0–57.28
Phosphate (μM)	8-Nov-09/2-Jan-11 (54)	9.25 (5.84)	3.52–35.86
Silicate (μM)	8-Nov-09/2-Jan-11 (54)	70.24 (43.62)	2.62–157.92
Ammonium (μM)	8-Nov-09/2-Jan-11 (54)	21.90 (23.66)	0.13–78.94
Urea (μM)	24-Jan-10/2-Jan-11 (45)	1.79 (1.19)	0.33–4.45
DON (μM)	8-Nov-09/2-Jan-11 (54)	111.42 (45.45)	15.90–223.96
DOP (μM)	8-Nov-09/2-Jan-11 (54)	2.00 (1.43)	0.0–6.55
NPOC ($mg L^{-1}$)	8-Nov-09/2-Jan-11 (54)	23.52 (11.07)	12.02–51.63
Total dissolved N (μM)	8-Nov-09/2-Jan-11 (54)	31.49 (28.53)	0.80–88.54
Total dissolved phosphorus (μM)	8-Nov-09/2-Jan-11 (54)	2.00 (1.43)	0.69–7.00

determined using Mystat 12 (SPSS). Canonical correlation was performed using CANOCO v.4.5 with the dependent variables total MC-LR (grab samples) and SPATT MC-LR, and the independent variables provided in Table 1, along with the molar nutrient ratios Si:N and N:P. Dates with missing values for environmental correlates (Table 1) were removed, resulting in $n = 52$ for the statistical analysis. Significance was set at $p < 0.05$ for all tests. After first testing the data using detrended correspondence analysis (DCA), redundancy analysis (RDA) was chosen as the preferred ordination method.

3. Results

3.1. HP20 adsorption characteristics

Laboratory-based adsorption profiles for microcystins are presented in Figs. 2 and 3. Since all four microcystin congeners exhibited nearly identical absorption characteristics, all points were combined and fit to a two-phase exponential decay function. The fit was excellent, with an r^2 value of 0.94. Initial concentrations varied by about a factor of 3 (61–184 $ng L^{-1}$) but there was no apparent bias in the adsorption profiles when normalized to the initial concentration. Control samples (no SPATT bag added) exhibited no signs of loss due to adsorption to the glass sample bottles or degradation during the incubations. Laboratory trials demonstrate that toxin is rapidly (hours) adsorbed to HP20 (Figs. 2 and 3) in both Pinto Lake and seawater. Saturation testing provided a value of 18,400 ng/g resin (equal parts MC-RR, MC-YR, MC-LR). During the Pinto Lake time series the maximum dissolved MC-LR measured from grab samples was 66.4 $ng L^{-1}$ and the maximum total (dissolved and particulate) value was 1425 $ng L^{-1}$ (25-October-2009). Corresponding SPATT extracts of 736 and 895 ng/g were obtained for the week before and after that sampling date, well below the saturation threshold obtained in the laboratory.

3.2. Desorption and recovery

Extraction efficiencies from laboratory samples were excellent, with 100% recovery using sequential 50% MeOH extractions (Fig. 4). There was no loss of toxin when rinsing with relatively large (100–500 mL) volumes of Milli-Q water, nor was it necessary to adjust the pH of the extraction solution. In contrast to the adsorption curves, there was a clear difference between MC-RR and the other congeners, with MC-RR exhibiting much lower sequential recoveries but with 90–100% recovery when using 250 mL 50% MeOH. A standard extraction protocol utilizing sequential extractions of 10, 20, 20 mL 50% MeOH was chosen for routine analysis of field samples resulting in 58–100% recovery based on the laboratory desorption studies. Desorption carried out using filtered seawater as an adsorption matrix followed by MQ rinses of the SPATT resin prior to extraction showed no difference compared to the use of filtered Pinto Lake water (data not shown).

Since ELISA kits are commonly used for measuring microcystins, a subset of SPATT extracts from the adsorption/desorption tests were analyzed using Abraxis and Envirologix kits. The Envirologix kit showed some suppression of the standard curve (reduced dynamic range) when directly analyzing 50% MeOH; this was compensated for by using standards in the same matrix, albeit with reduced kit sensitivity. The Abraxis kit failed with samples that contained >5% MeOH.

3.3. Field deployment

The results of SPATT field deployment at the Pinto Lake boat dock are shown in Fig. 5, together with other environmental

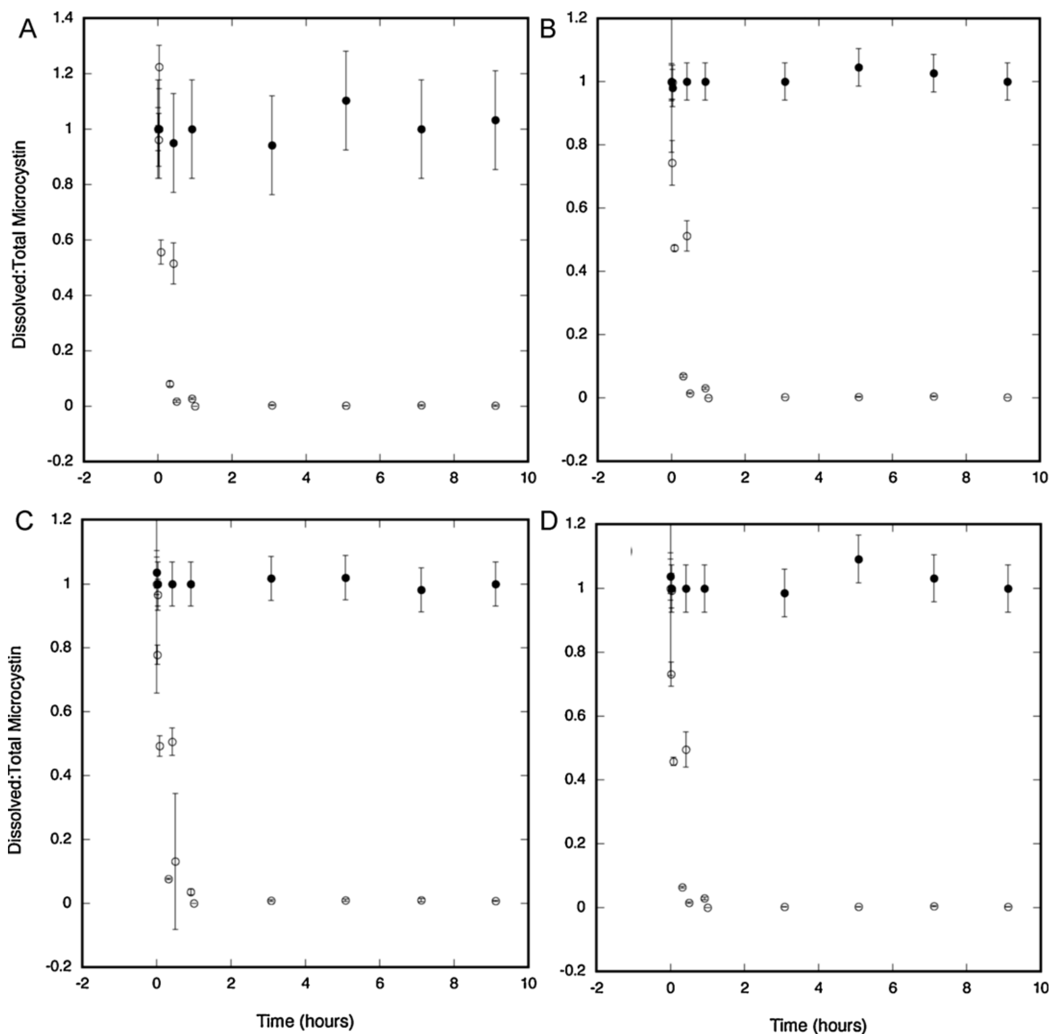

Fig. 2. Adsorption profiles normalized to the initial (control) concentrations for HP20 SPATT bags assessed in the laboratory. Congener-specific adsorption using filtered Pinto Lake water control (no SPATT) treatment data are also provided (open symbols). (A) MC-RR, (B) MC-YR, (C) MC-LR, and (D) MC-LA. Error bars represent SD of three replicates.

parameters. Since the predominant toxin was MC-LR, other compounds are not reported (but were occasionally detectable in both SPATT and grab samples). During the 16-month deployment period SPATT always detected MC-LR in Pinto Lake. In contrast, 42% of the time there were undetectable levels of microcystins in the grab samples. Despite this discrepancy there was good correlation between the two toxin measurements when comparing time-averaged grab samples (beginning/end of each SPATT deployment) versus SPATT (Spearman $r = 0.735$, $p < 0.001$). Therefore for canonical correlation analysis the two toxin measurements were treated as dependent variables in the analysis.

3.3.1. Environmental characteristics

Central California exhibits moderate seasonality with warmer summer temperatures and a rainy winter season (Fig. 5). In Pinto Lake, total phosphorus concentrations never reached zero, NPOC was elevated relative to other lakes (Mulholland, 2003), and nitrogen compounds were nearly always detectable (Table 1 and Fig. 5). Biomass as chlorophyll was also extreme, averaging 197.98 $\mu g\,L^{-1}$ with a peak value of 2975 $\mu g\,L^{-1}$ during a nearly monospecific *Microcystis* bloom. These characteristics place Pinto Lake in the eutrophic to hypereutrophic category for lakes (Carlson, 1977).

The time series at Pinto Lake captured two large toxin events in autumn 2009 and 2010 corresponding to maximal water and air

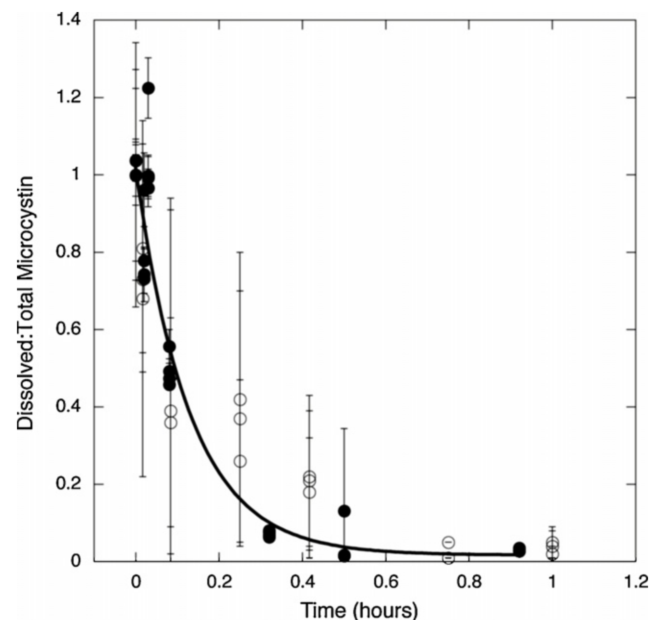

Fig. 3. The data in Fig. 2 are plotted together (solid symbols) and fit with a two-phase exponential decay function ($r^2 = 0.94$). Open symbols represent the same data analysis applied to the saltwater treatment.

temperature, with lower but persistent levels of toxins through the remainder of the series. *Microcystis* was present during the entire period but was not necessarily dominant; for much of 2010 the dominant organism was *Aphanizomenon* with a mixed diatom/dinoflagellate assemblage in spring (*Ceratium, Asterionella, Fragilaria*, and *Dinobryon* were all common). Other organisms of note included *Anabaena*, *Planktothrix*, and *Lyngbya*.

3.3.2. Statistical relationships

The best single predictor for toxin loads in Pinto Lake was biomass as chlorophyll concentration (r = 0.779, 0.664 for grab and SPATT samples respectively). Canonical correlation (RDA) identified both chlorophyll and TDN as significantly correlated to toxin concentrations (p = 0.05) with the first major axis including 8

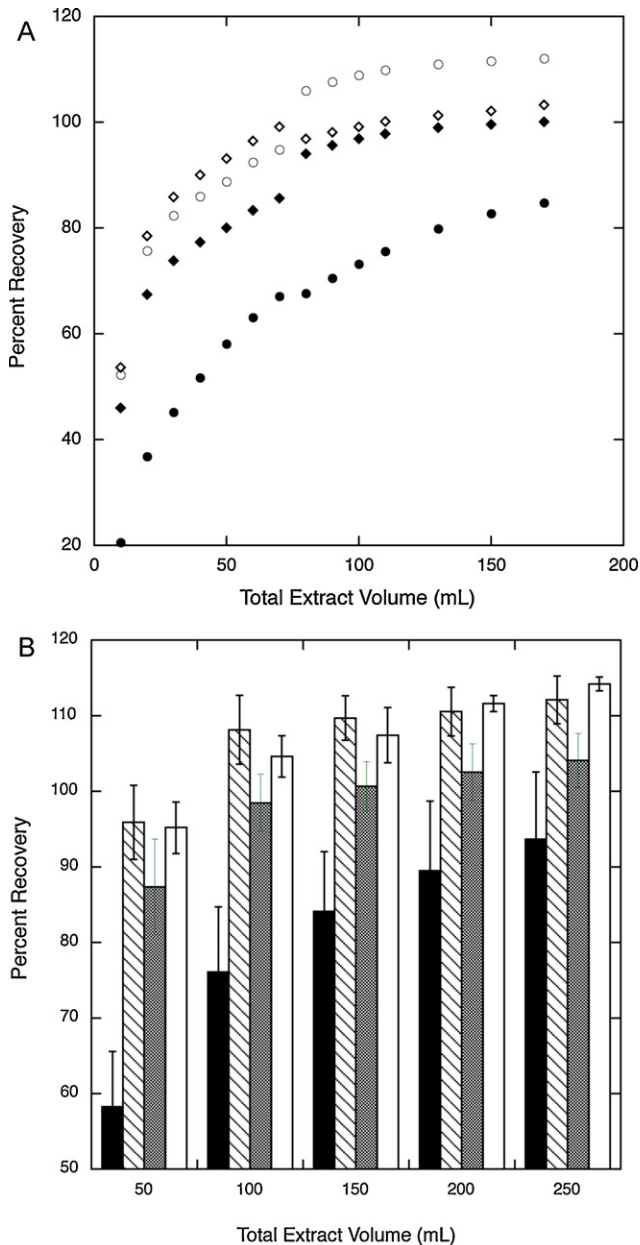

Fig. 4. Sequential desorption (recovery) data are presented. (A) MC-RR (solid circles), MC-YR (open circles), MC-LR (solid diamonds), and MC-LA (open diamonds) recovery for sequential 10 mL 50% MeOH extractions. (B) A separate set of SPATT bags were extracted using sequential 50 mL aliquots for MC-RR (black), MC-YR (hashed), MC-LR (grey), and MC-LA (white); error bars represent SD from 3 replicates.

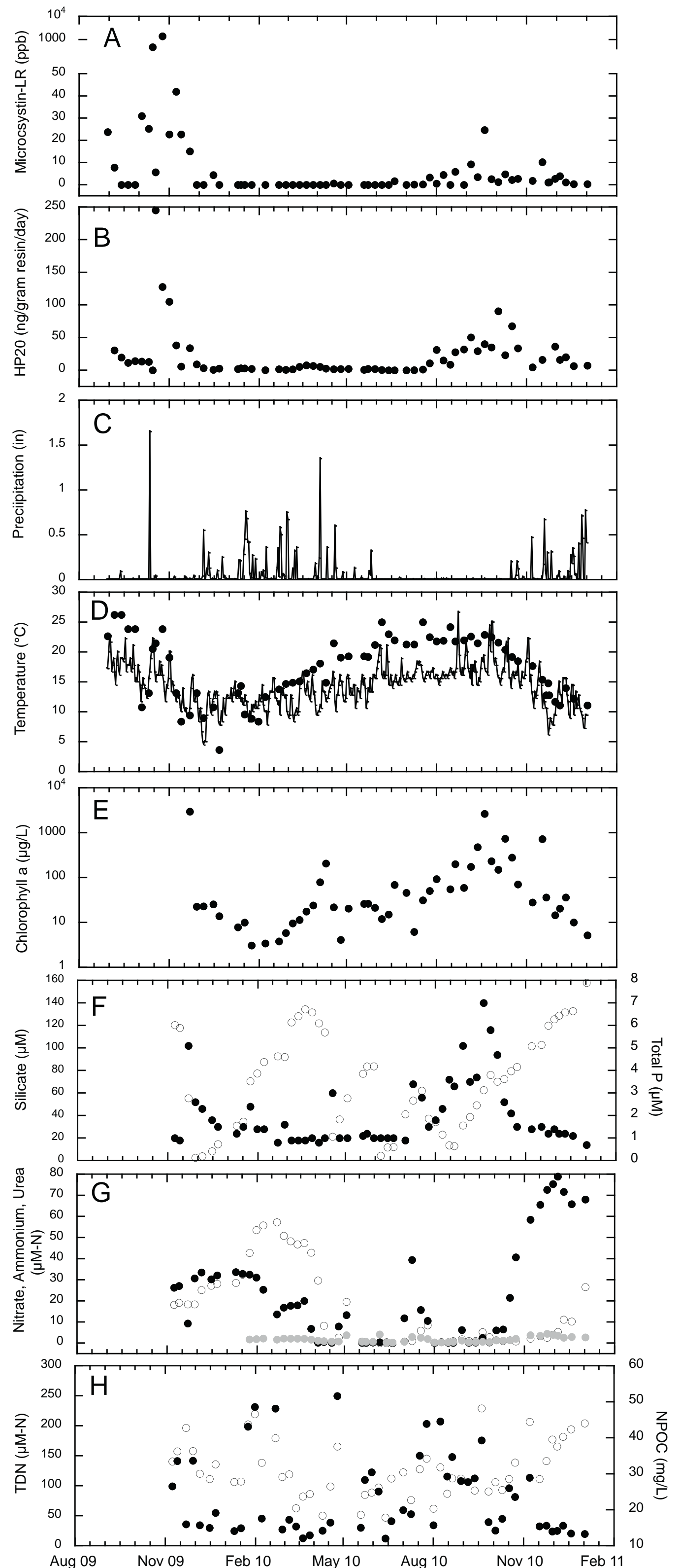

Fig. 5. Data from the Pinto Lake time series are presented; see Table 1 for date ranges. (A) Total MC-LR from grab samples; (B) HP20 SPATT MC-LR (symbols represent the date of recovery for each SPATT); (C) Precipitation recorded at the Salinas Municipal Airport; (D) mean air temperature (solid line) from the Salinas Municipal Airport and water temperature from grab samples (solid circles); (E) chlorophyll *a*; (F) silicate (open symbols) and total dissolved phosphorus (solid symbols); (G) nitrate (open symbols), ammonium (black symbols) and urea (grey symbols) displayed as µM-N; (H) total dissolved nitrogen (open symbols) and non-purgeable organic carbon (solid symbols).

Table 2
Standardized coefficients for variables from the first significant canonical environmental axis describing the relationship between total MC-LR (grab samples) and SPATT MC-LR as dependent variables. Italicized variables are significant; variables are listed as a function of relative importance in the combined canonical axis (highest to lowest), which is not necessarily indicative of the linear responses between individual variables and toxin concentrations. The last two rows provide the total variance explained and the cumulative toxin–environment relationship.

Variable	Correlation	Weighted Average	*p*-Value
Phosphate	−0.443	1.88	0.17
Chlorophyll	0.141	3.24	0.03
TDN	−0.390	4.02	0.05
Water temperature	0.124	2.88	0.27
NPOC	−0.312	2.54	0.62
Si:N ratio	−0.133	1.76	0.34
Silicate	−0.313	3.35	0.38
N:P ratio	−0.382	0.89	0.19
Variance explained:		51.7%	
Toxin–environmental variable correlation:		89.8%	

environmental variables (Table 2). Of these variables, none exhibited simple correlations >0.7 other than chlorophyll. The first major axis was significant ($p < 0.001$) and captured 51.7% of the variance in the toxin data. The second major axis was not significant and only marginally improved the overall analysis (57.6% total variance explained) and is therefore not discussed further. Toxin concentrations were positively associated with chlorophyll and water temperature, and negatively associated with several nutrient variables including P, TDN, Si, NPOC, and the Si:N and N:P ratios.

Despite the positive loading in canonical correlation for water temperature, the direct correlation was weak (polynomial fit; $p > 0.05$) and toxins were present in Pinto Lake at all times of the year (Figs. 5 and 6). Water temperature was also poorly correlated with chlorophyll ($n = 52$, $r = 0.191$). Although toxins generally increased above 18 °C water temperature, a substantial fraction of the positive toxin values occurred at 10–12 °C (Fig. 6), temperatures not generally conducive to growth of *Microcystis* (cf. Paerl and Huisman, 2009).

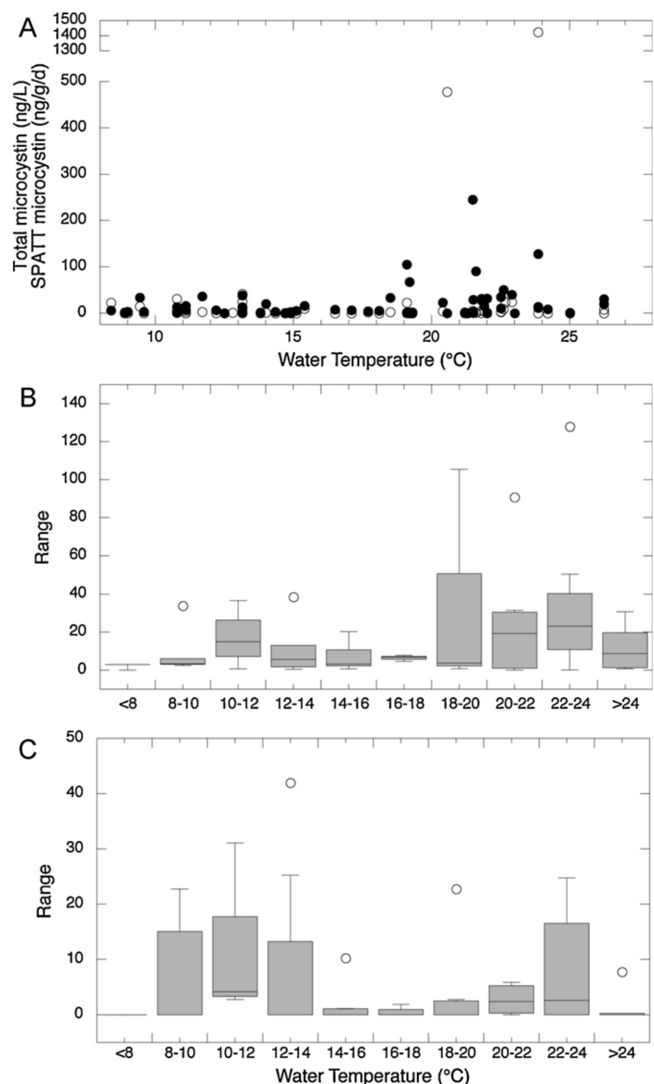

Fig. 6. Microcystin concentration as a function of water temperature. (A) Total MC-LR (open circles) and SPATT MC-LR (solid circles). (B) Total MC-LR represented as a boxplot with 2-degree bins. (C) SPATT MC-LR represented as a boxplot with the data binned to 2 °C intervals.

4. Discussion

The results from this study demonstrate that SPATT constructed with DIAION HP20 resin can be used successfully to track microcystins in the freshwater environment. SPATT has also been deployed and analyzed for microcystins within the Pajaro River plume (ocean) and at the Santa Cruz Municipal Wharf (ocean) as described in Miller et al. (2010). For similar assessment of passive samplers other studies have evaluated multiple resin choices (e.g. Lane et al., 2010; MacKenzie, 2010; Wood et al., 2011). Here only one resin, HP20, was tested for two reasons. First, as previously reported (Lane et al., 2010) HP20 SPATT is inexpensive and widely used for passive sampling of other toxins, thus simplifying deployment when multiple toxins in multiple environments are being monitored. Second, HP20 exhibited excellent adsorption and desorption characteristics, achieving 100% recovery for the four common congeners tested and with 60% (MC-RR) to >80% (MC-LR, MC-YR, MC-LA) recovery using a streamlined protocol with reduced volume. This compares favorably with recovery rates from POCIS samplers using Oasis HLB resin (89 and 94% recovery for MC-RR, MC-LR; Kohoutek et al., 2010), is similar to the recovery for domoic acid (>99% using HP20) reported by Lane et al. (2010), and is substantially better than the recovery (31–45%) for anatoxin-a using SPATT bags with various resins as reported by Wood et al. (2011). There was no loss of microcystins prior to extraction (e.g. with MQ rinses), and no special precautions were necessary during transport and short-term or extended storage.

As discussed in Lane et al., 2010, SPATT deployments should be considered semi-quantitative in that resin toxin loads cannot be directly compared to quantitative toxin concentrations in the environment, nor can SPATT adsorption efficiencies in the field be determined solely by laboratory trials. However, since field deployments used a standardized protocol data should be internally consistent providing a time series that is directly comparable to both the environmental correlates and the traditional grab samples. While SPATT and grab samples did not exhibit identical patterns during the time series (most notably, SPATT detected toxin during 100% of the deployments versus 52% positive values from the grab samples), there was a good correspondence between the two measurements, both statistically and qualitatively. As reported by Lane et al. (2010) HP20 SPATT are also inexpensive (~$1.30 per sampler), easy to manufacture, and versatile. As reported here, SPATT samples can also be analyzed by ELISA after dilution or with standards using the appropriate matrix. Both Abraxis and Envirologix kits performed adequately when the SPATT extracts were diluted 10-fold or greater (Student's

t-test, $p < 0.05$ for ELISA versus LC/MS samples). Although not directly tested as part of this study, microcystin SPATT extracts could also be evaporated to dryness and reconstituted in ELISA buffer or other matrices (Mekebri et al., 2009). Therefore while other combinations of resin and extraction protocols may exhibit improved characteristics, SPATT deployed with HP20 provides a robust monitoring method for microcystins in both fresh and saltwater and is complementary to previous uses of SPATT (MacKenzie, 2010).

Using the combination of SPATT and grab samples, several items of interest are apparent in the Pinto Lake time series. First, toxin is present year-round in this lake, a fact that would easily be missed from traditional grab samples. While the concentrations for much of the year are below the World Health Organization regulatory guideline of 1 $\mu g\,L^{-1}$ for polished drinking water and 20 $\mu g\,L^{-1}$ for recreational exposure (WHO, 1998), relatively little is known about chronic and sub-chronic exposure to these compounds. However Gilroy et al. (2000) summarize evidence for potential harmful impacts in humans to low-level exposure, including promotion of liver cancer in humans during long-term exposure through drinking water. Bio-accumulation of these compounds is also well documented in fresh water organisms (Lehman et al., 2010) and increasingly documented in estuarine and coastal organisms (Garcia et al., 2010; Miller et al., 2010). The persistent occurrence of microcystins in a recreational lake should thus be of some concern despite the low average levels of toxin.

A second item of interest from this time series is the presence of toxin during periods well below the documented thermal optimum for *Microcystis*. In the San Francisco Estuary, *Microcystis* has been reported to prefer high light, warm, shallow, eutrophied waters (Lehman et al., 2005) and was absent at water temperatures $<20\,^{\circ}C$ (Lehman et al., 2008). Similarly, numerous studies relate the dominance of *Microcystis* to the annual maximum water temperature (Paerl, 1988; Paerl et al., 2001; Davis et al., 2009), and link this to potential shifts caused by global warming (Paerl and Huisman, 2008; Paul, 2008; Paerl and Huisman, 2009). Davis et al. (2009) further demonstrated that warm water may favor toxic strains of *Microcystis* relative to non-toxic strains. Pinto Lake is consistent with this. However, while warm, high light conditions may be preferred, resulting in large blooms and corresponding elevated toxin levels in autumn 2009 and autumn 2010, warm temperatures are not a prerequisite for toxin occurrence. It is important to note that in this study algal species were not quantified so it is possible that there were other, cold-tolerant cyanobacteria present that were also producing microcystins. Of the other cyanobacteria routinely observed in Pinto Lake, *Planktothrix* and *Anabaena* have also been reported to produce microcystins (Carmichael, 2001). However, *Microcystis* was observed year-round and is capable of surviving in suboptimal temperature conditions (Brunberg and Blomqvist, 2002), while there were no obvious correlations between presence of non-*Microcystis* CHAB organisms and toxin concentration, including during high-biomass blooms of *Anabaena*, strongly suggesting that the toxins were associated with *Microcystis*.

The third item of interest is the relationship (from canonical correlation) between eutrophic status and toxin accumulation. Chlorophyll *a* was the single best predictor of toxin concentration from this time series, similar to the study of Lehman et al. (2010) who reported positive correlations between both chlorophyll and *Microcystis* abundance and total microcystins for the San Francisco Estuary. The first principal axis of the canonical correlation also identified a number of nutrient parameters, including N, Si, and P. *Microcystis* is not capable of N_2 fixation, and requires combined N sources to support growth, unlike *Aphanizomenon* which frequently co-occurred during bloom events in Pinto Lake and which was often the dominant organism in grab samples during 2010. While TDN was significantly correlated, specific N compounds were not retained in the final model. This could be due to the rather limited data set (n = 45–54) but may also reflect the generalist behavior of *Microcystis*. Moisander et al. (2009) reported that multiple forms of N stimulated both *Microcystis* growth and microcystin production in northern California Klamath river reservoirs, while Davis et al. (2010) reported that toxic strains of *Microcystis* were proportionally more abundant than non-toxic strains at elevated N and inorganic P concentrations in a series of New York lakes. *Microcystis* is also known to be flexible in its use of both dissolved organic nitrogen and phosphorus (Paerl and Huisman, 2008; Moisander et al., 2009; Davis et al., 2010). The significant correlations between toxin concentrations, TDN, and chlorophyll *a* suggest that a primary target for management and mitigation strategies in this system should be nutrient loading.

5. Summary

Solid Phase Adsorption Toxin Tracking using DIAION HP20 resin provides a robust way to monitor microcystin concentrations in freshwater and marine systems. Laboratory-based adsorption and recovery characteristics are near ideal, and SPATT manufacture, deployment, and processing provides a convenient and inexpensive method for monitoring toxins. An extended field analysis demonstrated good correspondence with traditional toxin sampling methods, but with enhanced sensitivity at low ambient toxin concentrations. Although the primary focus of this study was to evaluate the use of SPATT samplers with DIAON HP20, the time series from Pinto Lake also highlighted the year-round occurrence of toxins, and implicates nutrient loading as a primary correlate for this system.

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APPENDIX O

HACH Method 8203

Alkalinity

Alkalinity

DOC316.53.01166

Phenolphthalein and Total Alkalinity

Method 8203

10 to 4000 mg/L as $CaCO_3$

Digital Titrator

Scope and Application: For water, wastewater and seawater.

Test preparation

Before starting the test:

Four drops of Bromcresol Green-Methyl Red Indicator Solution[1] can be substituted for the Bromcresol Green-Methyl Red Indicator Powder Pillow.
Four drops of Phenolphthalein Indicator Solution[1] can be substituted for the Phenolphthalein Indicator Powder Pillow.
For added convenience when stirring, use the TitraStir® stirring apparatus[1].
meq/L Alkalinity = mg/L as $CaCO_3$ ÷ 50

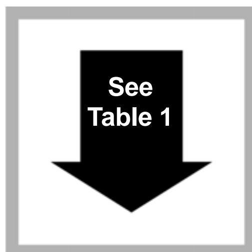
[1] See *Optional reagents and apparatus*.

Collect the following items:

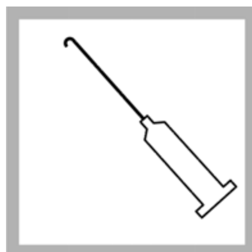
Description	Quantity
Bromcresol Green-Methyl Red Indicator Powder Pillow	1 pillow
Phenolphthalein Indicator Powder Pillow	1 pillow
Sulfuric acid titration cartridge (see *Range-specific information*)	1 cartridge
Digital titrator	1
Delivery tube for digital titrator	1
Graduated cylinder	1
Erlenmeyer flask, 250-mL	1

See *Consumables and replacement items* for reorder information.

Alkalinity

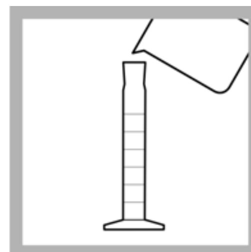


1. Select a sample volume and titration cartridge from the *Range-specific information* table.



2. Insert a clean delivery tube into the titration cartridge. Attach the cartridge to the titrator.

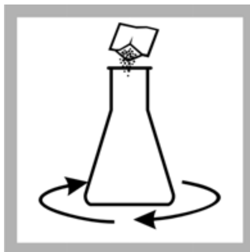
3. Turn the delivery knob to eject air and a few drops of titrant. Reset the counter to zero and wipe the tip.



4. Use a graduated cylinder or pipet to measure the sample volume from the *Range-specific information* table.

Alkalinity (continued)

5. Transfer the sample into a clean, 250-mL Erlenmeyer flask. If the sample volume is less than 100 mL, dilute to approximately 100 mL with deionized water.



6. Add the contents of one Phenolphthalein Indicator Powder Pillow. Swirl to mix.

If the solution turns pink, proceed to step 7. If the solution is colorless, the Phenolphthalein (P) alkalinity is zero. Proceed to step 9.

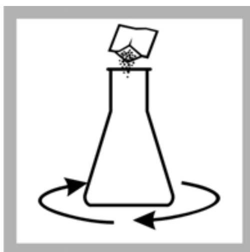
7. Place the delivery tube into the solution and swirl the flask. Turn the knob on the titrator to add titrant to the solution. Continue to swirl the flask and add titrant until the color changes from pink to colorless.

Write down the number of digits displayed on the counter.

8. Use the multiplier in the *Range-specific information* table to calculate the concentration:

digits x multiplier = mg/L as $CaCO_3$ P alkalinity

Example: 100 mL of sample was titrated with the 0.1600 N cartridge and 250 digits were used to reach the endpoint. The concentration is 250 x 0.1 = 25 mg/L as $CaCO_3$



9. Add the contents of one Bromcresol Green-Methyl Red Indicator Powder Pillow. Swirl to mix.

10. Continue the titration with sulfuric acid to a light pink color.

Write down the number of digits displayed on the counter.

Note: A pH meter may be used to titrate to a specific pH as required by sample composition. See the *End point pH* table.

11. Use the multiplier in the *Range-specific information* table to calculate the concentration:

digits x multiplier = mg/L as $CaCO_3$ total alkalinity

Example: 100 mL of sample was titrated with the 0.1600 N cartridge and 250 digits were used to reach the endpoint. The concentration is 250 x 0.1 = 25 mg/L as $CaCO_3$

Table 29 Range-specific information

Range (mg/L as $CaCO_3$)	Sample volume (mL)	Titration cartridge (N H_2SO_4)	Multiplier
10–40	100	0.1600	0.1
40–160	25	0.1600	0.4
100–400	100	1.600	1.0
200–800	50	1.600	2.0
500–2000	20	1.600	5.0
1000–4000	10	1.600	10.0

Table 30 End point pH

Sample composition	Total alkalinity	Phenolphthalein alkalinity
Alkalinity about 30 mg/L	pH 4.9	pH 8.3
Alkalinity about 150 mg/L	pH 4.6	pH 8.3
Alkalinity about 500 mg/L	pH 4.3	pH 8.3
Silicates or phosphates present	pH 4.5	pH 8.3
Industrial wastes or complex system	pH 4.5	pH 8.3
Routine or Automated Analyses	pH 4.5	pH 8.3

Interferences

Interfering substances lists substances that can interfere with this test.

Table 31 Interfering substances

Interfering substance	Interference level
Chlorine	Chlorine at levels above 3.5 mg/L may cause a yellow-brown color when the Bromcresol Green-Methyl Red Powder Pillow is added. Add one drop of 0.1 N Sodium Thiosulfate to the sample to remove chlorine before starting the test.
Color or turbidity	Color or turbidity can mask the color change of the end point. Use a pH meter instead of the color indicators and titrate to a pH of 8.3 for phenolphthalein acidity. For total alkalinity see *End point pH* for the correct end point pH.

Sample collection, preservation and storage

- Collect samples in clean plastic or glass bottles. Fill completely and cap tightly.
- Prevent excessive agitation or prolonged exposure to air. Complete the test procedure as soon as possible after collection for best accuracy.
- The sample can be stored for at least 24 hours if cooled to 4 °C (39 °F) or below.
- Warm to room temperature before the test is started.

Alkalinity relationship table

Total alkalinity primarily includes hydroxide, carbonate and bicarbonate alkalinities. The concentration of these alkalinities in a sample may be determined when the phenolphthalein and total alkalinities are known (see *Alkalinity relationships*).

To use the table follow these steps:

g. Does the phenolphthalein alkalinity equal zero? If yes, use Row 1.

h. Does the phenolphthalein alkalinity equal total alkalinity? If yes, use Row 2.

i. Divide the total alkalinity by 2 to give one-half the total alkalinity.

j. Select Row 3, 4 or 5 based on comparing the result of step c (one-half total alkalinity) with the total alkalinity.

k. Perform the required calculations in the appropriate row, if any.

l. Check your results. The sum of the three alkalinity types will equal the phenolphthalein alkalinity.

For example:
A sample has 170 mg/L as $CaCO_3$ phenolphthalein alkalinity and 250 mg/L as $CaCO_3$ total alkalinity. What is the concentration of hydroxide, carbonate and bicarbonate alkalinities?

The phenolphthalein alkalinity does not equal 0 (it is 170 mg/L), see step g.

The phenolphthalein alkalinity does not equal total alkalinity (170 mg/L vs. 250 mg/L), see step h.

One-half of the total alkalinity (250 g/L) equals 125 mg/L. Because the phenolphthalein alkalinity (170 mg/L) is greater than one-half the total alkalinity (125 mg/L), select row 5.

The hydroxide alkalinity is equal to:
2 x 170 = 340

340 – 250 = 90 mg/L hydroxide alkalinity

The carbonate alkalinity is equal to:
250 – 170 = 80

80 x 2 = 160 mg/L carbonate alkalinity

The bicarbonate alkalinity equals 0 mg/L.
Check: (See step l)

90 mg/L hydroxide alkalinity + 160 mg/L carbonate alkalinity + 0 mg/L bicarbonate alkalinity = 250 mg/L

The above answer is correct; the sum of each type equals the total alkalinity.

Table 32 Alkalinity relationships

Row	Sample result	Hydroxide alkalinity equals:	Carbonate alkalinity equals:	Bicarbonate alkalinity equals:
1	Phenolphthalein Alkalinity = 0	0	0	Total Alkalinity
2	Phenolphthalein Alkalinity equal to Total Alkalinity	Total Alkalinity	0	0
3	Phenolphthalein Alkalinity less than one-half of Total Alkalinity	0	Phenolphthalein Alkalinity times 2	Total Alkalinity minus two times Phenolphthalein Alkalinity
4	Phenolphthalein Alkalinity equal to one-half of Total Alkalinity	0	Total Alkalinity	0
5	Phenolphthalein Alkalinity greater than one-half of Total Alkalinity	2 times Phenolphthalein Alkalinity minus Total Alkalinity	2 times the difference between Total and Phenolphthalein Alkalinity	0

Accuracy check

End point confirmation

Use a buffer pillow with the same pH as the end point with the indicator to make sure the end point color is accurate.

- Phenolphthalein alkalinity—Add 50 mL of deionized water to a flask. Add one pH 8.3 buffer powder pillow and one Phenolphthalein Indicator Powder Pillow and swirl to mix. Use this solution for comparison during the titration with the sample.
- Total alkalinity—Add 50 mL of deionized water to a flask. Add one pH 4.5 buffer powder pillow and one Bromcresol Green-Methyl Red Indicator Powder Pillow and swirl to mix. Use this solution for comparison during the titration with the sample.

Standard additions method (sample spike)

Required for accuracy check:

- Alkalinity Voluette® Ampule Standard Solution, 0.500 N
- Ampule breaker
- TenSette Pipet, 0.1–1.0 mL and Pipet Tips

1. Open the standard solution ampule.
2. Use the TenSette Pipet to add 0.1 mL of the standard to the titrated sample. Swirl to mix.
3. Titrate the spiked sample to the end point. Write down the amount of titrant that was used to reach the end point.
4. Repeat steps 2 and 3, using two more additions of 0.1 mL. Titrate to the end point after each addition.
5. Each 0.1 mL of standard that was added will use approximately 25 digits of the 1.600 N titration cartridge or 250 digits of the 0.1600 N titration cartridge to reach the endpoint. If more or less titrant was used, there may be an interference (see *Interferences*) or the concentration of the titrant has changed.

Summary of method

The sample is titrated with sulfuric acid to a colorimetric end point corresponding to a specific pH. Phenolphthalein alkalinity is determined by titration to a pH of 8.3, as evidenced by the color change of phenolphthalein indicator and indicates the total hydroxide and one half the carbonate present. M (methyl orange) or T (total) alkalinity is determined by titration to a pH between 4.3 and 4.9 and includes all carbonate, bicarbonate and hydroxide. Alternatively, total alkalinity end points may be determined by using a pH meter and titrating to the specific pH required for the sample composition.

Consumables and replacement items

Required reagents

Description	Quantity/Test	Unit	Catalog number
Alkalinity Reagent Set (approximately 100 tests)			2271900
(1) Bromcresol Green-Methyl Red Powder Pillows	1 pillow	100/pkg	94399
(1) Phenolphthalein Indicator Powder Pillows	1 pillow	100/pkg	94299
(1) Sulfuric Acid Titration Cartridge, 0.1600 N	varies	each	1438801
(1) Sulfuric Acid Titration Cartridge, 1.600 N	varies	each	1438901

Required apparatus

Description	Quantity/Test	Unit	Catalog number
Digital Titrator		each	1690001
Flask, Erlenmeyer, graduated, 250-mL	1	each	50546
Graduated cylinder—select one or more based on range:			
Cylinder, graduated, 10-mL	1	each	50838
Cylinder, graduated, 25-mL	1	each	50840
Cylinder, graduated, 50-mL	1	each	50841
Cylinder, graduated, 100-mL	1	each	50842

Recommended standards

Description	Unit	Catalog number
Alkalinity Standard Solution, Voluette® Ampule 0.500 N Na_2CO_3, 10-mL	16/pkg	1427810

Optional reagents and apparatus

Description	Unit	Catalog number
Buffer Powder Pillows, pH 4.5	25/pkg	89568
Buffer Powder Pillows, pH 8.3	25/pkg	89868
Stir bar, octagonal 28.6 mm x 7.9 mm	each	2095352
TenSette Pipet, 0.1 to 1.0 mL	each	1970001
Water, deionized	500 mL	27249
Pipet, volumetric, Class A, 10 mL	each	1451538
Pipet, volumetric, Class A, 20 mL	each	1451520
Pipet Filler, safety bulb	each	1465100
Bottles, sampling, poly, 500 mL	each	2087079
Bromphenol Green-Methyl Red indicator solution	100 mL MDB	2329232
Phenolphthalein Indicator solution, 5 g/L	100 mL MDB	16232
pH meter	each	—
TitraStir stir plate, 115 Vac	each	1940000
TitraStir stir plate, 230 Vac	each	1940010

FOR TECHNICAL ASSISTANCE, PRICE INFORMATION AND ORDERING:
In the U.S.A. – **Call toll-free 800-227-4224**
Outside the U.S.A. – **Contact the HACH office or distributor serving you.**
On the Worldwide Web – **www.hach.com**; E-mail – **techhelp@hach.com**

HACH COMPANY
WORLD HEADQUARTERS
Telephone: (970) 669-3050
FAX: (970) 669-2932

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APPENDIX P

Field sheets and checklists

SWAMP Field Data Sheet for 2013 Cyanotoxin Screening of Lakes/Reservoirs and Coastal Wetlands

Project CODE: RWB9_CyanoBac_2013 | Date (mm/dd/yyyy): ___/___/______

Station ID: ___ ___ ___ ___ ___ ___ ___ ___ ___ | Arrival Time: ___________ | Departure Time: ___________

Field Crew: | Time recorded on all samples: ___________

HABITAT OBSERVATIONS (Collection Method = Habitat_generic)

SITE ODOR: None, Sulfides, Sewage, Petroleum, Smoke, Other ________

WADEABILITY: Y / N/ Unk

SKY CODE: Clear, Partly Cloudy, Overcast, Fog, Smoky, Hazy

OTHER PRESENCE: Vascular, Nonvascular, OilySheen, Foam, Trash, Other ________

DOMINANT SUBSTRATE: Bedrock, Concrete, Cobble, Gravel, Sand, Mud, Unk, Other ________

WATERCLARITY: Clear (see bottom), Cloudy (> 4" vis), Murky (<4" vis)

WATERODOR: None, Sulfides, Sewage, Petroleum, Mixed, Other ________

WATERCOLOR: Colorless, Green, Yellow, Brown

PRECIPITATION: None, Fog, Drizzle, Rain, Snow

PRECIPITATION (last 24 hours):

EVIDENCE OF FIRES: No, <1 year, <5 years

OVERLAND RUNOFF (Last 24 hours): None, Light, Moderate, Heavy, Unknown

OBSERVED FLOW: NA, Dry Waterbody Bed, No Obs Flow, Isolated Pool, Trickle (<0.1 cfs), 0.1-1cfs, 1-5cfs, 5-20cfs, 20-50cfs, 50-200cfs, >200cfs

QUANTA READINGS

Temperature ___________ °C	**DO** ___________ mg/L	**Conductivity** ___________ µS/cm
pH ___________	**DO** ___________ %	**Salinity** __________ ppt

SAMPLES TAKEN

Nutrient Samples			**Particulate Samples**					**SPATT**		**Salinity**
TN/TP	DIN	Alk	Chl a	Cyanotoxin	Pigments	Particulate Nitrogen (PN)	Particulate Phosphorous (PP)	SPATT Bag	SPATT Bag	Estuary Salinity Reading
Whole Water	Syringe Filtered	Hach Kit	250 mls filtered	500 mls filtered	250 mls filtered	100 mls filtered	100 mls filtered	Deployed	Retrieved	‰

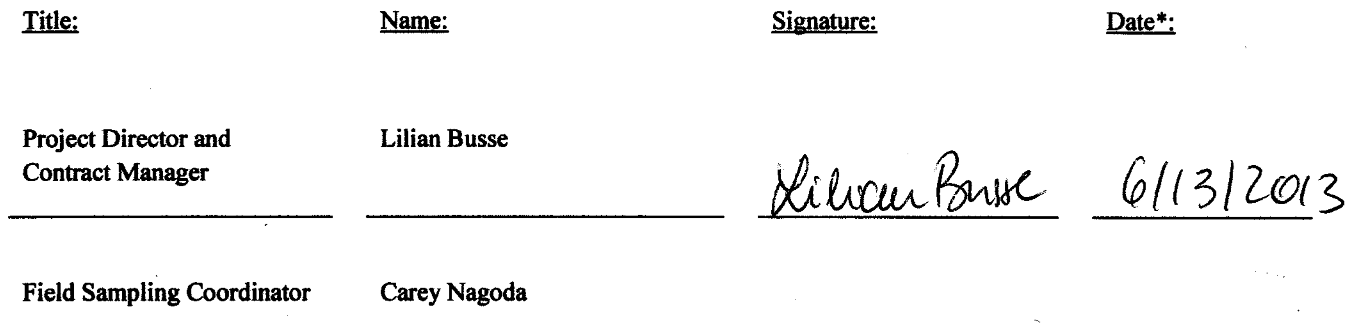
SITE	RECON DATE	RECON SAMPLES SHIPPED WPCL	SAMPLE DATE #1	***SCCWRP*** SAMPLES SHIPPED	***WPCL*** SAMPLES SHIPPED	***UC Santa Cruz*** SAMPLES SHIPPED	SAMPLE DATE #2	***SCCWRP*** SAMPLES SHIPPED	***WPCL*** SAMPLES SHIPPED	***UC Santa Cruz*** SAMPLES SHIPPED	SAMPLE DATE #3	***SCCWRP*** SAMPLES SHIPPED	***WPCL*** SAMPLES SHIPPED	***UC Santa Cruz*** SAMPLES SHIPPED
LAKES/RESERVOIRS														
Lake Henshaw 903PLH214		N/A												
Vail Lake 902TV0111	6/22/2013	N/A	7/20/2013				8/24/2013				9/28/2013			
Lake Sutherland 905PLS198		N/A												
Lake Hodges 905PLH070		N/A												
Cuyamaca Reservoir 907CUYRES	4/25/2013	N/A												
Morena Reservoir 911PMR110		N/A												
El Capitan Reservoir 907PEC062		N/A												
Miramar Reservoir 906PLM142		N/A												
Lake Murray 907LKMURR		N/A												
Lower Otay Reservoir 910PLO182		N/A												
COASTAL WETLANDS														
Agua Hedionda Lagoon 904AGHDLG	5/29/2013													
San Diego River Estuary 907SDRVES	5/30/2013	5/30/2013	7/3/2013				8/8/2013				9/5/2013			
Buena Vista Lagoon 904TB0047	5/29/2013													
San Elijo Lagoon 904SNELLG	5/22/2013	5/22/2013	7/1/2013				8/6/2013				9/3/2013			
Los Penasquitos Lagoon 906LSPNLG	5/22/2013	5/22/2013	7/1/2013				8/6/2013				9/3/2013			
San Diego Bay (Sweetwater) 908SDBYSW	5/20/2013	5/22/2013	7/2/2013				8/7/2013				9/4/2013			
San Diego Bay (Otay) 908SDBYSS	5/20/2013	5/22/2013	7/2/2013				8/7/2013				9/4/2013			
San Diego Bay (open, by NTC) 908SDBNTC	5/30/2013	5/30/2013	7/2/2013				8/7/2013				9/4/2013			
Mission Bay 90606MISS	5/30/2013	5/30/2013	7/3/2013				8/8/2013				9/5/2013			
Tijuana Estuary 911TJRVES	5/20/2013	5/22/2013												

APPENDIX Q

Approval signatures

APPROVAL SIGNATURES

SAN DIEGO REGIONAL WATER QUALITY CONTROL BOARD (SD RWQCB):



Title:	Name:	Signature:	Date*:
Project Director and Contract Manager	Lilian Busse	Lilian Busse	6/13/2013
Field Sampling Coordinator	Carey Nagoda		

STATE BOARD (SWRCB):

Title:	Name:	Signature:	Date*:
SWAMP Database Manager (SWRCB/SWAMP)	Stacey Swenson		
SWAMP QA Officer (SWRCB/SWAMP)	Beverly van Buuren		

SOUTHERN CALIFORNIA COASTAL WATER RESEARCH PROJECT (SCCWRP):

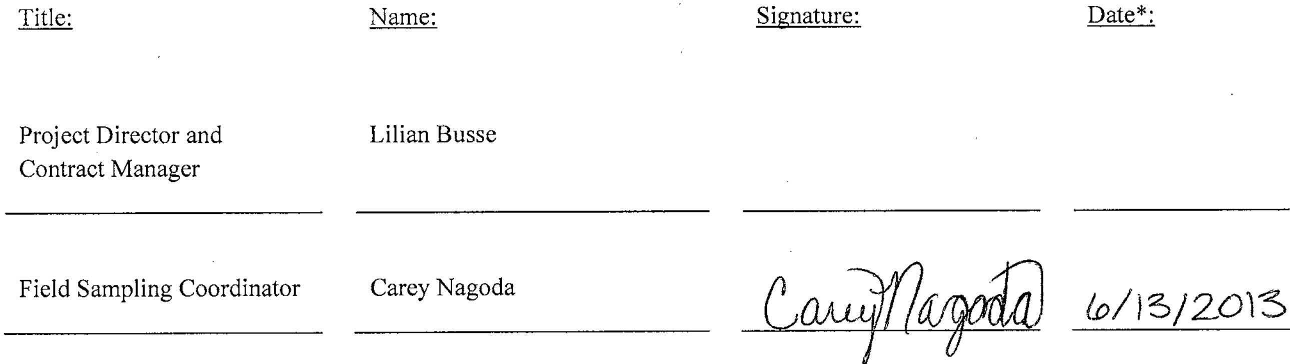
Title:	Name:	Signature:	Date*:
Project Manager	Meredith Howard		

CALIFORNIA DEPARTMENT OF FISH AND WILDLIFE – WATER POLLUTION CONTROL LABORATORY (DFW-WPCL):

Title:	Name:	Signature:	Date*:
WPCL QC Officer	Gail Cho		

APPROVAL SIGNATURES

SAN DIEGO REGIONAL WATER QUALITY CONTROL BOARD (SD RWQCB):



Title:	Name:	Signature:	Date*:
Project Director and Contract Manager	Lilian Busse		
Field Sampling Coordinator	Carey Nagoda	Carey Nagoda	6/13/2013

STATE BOARD (SWRCB):

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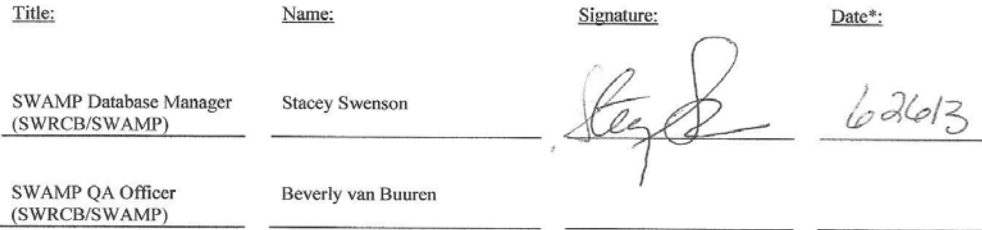

Title:	Name:	Signature:	Date*:
SWAMP Database Manager (SWRCB/SWAMP)	Stacey Swenson	[signature]	6/26/13
SWAMP QA Officer (SWRCB/SWAMP)	Beverly van Buuren		

SOUTHERN CALIFORNIA COASTAL WATER RESEARCH PROJECT (SCCWRP):

Title:	Name:	Signature:	Date*:
Project Manager	Meredith Howard		

CALIFORNIA DEPARTMENT OF FISH AND WILDLIFE – WATER POLLUTION CONTROL LABORATORY (DFW-WPCL):

Title:	Name:	Signature:	Date*:
WPCL QC Officer	Gail Cho		

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Page 3 of 224

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Title:	Name:	Signature:	Date*:
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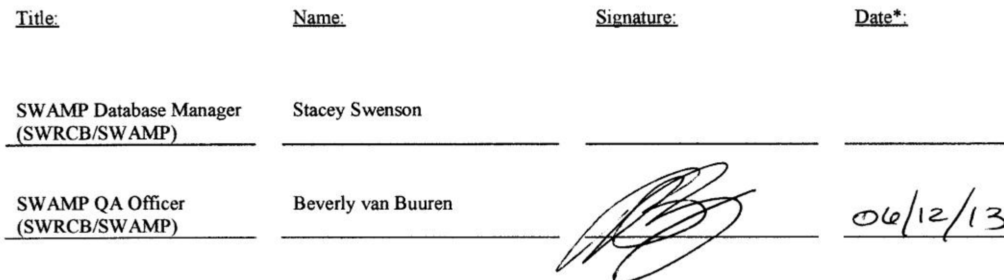
SWAMP QA Officer (SWRCB/SWAMP)	Beverly van Buuren		06/12/13

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Project Manager	Meredith Howard		

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Title:	Name:	Signature:	Date*:
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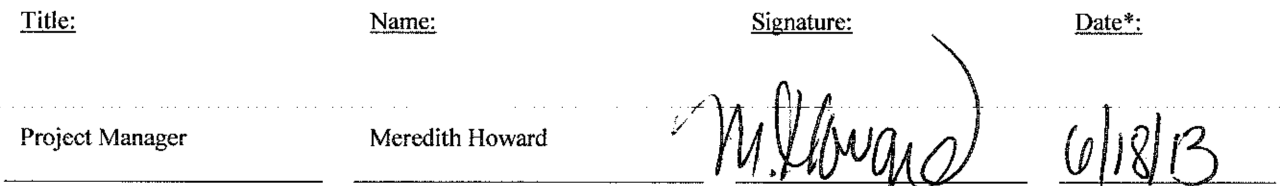

Title:	Name:	Signature:	Date*:
Project Manager	Meredith Howard	M. Howard	6/18/13

CALIFORNIA DEPARTMENT OF FISH AND WILDLIFE – WATER POLLUTION CONTROL LABORATORY (DFW-WPCL):

Title:	Name:	Signature:	Date*:
WPCL QC Officer	Gail Cho		

APPROVAL SIGNATURES

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WPCL QC Officer	Gail Cho	Gail Cho	6/26/13