SAMPLING AND ANALYSIS PLAN – PHASE II FOR TOXIC CYANOBACTERIA IN LAKE WASHINGTON, LAKE SAMMAMISH, AND LAKE UNION

SUBMITTED TO:

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NAME OF PROJECT: Sampling and Analysis Plan for Toxic Cyanobacteria in Lake Washington, Lake Sammamish, and Lake Union.

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421235 – ROUTINE MAJOR LAKES SAMPLING AND ANALYSIS
421395 – SWIMMING BEACHES SAMPLING AND ANALYSIS

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TABLE OF CONTENTS

LIST OF TABLES

1 Introduction

King County has collected samples for analysis of the cyanotoxin, microcystin, in selected King County lakes during summer and fall of 2002 through 2004. This Phase II Sampling and Analysis Plan (SAP) describes the revised scope of work, field sampling procedures, and laboratory analytical requirements for the study as it will be performed during 2005 and 2006.

Mass accumulations or "blooms" of cyanobacteria in freshwater ecosystems are primarily caused by nutrient, particularly phosphorus (P), enrichment. Cyanobacteria blooms can cause surface scums, decreased water column transparency, dissolved oxygen depletion and unpalatable drinking water due to taste and odors. Some cyanobacteria also produce toxic compounds ("cyanotoxins") that have caused livestock, wildlife and pet fatalities worldwide (reviewed by Carmichael 1994; Chorus 2001).

Cyanotoxins include a broad, diverse range of chemicals and mechanisms of toxicity (Carmichael 1994; Sivonen and Jones 1999). Major classes of cyanotoxins include the cyclic peptides, which are primarily hepatotoxins (microcystins and nodularins); alkaloids and an organophosphate, which are strong neurotoxins (anatoxin-a, anatoxin-a (S), and saxitoxins); a cyclic guanide alkaloid, which inhibits protein synthesis (cylindrospermopsin); lipopolysaccharides, which have pyrogenic properties; and dermatoxic alkaloids (aplysiatoxins and lyngbyatoxins) (Chorus 2001).

The hepatotoxins are of particular concern due to their prevalence and potential to harm animals and humans. Hepatotoxins damage liver tissues, and at high doses can cause liver failure and death (Carmichael 1994). Hepatotoxins with seven amino acids are called microcystins (produced by species of *Microcystis, Planktothrix,* and *Anabaena*) and those with five amino acids are nodularins (produced by *Nodularin spumigena*). The mechanism of toxicity involves the inhibition of the specific protein phosphatase enzymes possessed by all eukaryotic cells. In addition, microcystins are suspected tumor-promoters and teratogens (Falconer 1998). These toxins have been associated with elevated rates of primary liver cancer in people drinking waters with high densities of cyanobacteria (Yu 1989).

In a comparison of surveys from different countries, Chorus (2001) found that microcystins were more frequently detected than anatoxins in cyanobacterial bloom samples. Microcystins were detected in at least 60% of all samples investigated in the Danish, German, Portuguese and Korean studies. In the Czech study, microcystins were detected in 90% of all samples. Neurotoxicity was documented in one quarter or less of the bloom samples investigated in these studies (Chorus 2001). Thus, investigations of cyanotoxicity and the development of public health guidelines have focused on microcystins due to their widespread occurrence and potential for chronic toxicity.

1.1 Project Background

Toxic cyanobacteria have been increasingly detected in western Washington lakes since the first documented toxic episode in American Lake in 1989 (Jacoby et al. 1994). Since

then, toxic cyanobacteria have been detected in Steilacoom Lake (Jacoby et al. 2000), American Lake, Green Lake, Lake Waughop, and Lake Sammamish, resulting in several lake closures. Microcystins were measured at concentrations of approximately 500 µg g-1 dry weight cyanobacteria and may have been responsible for the death of a pet dog and the reported illnesses of several young children who swam in Lake Sammamish during a toxic bloom in September 1997.

The toxic bloom of *Microcystis aeruginosa* in Lake Sammamish during 1997 and subsequent detection of microcystins by Johnston and Jacoby (Johnston and Jacoby 2003) in 1999 prompted King County to develop a sampling and analysis plan for the measurement of microcystins in other King County lakes in 2002. Revisions to this plan, as included in this Phase II SAP, are based upon the results of the County's 2002-2004 efforts as discussed below.

1.2 Summary of Studies Prior to 2002

Few studies of cyanotoxicity have been conducted in Washington State lakes (e.g., Jacoby *et al*. 1994; Jacoby *et al*. 2000). Cyanobacterial activity and environmental conditions that may promote toxic cyanobacteria were investigated in Lake Sammamish during summer and fall 1999 (Johnston and Jacoby 2003). Microcystins were detected using enzyme linked immunosorbent assay (ELISA) during late August and early September 1999 despite low cyanobacterial abundance. In contrast to other studies in which concentrations varied substantially throughout a lake (Carmichael & Gorham 1981; Jacoby et.al. 1994) similar microcystin concentrations were detected throughout Lake Sammamish at all depths, ranging between 0.19 to 3.8 µg L^{-1}, with the exception of the boat launch where a surface concentration reached 43 µg L^{-1}.

During the toxic episodes in 1997 and 1999, *Microcystis* was associated with a stable water column, increased surface total phosphorus concentrations (> 10 µg L^{-1}), surface temperatures greater than 22°C, high total nitrogen to phosphorus ratios (> 30) and increased water column transparency (up to ~5.5 m). Microcystin:chlorophyll-*a* (chl-*a*) ratios varied from 0.4 to 6.4, with higher ratios in the hypolimnion during the toxic episode in 1999. Migration of *Microcystis* and *Anabaena* occurred in both the deep and shallow portions of the lake. Migration rates were more than 2 times higher at the shallow station, and the migrating cyanobacteria were dominated by *Microcystis* (89 to 99% of the total biovolume) at both stations. External loading of nutrients due to the large storm event that preceded the 1997 toxic episode may have provided the nutrients needed to fuel that bloom. Despite the lack of rain and subsequent external runoff, toxic *Microcystis* occurred in 1999. The migration of *Microcystis* from the nutrient-rich sediments may have contributed to the toxic population detected in 1999.

1.3 King County 2002 – 2004 Microcystin Monitoring Results

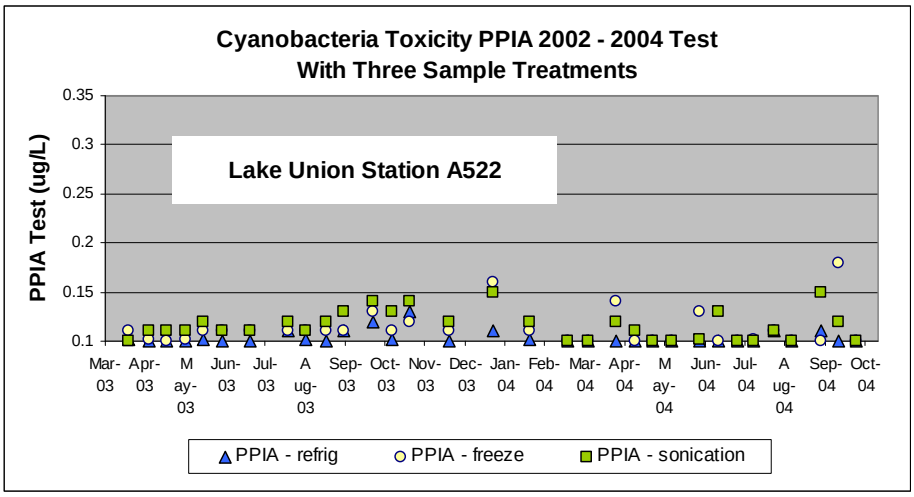
King County had two parallel microcystin monitoring efforts in 2002 through 2004. The first County monitoring effort included samples collected at all 25 routinely monitored Major Lake stations beginning in spring 2002. These samples were processed using a

0.45 μm syringe-filter and analysis with the ELISA method. No microcystins were detected using these protocols.

The second effort, as described in the 2002 SAP, was focused on determination of environmental conditions that may trigger toxin production and the evaluation of microcystin extraction methods. This effort involved the monitoring of six stations in lakes Union, Washington, and Sammamish. Samples from this effort were either refrigerated or treated with one of two extraction processes (freezing and sonication) prior to analysis by protein phosphatase inhibition assay (PPIA) and ELISA. None of the 193 samples analyzed using the ELISA test had detectable levels of microcystins. Samples analyzed using the PPIA method had microcystin concentrations above 0.1 μg/L detection limit in 72 of the refrigerated samples, 107 of the frozen samples, and 111 of the sonicated samples.

As found in the 1999 study on Lake Sammamish, the highest concentrations of microcystins generally occurred in late September (Figure 1a to 1e). The maximum microcystin concentration in Lake Washington (0.62μg/L) was measured on September 20, 2004 at station 0852. In Lake Sammamish, the highest microcystin concentration (0.22μg/L) was measured at station 0611 on August 2, 2004. The highest microcystin concentration in Lake Union (0.18 μg/L) occurred on September 20, 2004.

Microcystin:chl-*a* ratios were very low, ranging from 0.007 to 0.228 μg/L. Complete results of the phytoplankton biovolume and enumeration were not available at the time of this Phase II SAP. However, a cursory look at some of the reports indicates that *Anabaena* sp. was present in the majority of samples in which microcystins were detected. A full analysis will be completed when all reports from Water Environmental, Inc. are received.


Cyanobacteria Toxicity PPIA 2002 - 2004 Test
With Three Sample Treatments
Lake Union Station A522
PPIA Test (ug/L)
0.35
0.3
0.25
0.2
0.15
0.1
Mar-03 Apr-03 May-03 Jun-03 Jul-03 Aug-03 Sep-03 Oct-03 Nov-03 Dec-03 Jan-04 Feb-04 Mar-04 Apr-04 May-04 Jun-04 Jul-04 Aug-04 Sep-04 Oct-04
PPIA - refrig
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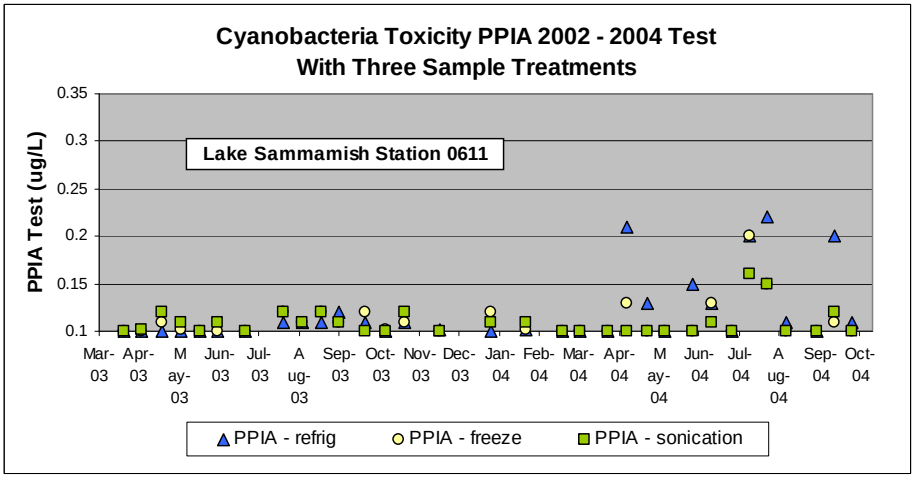
PPIA - sonication


Cyanobacteria Toxicity PPIA 2002 - 2004 Test
With Three Sample Treatments
Lake Sammamish Station 0611
PPIA Test (ug/L)
0.35
0.3
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0.2
0.15
0.1
Mar-03 Apr-03 May-03 Jun-03 Jul-03 Aug-03 Sep-03 Oct-03 Nov-03 Dec-03 Jan-04 Feb-04 Mar-04 Apr-04 May-04 Jun-04 Jul-04 Aug-04 Sep-04 Oct-04
PPIA - refrig
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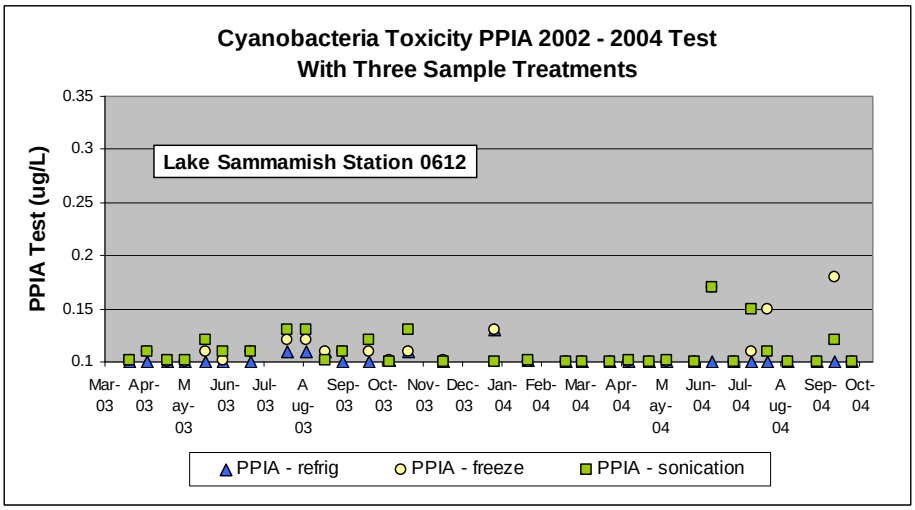
PPIA - sonication


Cyanobacteria Toxicity PPIA 2002 - 2004 Test
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Lake Sammamish Station 0612
PPIA Test (ug/L)
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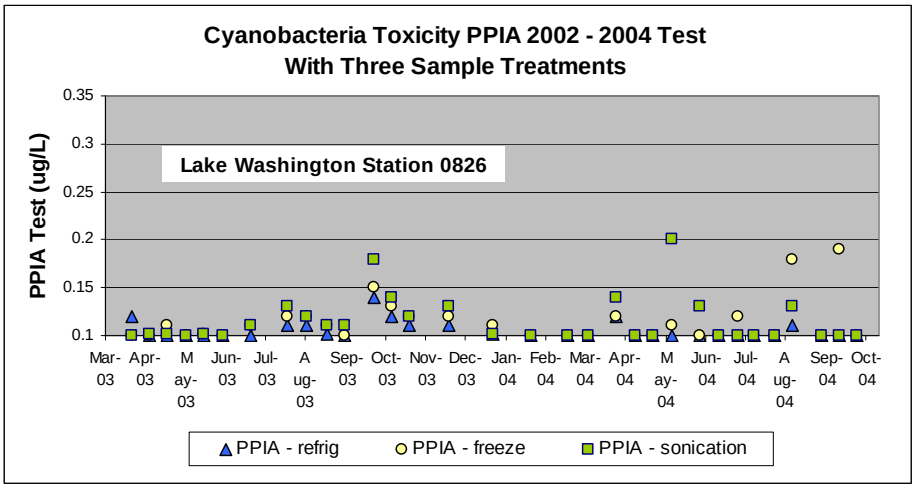
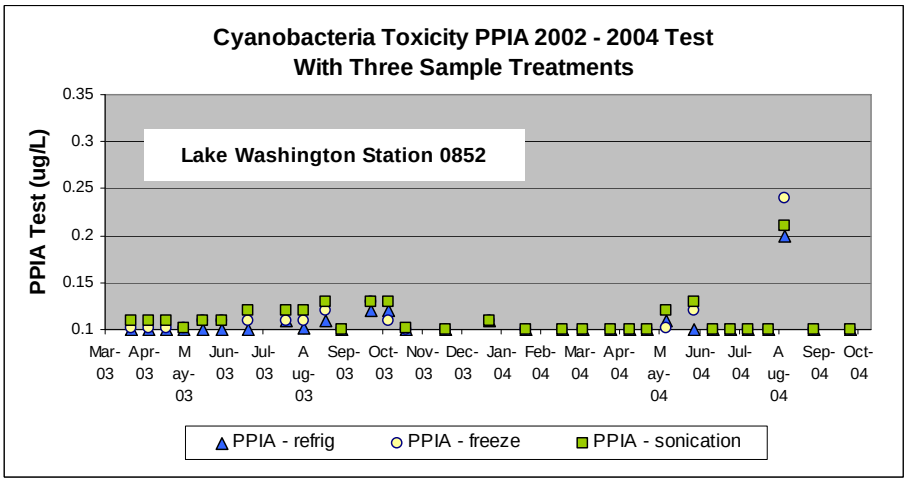
0.3
0.25
0.2
0.15
0.1
Mar-03 Apr-03 May-03 Jun-03 Jul-03 Aug-03 Sep-03 Oct-03 Nov-03 Dec-03 Jan-04 Feb-04 Mar-04 Apr-04 May-04 Jun-04 Jul-04 Aug-04 Sep-04 Oct-04
PPIA - refrig
PPIA - freeze
PPIA - sonication





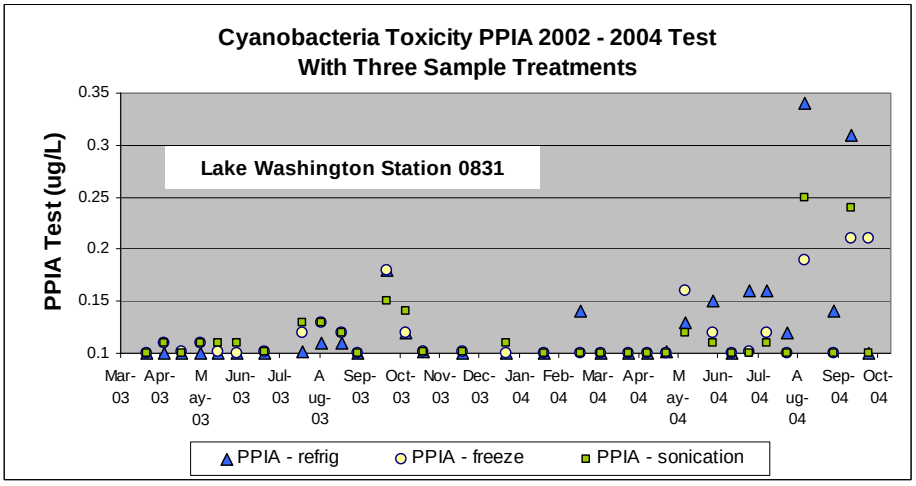


Figure 1a to 1f. Results of the Cyanobacteria Toxicity PPIA 2002-2004 Tests.

1.4 Regulatory Status of Cyanotoxin Criteria and Guidelines

The U.S. Environmental Protection Agency, the State of Washington Department of Ecology (Ecology), and the Washington State Public Health Department have not promulgated water quality standards for cyanotoxins. Thus, the increased detection of cyanotoxins in water bodies worldwide generates a complex challenge for water resource managers. The World Health Organization (WHO) has proposed that microcystin concentrations should not exceed 1 $\mu g\ L^{-1}$ in drinking water (WHO 1998; Chorus & Bartram 1999).

Chorus (2001) performed a risk assessment on how much scum could kill a small child based on a worst-case scenario using toxicological data and microcystin concentrations. In this assessment, she assumed that the acute oral toxicity of microcystin-LR in mice (LD_{50} 5 to 10.9 mg kg^{-1} body weight) applies to humans and an intracellular microcystin concentration of 3 mg mL^{-1} of cell material was present in the scum. For a 10-kg child, 50 mg microcystin could be a lethal dose, which would correspond to 17 mL of pure cell material. Extending this analysis to a high microcystin concentration of 24 mg L^{-1} (measured in a scum sample from Germany), Chorus (2001) estimated that a toxic dose for a 10-kg child would require swallowing about 2 L of water. If the scum is more toxic or the cell density of *Microcystis* in the scum is higher, a lethal dose could occur at lower volumes of ingested water (e.g., with a fivefold increase in toxicity and a four-fold higher cell density, 0.1 L of water could be lethal). She concludes that although acute microcystin poisonings are possible at the concentrations measured in recreational water bodies, they are unlikely. However, liver damage in people exposed to high microcystin concentrations during swimming is not unlikely, particularly if exposure is repeated or prolonged. The implications are most severe for children who tend to play in shallow areas where dense scums accumulate. The long-term consequences of these exposures in terms of liver damage and human health are uncertain.

Several countries have developed guidelines for recreational exposure to cyanotoxins. The focus of these guidelines is on microcystins due to their widespread occurrence and potential for chronic toxicity (Chorus *et al*. 2000; Chorus 2001). For example, the Federal Environmental Agency of Germany recommends posting warning signs and conducting a remedial investigation if chl-*a* exceeds 40 $\mu g\ L^{-1}$ and cyanobacteria are dominant. If chl-*a* exceeds 150 $\mu g\ L^{-1}$ or if total microcystin concentrations exceed 100 $\mu g\ L^{-1}$, closure of the swimming beach is recommended until the bloom declines. WHO has issued guidelines that identify three levels of hazards based on cyanobacteria abundance as measured by concentrations of chl-*a* or cell densities (Fastner *et al*. 1999). However, the large variability in the microcystin:chl-*a* ratios found in several studies (reviewed by Chorus 2001) indicates that direct measurement of microcystins is a more accurate measure of the potential toxicity of cyanobacteria. In this regard, microcystin:chl-*a* ratios in Lake Sammamish ranged from 0.4 to 6.4, with higher ratios (mean = 3.20) in the hypolimnion during the toxic episode in 1999 (Johnston and Jacoby 2003).

1.5 Study Area Description

Initial routine sampling for microcystin analysis using the ELISA procedure was initiated in spring of 2002. This Phase II SAP builds upon the more focused and thorough approach that began in May, 2003. Originally this focused study included deep mid-lake stations in lakes Washington, Sammamish, and Union. Sampling locations have been modified in this SAP to include several nearshore stations and swimming beaches on these lakes. All sites are currently active in the Major Lakes monitoring program (see Major Lakes Monitoring Program SAP, King County, forthcoming in 2005) or are part of the seasonal Swimming Beach Monitoring Program. If blooms are detected in other regional lakes, additional grab samples may be collected from those lakes and analyzed. If bloom samples are collected at other than established stations, coordinates will also be collected.

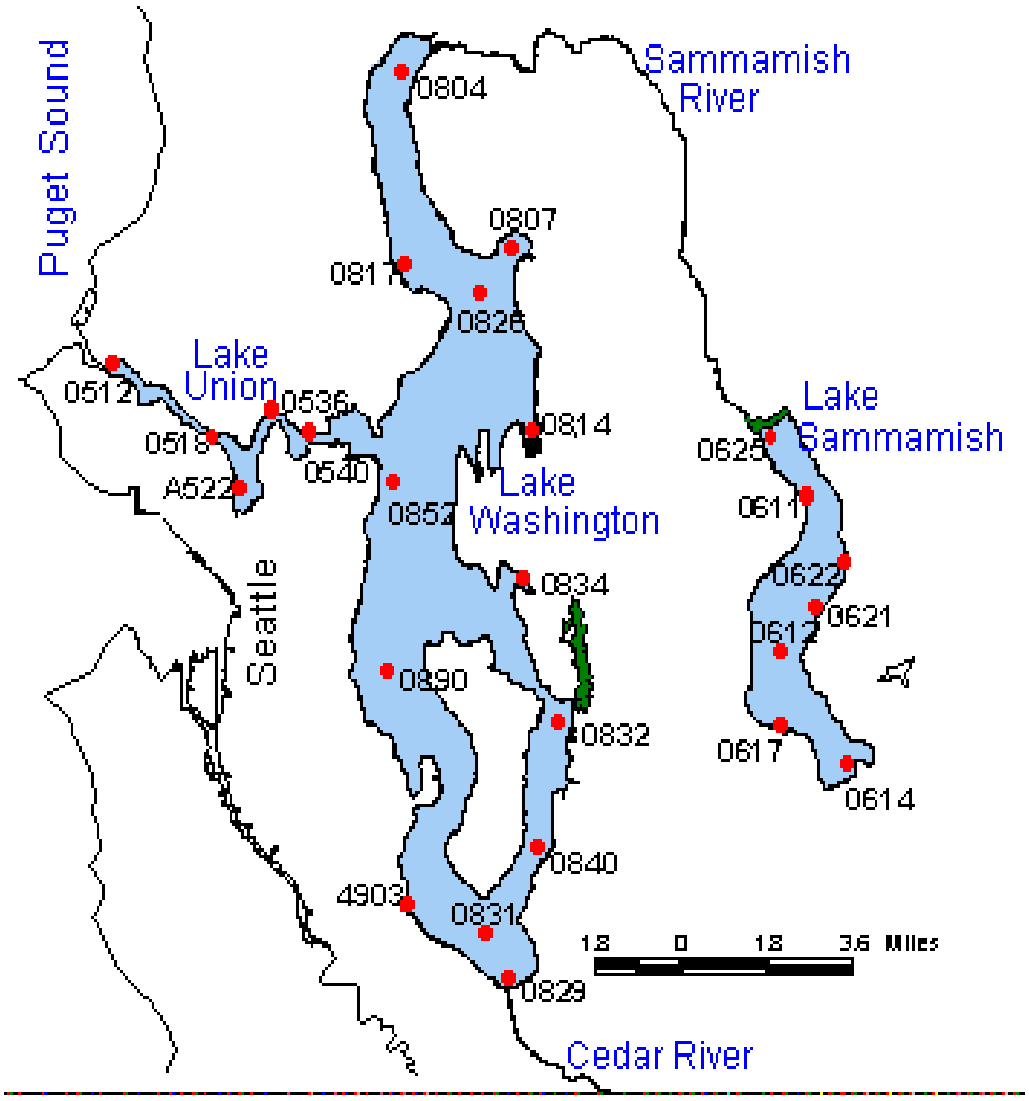


Figure 2. Major Lakes Ambient Monitoring Program Sampling Locations.

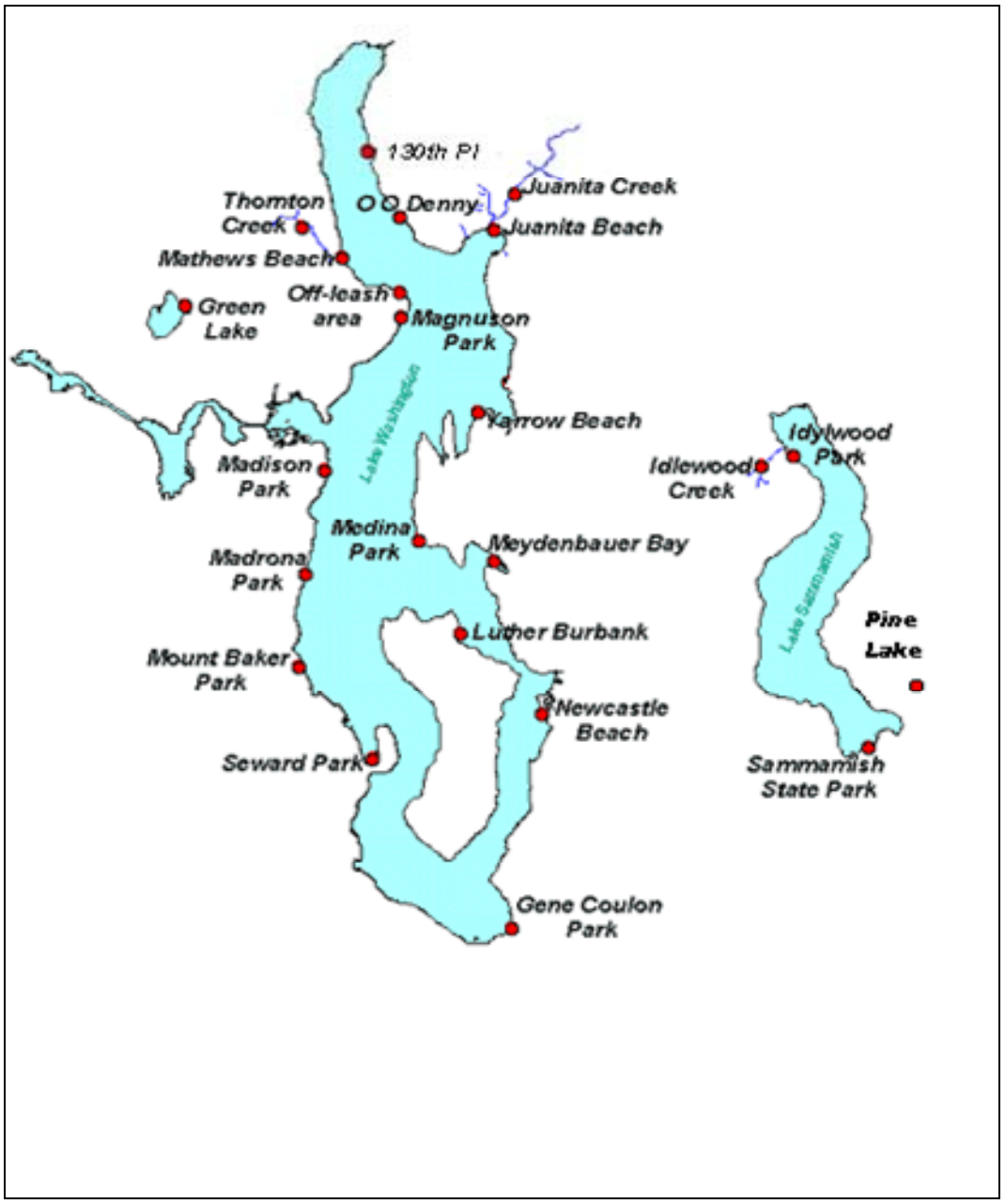


Figure 3. Swimming Beach Monitoring Program Sampling Locations.

1.6 Project Objectives

The objectives of the Toxic Cyanobacteria in Lake Washington, Lake Sammamish and Lake Union Study are to:

- Conduct a baseline screening study of microcystin concentrations in selected King County lakes during spring through fall
- Establish relationships between microcystins and other indicators of cyanobacteria biomass [e.g., (chl-*a*, cell densities, fluorometer readings]
- Assess human health risks associated with exposure to microcystins during recreational use of King County lakes.

The study will provide decision-makers with information and recommendations regarding recreational water use during cyanobacterial blooms and will lead to improved management of King County lakes for the protection of aquatic life and human health.

2 Study Design

2.1 Approach

This survey is primarily designed to evaluate the potential for cyanobacterial toxicity and the presence/absence of cyanobacterial toxins, and secondarily to estimate concentrations and geographic extent of the toxicity, should it be present. The assessment of cyanotoxins will focus on microcystins due to their widespread occurrence and potential for chronic toxicity. Microcystins will be measured in three water bodies in King County's Major Lakes Program (i.e., Lakes Sammamish, Washington and Union). The monitoring efforts described in this Phase II SAP will begin in March 2005. After two years of monitoring microcystins, the monitoring program will be re-evaluated and the sampling design optimized. At that time, the SAP will be revised or amended as needed. **NOTE**: the onset of this monitoring effort will cancel the previously ongoing cyanobacteria toxicity testing at Routine Major Lakes Sites.

Sample collection will utilize the combined efforts of the Routine Major Lakes Sampling Program and the Swimming Beach Monitoring Program. Table 1 lists the specific sampling sites for microcystin analysis. Table A (attached) lists all Major Lake sampling sites and swimming beach sites included in this study, and illustrates how microcystin and quantitative phytoplankton sample collection is coordinated with these programs. The Major Lakes Sampling Program collects samples twice per month from March through October. Swimming Beach Monitoring occurs weekly from mid-May through mid-September. Coordination with both programs will provide for weekly sample collection throughout most of the productive growing season and for better tracking of microcystin production in the lakes.

An example of the May through September monthly sampling scenario is as follows:

- Week 1 13 Routine Major Lake sampling sites
- Week 2 10 Swimming Beach sampling sites
- Week 3 13 Routine Major Lake sampling sites

- Week 4 10 Swimming Beach sampling sites, etc.

During the months of March, April and October, when the Swimming Beach Monitoring is not taking place, sampling will occur at the Routine Major Lakes sites only. During months when there are 3 weeks between lake sampling, samples will be collected at the Swim Beach sites.

Table 1. Summary of Cyanobacteria Toxicity Sampling Design.

Routine Major Lake Sampling Site	Samples per visit	Visits per year (March - October)	**Swim Beach Sampling Site**	Samples per visit	Visits per year (mid-May-mid-Sept)
0807	1	16	0806SB – Juanita	1	10
0826[a]	1	16	0826SB – Magneson	1	10
0814	1	16	0825SB – Yarrow	1	10
0834	1	16	0834SB – Meydenbauer	1	10
0832	1	16	083930SB – Newcastle	1	10
0831[a]	1	16	0828SB – Gene Coulon	1	10
0852[a]	1	16	0852SB – Madison	1	10
0817	1	16	0818SB - Matthews	1	10
0625	1	16	0602SB – Idylwood	1	10
0611[a]	1	16	---	---	---
0614	1	16	0615SB –Lk Samm State Park	1	10
0612[a]	1	16	---	---	---
A522[a]	1	16	---	---	---
Field Replicate (one station every other event)					
0852[a]	alternate visits	8	0806SB	alternate visits	5
TOTAL[b]		216			105

Laboratory Replicate (one station every other event)					
0852	alternate visits	8	0806SB	alternate visits	5

a. Indicates integrated composite sample. All other samples are discrete surface grabs. See Section 2.3.1.

b. Total number of routinely collected samples for microcystin analysis. Chl-*a*/pheo-*a* and quantitative phytoplankton analysis will be carried out at Routine Major Lake Stations during all sampling events. Additional samples will be collected from swimming beach sites and archived for possible future analysis as per item #2 in Section 2.1. Up to 10 samples per bloom event may be collected and evaluated, with the possibility that additional samples beyond these 10 may be requested, as per items #2 and #3, Section 2.1.

The following three types of sampling scenarios are included in this study:

1. Routine Major Lakes Sampling. Thirteen sampling sites were selected at Routine Major Lake monitoring locations in order to relate cyanobacterial data to other lake data. At least one deep station is included in each lake and the rest are nearshore sites that are within close proximity to selected swimming beaches monitored by the County (Table 1 and Table A).

   An aliquot of the sample collected as part of the routine sampling effort will be used for this study. Sample collection in the Routine Major Lakes program has been modified to incorporate use one of two methods – either an integrated composite sample, or a discrete surface sample. Table A identifies which sampling technique is used at each site in the overall Major Lakes Program. Section 2.3.1 describes the two sample collection methods.

   Microcystin will be measured by PPIA using the extraction methods described in this Phase II SAP. Chl-*a*/pheo-*a* (pheophytin *a*) analysis will be conducted on all thirteen of the Major Lake samples as part of this Routine Major Lakes Monitoring effort. See Major Lakes Monitoring Program SAP for further discussion. **NOTE** that quantitative phytoplankton enumeration and identification is being performed for all thirteen samples collected from the Major Lake stations as part of this focused Toxic Cyanobacteria Study (Table 1 and Table A).

2. Swimming Beach Monitoring. The second component of this sampling scenario will be conducted by the laboratory's Environmental Services Section (ESS) staff as part of the Swimming Beaches Monitoring Program. Ten stations that are part of the seasonal monitoring for fecal coliform bacteria will be included in this round of the focused Toxic Cyanobacteria Study (Table 1).

   Sufficient sample volume will be collected for microcystin testing and quantitative phytoplankton identification and enumeration. Quantitative phytoplankton identification and enumeration samples will be archived and analyzed if determined necessary by high microcystin concentrations. If toxins are present, quantitative phytoplankton identification and enumeration may be determined using the same methodology as for the Routine Major Lakes sampling effort. Sample collection will be a surface dip.

In addition, ESS will routinely visually inspect the waters at other swimming beach stations for cyanobacteria blooms while conducting the Swimming Beaches program. Up to 10 samples may be collected per bloom event, followed by Project Manager evaluation, and subsequent decisions regarding appropriate next steps.

3. Bloom Sampling. Focused sampling efforts will be made to collect scums or accumulations of cyanobacteria if they are present within the visual distance of routine lakes sampling sites (see 1 above) or as part of the Small Lakes Monitoring Program. A bloom will be defined by a visually observable accumulation of phytoplankton in the water column or as a surface accumulation. Coordinates will be obtained for these grab samples and a LIMS locator created. New locator names will be consistent with the naming convention system established for the Major Lakes and/or Small Lakes Programs. Up to 10 samples may be collected during a bloom event, at which time the Toxic Cyanobacteria Study Project Manager will evaluate such data as is available and discuss with the laboratory available options for proceeding with the bloom investigation.

   Sufficient volume will be collected for toxicity testing, as well as chl *a*/pheo-*a*, and phytoplankton quantitative enumeration and identification, if necessary. Microcystin will be measured by ELISA and PPIA on these discrete samples using the extraction methods described in this Phase II SAP. If toxins are present, chl–*a*/pheo-*a* and quantitative phytoplankton identification and enumeration may be determined using the same methodology as for the Routine Major Lakes sampling effort. See Major Lakes Monitoring Program SAP for further discussion.

2.2 Timeline

As noted, initial routine sampling and analysis of microcystins by ELISA was implemented in spring, 2002. This was followed by the more focused approach (detailed in the previous Cyanotoxicity SAP) from May 2003 and through November 2004. This Phase II SAP will be implemented from March 2005 and continue through October 2006. Samples are not collected for this study between November – February. Previous sampling programs will be discontinued.

2.3 Sampling Procedures

Protocols for the sampling and analysis of microcystins do not currently exist. However, a working group of the International Organization for Standardization is currently developing such protocols (Chorus, personal communication, April 24, 2002). The following sampling procedures are based on methods of Carmichael (2001), Chorus (2001), Johnston and Jacoby (2002).

Table 2. Sample Container & Preservation Requirements

Parameter	Matrix	Container	Preservation	Hold time
Quantitative Phytoplankton	Liquid	60-mL Glass wrapped in foil	Lugol's solution	365 days
Chlorophyll- *a* (in lab) (CHLA)	Liquid	1-L HDPE, AWM	4°C	1 day for filtration 28 days for analysis
Pheophytin- *a* (in lab) (PHEO)	Liquid	1-L HDPE, AWM (same bottle as collected for lab analysis of chlorophyll- *a*)	4°C	1 day for filtration 28 days for analysis
Microcystins ELISA (MLR-ELISA)	Liquid	250- ml Glass, AWM widemouth	4°C	24 to 48 hours then freeze
Microcystins PPIA (MLR-PPIA)	Liquid	250- ml Glass, AWM widemouth (same bottle as collected for MLR-ELISA)	4°C	24 to 48 hours then freeze
Microcystins HPLC	Liquid	1-L Teflon	4°C	ASAP

Notes:
AWM – Amber wide mouth bottle

HDPE – High density polyethylene bottle
HPLC – High performance liquid chromatography
PP – Polypropylene
VOA – Volatile organics analysis

2.3.1 Water sample collection and storage procedure to test for toxins:

Samples will be collected using the site-specific collection method identified above in Section 2.1 (e.g., integrated composite, discrete surface, or surface grab).

Integrated Composite technique: Vertically integrated composite samples are collected using a weighted length of ¾-inch tygon tubing let down vertically through the water

column as done for the Routine Major Lakes sampling program. This tube is marked so that when fully extended, the distance from the mark at the water surface to the end of the tube is 10 m. The tube is plugged at the surface and at the submerged end by a check valve and retrieved. The tube contains a vertically integrated sample of the lake from surface to 10 meters. The sample is decanted into a stainless steel bowl and homogenized before sub-sampling for microcystin, chl-*a*, pheo-*a* and phytoplankton enumeration. If more than one tube is collected, combine the water in the steel bowl prior to filling sample containers. Aliquots for microcystin analysis will be poured into a 250-mL glass, AWM bottle, leaving some headspace for freezing. The sample bottle should not be pre-rinsed with sample.

Discrete Surface Samples: Discrete surface samples are grab samples collected 1 m below the water surface using Scott bottles or Niskin bottles on the CTD rosette.

Swimming Beach surface grabs: For surface grabs, fill the 250-mL glass, AWM bottle by dipping the bottle mouth-down into the water. With a sweeping arch, collect water from approximately 2 feet below the surface, leaving a headspace.

- Label the bottles if not pre-labeled.
- Place the sample bottles in a cooler with ice packs (no preservative required).
- Subsamples will be removed from the 250-mL glass bottle and frozen within 24 to 48 hours of arrival at the King County Environmental Laboratory. Bottles and vials should be slanted to prevent breakage during freezing. Samples must be stored frozen for a minimum of 12 hours to insure complete freezing of the sample.
- Periodically, one additional 1-L Teflon bottle will be collected for confirmatory HPLC analysis of microcystins. This bottle will be kept at 4˚C and delivered ASAP to the subcontracted laboratory for analysis.

2.3.2 Water sample collection and storage procedure for quantitative identification of cyanobacteria.

Quantitative cyanobacteria identification and enumeration will be conducted at the thirteen Major Lake stations as part of this Phase II SAP for Toxic Cyanobacteria (Table 1 and Table A). Quantitative phytoplankton identification and enumeration will be subcontracted out to Maribeth Gibbons at WATER Environmental, Inc.

In addition, samples for quantitative identification and enumeration will be collected and preserved at the designated Swimming Beach sites in the event that high microcystin concentrations warrant further investigation. A 60 mL aliquot will be collected and placed in properly labeled opaque bottles (typically 60 mL glass vials wrapped in aluminum foil) and preserved with a sufficient amount of concentrated Lugol's solution to turn the sample light red; typically eight drops. Care should be taken that samples are covered tightly and stored in the dark until analyzed.

In the event that algal blooms are sampled (as per #3 in section 2.1), samples will be collected and preserved as described above.

2.3.3 Water sample collection and storage procedure for chlorophyll *a*/pheophytin *a* analysis.

Samples are collected for chlorophyll *a*/pheophytin *a* analysis as part of the Major Lakes Program using either the integrated composite sampling or discrete surface sampling method identified for each site in Table A. In the event that algal bloom samples are collected as per #3 in Section 2.1, additional sample volume will need to be collected and preserved for possible chl-*a*/pheo-*a* analysis.

In general, samples should be stored in the dark at 4°C before filtration, which should take place ASAP and up to 1 day following collection. Filters are then stored in 90% acetone, in a foil-covered rack in a -20°C freezer (non frost-free) for up to 28 days prior to sonication and instrumental analysis. Once samples are filtered, it is preferred to store the samples on filters for at least two days prior to sonication and analysis to help facilitate extraction of chlorophyll from algae into the acetone medium.

See the Major Lakes Monitoring Program SAP for more details (King County 2005).

3 Laboratory Analysis

ELISA and PPIA assays are suitable for rapid and sensitive detection of microcystins. These methods are useful for preliminary toxin screening for both cyanobacterial samples and extra cellular microcystins in the water (Chu *et* al. 1990; Chorus 2001). ELISA is based on the structure of the microcystin molecule and requires antibodies against microcystins whereas PPIA is based on the toxic effects of microcystins. The PPIA method is preferred for waters that may contain toxic forms of microcystins and nodularins.

ELISA and PPIA are suitable as indicating tests for the analysis of extra cellular microcystins at concentrations below 1 µg/ L. ELISA is the most sensitive and simple method, but has the potential for false positive reactions (Chorus 2001). PPIA provides preliminary information on the toxicity of microcystins in comparison to the microcystin content measured by ELISA. For confirmation of microcystin, HPLC analysis is recommended (Chorus 2001).

The King County Environmental Laboratory has developed Standard Operating Procedures (SOP) for the measurement of microcystins using ELISA (SOP 04-02-009) and microcystins and nodularins using PPIA (SOP 04-02-012) in water.

3.1 Toxin Structure and Cross-Reactivity Analysis Summary

Microcystins are a group of cyclic heptapeptide hepatotoxins produced by species of the common bloom-forming genera of cyanobacteria including *Microcystis*, *Anabaena, Nostoc* and *Oscillatoria*. These toxins contain two variable L-amino acids, three D-amino acids and two unusual amino acids. There are now over 50 different microcystins which have been structurally characterized and which differ primarily in the two L-amino acids and methylation or demethylation of the two unusual amino acids. These microcystins all contain the Adda amino acid, which is essential for expression of their

biological activity. Nodularins are monocyclic pentapeptide liver toxins produced by the cyanobacterium *Nodularia*. Nodularins contain Adda but lack one of the L- and D-amino acids found in microcystins. Both microcystins and nodularin have been found to be potent inhibitors of protein phosphatase (PP) isozyme types 1 and 2A. The inhibitory action of the toxins on PP1 is considered a basis for their toxicity and forms the basis for the PP1 inhibition assay. Currently several methods have been developed to detect and quantify cyanotoxins. However, there is no single method that provides adequate monitoring for all cyanotoxins. Many of the microcystins and nodularins in environmental samples will be detected by a combination of the ELISA and PPIA methods.

3.1.1 Sample Preparation for Toxin Assay

To measure total microcystin concentrations (extra- and intracellular) in the water samples, sample preparation will include a cell-lysing step prior to analysis.

The objective of the cell-lysing is to generate a sample in which all microcystins (extra and intracellular) have been converted into a free form that can be measured by ELISA and PPIA, thus providing a close approximation of the total concentration in the ambient sample (extra and intracellular). The resulting concentration should be representative of a recreational exposure in which a swimmer ingests ambient water and cells as a combined dose. If samples were analyzed without lysing, results would be reported as *Free Microcystins.* Since all samples collected for this study will be analyzed following lysing, results will be reported as *Total Microcystins*. Note ELISA measures only free microcystin, not the amount chemically bound to the cell or molecular components such as protein phosphatase enzymes.

Established protocols for extraction are unavailable at this time. The 2002 - 2004 focused cyanobacteria toxin study utilized two techniques to evaluate their effectiveness in lysing – 1) freezing of samples for a minimum of 12 hours, or 2) sonication. Unfrozen but refrigerated controls were also analyzed, to provide data to evaluate the two options listed above. Evaluation of the two extraction methods was inconclusive due to low microcystin concentrations. For this 2005 – 2006 round of sampling, laboratory staff recommended combining extraction methods. Therefore, each sample will receive the following lysing process:

- 10-ml aliquots will be frozen for a minimum of 12 hours and then
- thawed at room temperature and then immediately sonicated (ultrasonic disruption) using the Vibra Cell Sonicator.
- Samples will be filtered through a 0.45 μm filter prior to analysis.

NOTE: Green pigments and associated substances in 0.45 μm filtrate can mask the presence of microcystins. Additional filtration to 5000 NMWL will be performed when the filtrate appears colored to remove pigments and associated substances that may interfere with the assay. Since the ELISA requires 50 μL per replicate, a scaled up version of the ultra filtration system, perhaps including centrifuge, may be most efficient

(see attachment for further discussion). The method detection limit (MDL) is 0.05 μg/L as microcystin-LR equivalents. MDL for the PPIA is 0.1 μg/L as microcystin-LR equivalents.

Holding times for microcystin analysis in frozen samples have not been established to date. Other studies have shown that microcystins do not readily degrade in frozen samples (Chorus, personal communication, April 24, 2002). Deep-freezing samples that have been freeze-dried will ensure sample preservation; however, even wet-frozen samples demonstrate no substantial loss in microcystin concentration over months or years. Storage of dried samples at air temperature should be avoided because absorbed moisture from the air may activate the bacteria (Chorus, personal communication, April 24, 2002). Based on KCEL SOP(s) 04-02-009 and 012, a conservative holding time for frozen samples of 7 days will be employed. Holding times for the filtrate at 4 ºC are being determined.

3.1.2 Microcystins– ELISA

The ELISA test kit uses polyclonal antibodies that bind either microcystins or a microcystin-enzyme conjugate. Microcystins in the sample compete with the microcystin-enzyme conjugate for a limited number of antibody binding sites. Since the same number of antibody binding sites are available on every test well, and each test well receives the same number of microcystin-enzyme conjugate molecules, a sample that contains a low concentration of microcystins allows the antibody to bind many microcystin-enzyme conjugate molecules. The result is a dark blue solution. Conversely, a high concentration of microcystins allows fewer microcystin-enzyme conjugate molecules to be bound by the antibodies, resulting in a lighter blue solution. The plate kit does not differentiate between microcystin-LR and other microcystin variants but detects their presence to differing degrees. At 50% inhibition the concentrations are: MC-LR 0.31 μg/L, MC-RR 0.32 μg/L, MC-YR 0.38 μg/L and NODLN 0.47 μg/L.

3.1.3 Microcystins –PPIA

The enzyme protein phosphatase is inhibited in a concentration-dependent manner by microcystins. Subsequent exposure of the enzyme to a substrate that forms a colored product reveals the degree of enzyme inhibition. Comparison of sample results with those of known standards quantifies the level of microcystins in the sample.

3.1.4 Microcystins - HPLC

A selected number of samples will be submitted to Water Management Laboratories Inc. in Tacoma, Washington, for confirmation of total microcystins by HPLC. The MDL for the HPLC analysis is < 0.1 μg/L as microcystin if provided with 100 mL of sample (personal communication with lab). A percentage of samples with microcystin concentrations exceeding 1.0 μg/L will be sent for confirmation whenever available.

3.2 Analytical Procedures

Samples will be analyzed using the procedures and detection limits listed in the table below.

Table 4. Laboratory Analysis Summary

Parameter	Reference	Method Detection Limit	Reporting Detection Limit
Microcystins by ELISA	KCEL SOP 04-02-009	0.05 µg/L	0.05 µg/L
Microcystins and Nodularins by PPIA	KCEL SOP 04-02-012	0.1 µg/L	0.1 µg/L
Confirmatory Microcystins by HPLC	WML Inc.	0.1 µg/L	0.1 µg/L
Chlorophyll *a*	EPA 446.0	0.5 µg/L	1.0 µg/L
Pheophytin *a*	EPA 446.0	1.0 µg/L	2.0 µg/L

4 Data Quality Objectives

The procedures and practices described in this study specific SAP are designed to generate data of sufficient quality to support decision making as discussed in the site specific SAP. Critical elements of laboratory data quality objectives are discussed in this section. Procedures to attain these data quality objectives are discussed throughout this document. In particular, Section 7.0, Quality Control Procedures, addresses many of the procedures necessary to obtain data that meet these data quality objectives.

4.1.1 Laboratory Precision

Laboratory precision will be assessed using laboratory duplicate QC samples. When both sample results are at or exceed the MDL the RPD (relative percent difference) should be less than 25 %. An RPD cannot be determined unless both values are at or above the MDL since no values are reported if <MDL. Note that the Method Detection Limit (MDL) and the Reporting Detection Limit (RDL) are the same for both the ELISA and PPIA.

The actual criteria for performing the RPD calculation and applying the control limits are based on at least one of the values being >RDL. If both results are <RDL, no calculation is applied and there are no expectations placed on the data with respect to precision.

If one value is >RDL and the other <MDL, a RPD is still calculated using zero for the less <MDL value.

A 25% RPD is applicable to chlorophyll-*a* but a 50% window is used for pheophytin-*a*.

4.1.2 Field Precision

Information regarding the precision of sampling procedures will be obtained by collecting field replicates. The data user should take the information obtained by collecting field replicates into account when making decisions based on data generated under this SAP.

4.1.3 Bias

Bias is an indicator of the accuracy of analytical data. For this project, laboratory control samples or blank spikes, whichever are available, will be used to assess bias. Results should be within 20% of the true value or within the criteria provided with the purchase of the control sample.

Bias will also be assessed by the evaluation of field blank and method blank data. Analytical results for method blanks should be less than the MDL.

The use of matrix spike recovery data will provide additional information regarding method performance on actual samples. The laboratory will use professional judgment regarding assessment of data quality and any subsequent action taken as a result of matrix spike recoveries.

4.1.4 Representativeness

This survey is primarily designed to evaluate the presence/absence of cyanobacterial toxicity, and secondarily to estimate concentrations and geographic extent of the toxin distribution, should it be present. Representative samples will be obtained through the following practices:

- The use of generally accepted sampling procedures will allow for the collection of representative samples.
- Subsampling within the King County Environmental Laboratory will be conducted according to lab standard operating procedures. These procedures are designed to obtain representative subsamples.

Note that additional practices to be used to obtain representative data are described in the site specific SAP; Major Lakes Monitoring Program SAP, King County, forthcoming in 2005.

4.1.5 Comparability

Data comparability will be obtained through the use of standard sampling procedures and analytical methods. Additionally, adherence to the procedures and QC approach contained in this SAP will provide for comparable data throughout the duration of this

project. Before making changes to sample collection, storage or analysis procedures, each must be evaluated to verify that comparability will not be compromised.

4.1.6 Completeness

Completeness will be evaluated by the following criteria:

- The number of usable data points compared to the projected data points as detailed in this SAP.
- Compliance with the data quality criteria as presented in this section.
- Compliance with specified holding times.

The goal for the above criteria is to obtain 100% data completeness. However, where data are not complete, decisions regarding re-sampling and/or re-analysis will be made by a collaborative process involving both data users and data generators. These decisions will take into account the project data quality objectives as presented above.

5 Data Reduction, Review, and Reporting

Data reduction, review and reporting will be performed under the King County Environmental Laboratory's standard operating procedures. Laboratory data will be provided to data recipients within 30 days of sample receipt. Data reports will include sufficient information to conduct the data assessment. Field measurements will also undergo standard review and reporting procedures. Data will be reported in the standard laboratory-reporting format. This includes an analytical result, MDL and RDL, if available. The reporting format and standard due dates for quantitative phytoplankton data will be defined by the contract that King County establishes with Water Environmental Services, Inc.

Protocols will be worked out with the King County Environmental Laboratory for the rapid turn around of selected samples in the event of a bloom episode that could have potential public health implications. Preliminary project data, required in the event of a bloom episode that could have potential public health implications, will be reported using KCEL Preliminary Data Reporting Form followed by final data as soon as practical.

Final project data will be presented to the project and program managers in a format that will include the following:

- King County Environmental Laboratory Comprehensive Reports consisting of spreadsheets of analytical and field parameters;
- Case narratives for ELISA and PPIA results prepared by the Aquatic Toxicology Section;
- Section narratives of chemistry and microbiology data including supporting QC documentation (provided by the King County Environmental Laboratory) in the event of analytical or data anomalies.

- A technical memorandum summarizing field sampling, analytical work and interpretation of the QC results (provided by the King County Environmental Laboratory).

- Cyanobacteria identification and biovolume determinations conducted by Water Environmental Services, Inc., as per contract and the Major Lakes Quantitative Phytoplankton SAP (King County DNRP 2003).

6 Project Organization

Project team members and their responsibilities are summarized below. All team members are staff of the King County Department of Natural Resources and Parks, Water and Land Resources Division.

Table 5. Project Team Members

Name/Telephone	Title	Affiliation	Responsibility
Katherine Bourbonais (206) 684-2382	Laboratory Project Manager	Environmental Laboratory	Coordination of analytical activities, lab QA/QC and data reporting.
Jeff Droker (206) 684-2309	Environmental Scientist	Environmental Laboratory	Coordination of sampling activities, field QA/QC, and field analyses.
Debra Bouchard (206) 263-6343	Water Quality Planner	Water & Land Resources	Project manager for the Toxic Cyanobacteria Study, coordination between lab, contracted phytoplankton specialist, and in-house specialist
Colin Elliott (206) 684-2343	Quality Assurance Officer	Environmental Laboratory	Overall project QA/QC.
Gabriela Hannach (206) 684-2301	Aquatic Toxicologist	Environmental Laboratory	Coordination of toxicity analysis
Jim Buckley (206) 684-2314	Aquatic Toxicologist	Environmental Laboratory	ELISA and PPIA method development
Duc Nguyen (206) 684-2377	Environmental Scientist	Environmental Laboratory	Coordination of chl-*a*/pheo-*a* analysis

7 Quality Control Procedures

7.1 Field Quality Control Procedures

Over the course of this project, field QC samples will be collected at the frequency listed below. It is recommended that a set of field QC samples be collected during the first sampling effort to provide an initial indication of field sampling precision and bias.

Table 6. Field Quality Control Samples

Type of Quality Control Sample	Description	Frequency
Field Blank	Reagent sample matrix that has been processed as a field sample, used as an indication of sampling process contamination.	Over the course of the project, 1 per sampling day, done at a random site. If more than 20 sites are collected in one day, 2 field blanks should be collected.
Field Replicate	A second sample generated from the same sampling location as the initial sample, but from a second sampler deployment. Used as an indicator of field sampling precision.	Over the course of the project, 1 per sampling event, done at a random site. If more than 20 sites are done in a day, 2 field replicates should be collected.

7.1.1 QC Practices for Field Measurements

Sampling for this Toxic Cyanobacteria Study is conducted concurrently with the Routine/Ambient Major Lakes Monitoring program. Therefore QA practices are covered under those SAPs.

7.2 Laboratory Quality Control Procedures

The King County Environmental Laboratory is accredited by the Washington State Department of Ecology. As a requirement of this accreditation, the lab is audited by the Washington State Department of Ecology. Additionally, the King County Environmental Laboratory participates regularly in US EPA inter-laboratory performance evaluation studies.

A number of samples will also be analyzed by HPLC for microcystins. Both ELISA and PPIA are suitable as indicating tests for the analysis of extra cellular microcystins, but ELISA has potential for false positives. Therefore, confirmatory analysis using a different determinative approach will provide information that can be used to evaluate ELISA data. The number and frequency of confirmatory sample analyses will be determined by the Project Manager.

7.2.1 Frequency of quality control samples

For samples analyzed at the King County Environmental Laboratory, the frequency of quality control samples to be performed for this project is shown in the following table. QC samples shown below may not be available for all lab analysis.

Table 7. Laboratory Quality Control Samples

Type of Quality Control Sample	Description	Frequency
Method Blank	An aliquot of clean reference matrix carried through the analytical process and used as an indicator of contamination.	1 per sample batch. Maximum sample batch size equals 20 samples.
Laboratory Control Sample	Solution of known analyte concentration, processed through the entire analytical procedure and used as an indicator of method accuracy and precision.	1 per sample batch, as available. Maximum sample batch size equals 20 samples.
Spike Blank	Known concentration of target analyte(s) introduced to clean reference matrix, processed through the entire analytical procedure and used as an indicator of method performance.	Used if a laboratory control sample is not available. 1 per sample batch. Maximum sample batch size equals 20 samples.

In addition to the QC samples specified above, the following QC samples will be performed on samples from this project at the frequency listed below:

Table 8. Additional Laboratory Quality Control Samples

Type of Quality Control Sample	Description	Frequency
Lab Duplicate	A second aliquot of a sample, processed concurrently and identically with the initial sample, used as an indicator of method precision.	Over the course of the project, 1 per 20 samples.

KCEL laboratory QC samples for chl–*a*/pheo-*a* and microcystins analysis and associated control limits are summarized below. These QC samples will be analyzed at a frequency of one per analytical batch 20 or fewer samples.

Table 9. Laboratory QC Requirements

Parameter	Method Blank	Duplicate RPD	Negative Control	CS % Recovery
Chl-*a*	<MDL	25%	NA	90 to 110 %
Pheo-*a*	<MDL	50%	NA	NA
Microcystins	<MDL		<0.1 ppb	NA

Notes:
CS- Check Standard (positive control equivalent to Laboratory Control Sample)
MDL – Method Detection Limit
NA – Not Applicable
RPD – Relative Percent Difference

7.3 Corrective Action

King County Environmental Laboratory standard operating practice is to detect and correct analytical difficulties during sample analysis. Should the lab have difficulty in meeting the data quality objectives outlined in this QA plan, the lab will work with the data user to develop and implement corrective action.

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9 Attachment –THE INFLUENCE OF PIGMENTS ON RESULTS IN THE PROTEIN PHOSPHATASE INHIBITION ASSAY

Lab Analysis conducted by Jim Buckley

020123 JB

In the ELISA for microcystins, it is not uncommon to observe anomalous %B values of greater than 100% for environmental samples. The same is true for the PPIA. When the samples from the *Aphanizomenon* bloom in Green Lake were analyzed by PPIA, samples filtered to 0.45 um retained a green color and yielded %B values much greater than 100%. This provided an opportunity to test for pigment removal with an ultrafiltration system consisting of a filter of 5000 nmwl and centrifugation at 15,000 g.

The following table shows results of three days of tests of several treatments of Green Lake SE (GL SE) and Aquatheater (GL AT) samples designed to identify the influence of pigments, probably mostly chlorophylls, on the results of PPIA. Values in bold print are from samples that were green in color following filtration to 0.45um.

Terms:
0.45 = filtration to 0.45 um with glass fiber prefilter and 0.45 um Millipore Millex filter
5000 = filtration to 5000 nominal molecular weight limit (nmwl) Millipore Ultrafree centrifuge unit
control = assay mixture including sample but without PP enzyme
blank = negative control without PP enzyme
centrifuge = 10 min. at 15,000g

Sample /Treatment	GL SE 1		GL SE 2		GL AT 1		GL AT 2	
	%B	ug/L	%B	ug/L	%B	ug/L	%B	ug/L
12-29-01								
0.45 + 5000	90	0.095	92	0.087	90	0.093	86	0.111
control	2		-15		3		4	
blank = 0.069								
1-8-02								
0.45	**373**	**0.000**	**442**	**0.000**	95	0.082	92	0.092
0.45 + 5000	94	0.085	95	0.081	91	0.094	91	0.095
control	-0.1		-1.0		-1.0		-4	
blank = 0.107								
1-10-02								
0.45	**391**		**489**					
control	**285**		**376**					
0.45 + centrifuge	**355**		**421**					
control	**257**		**332**					
blank = 0.075								

12-29-01
The samples were filtered to 0.45 (green) and then to 5000 (clear) and assayed for microcystins. Results showed microcystins present at 0.111 ug/L in the GL AT 2 sample. All other samples gave results < MDL of 0.1 µg/L. Control %B values were ≤ 4, indicating little or no apparent endogenous PP activity.

1-8-02
Samples from GL SE 1 and 2 filtered to 0.45 um only gave very high %B values (**373** and **442**) due to the OD from the green pigments and perhaps from other substances also. In comparison, samples GL AT 1

and 2, also filtered to 0.45 um, were clear and gave typical %B values (95 and 92). When the 0.45-filtered GL SE 1 and 2 samples were further treated by filtration to 5000 nmwl thereby removing the green color and putative other substances, the %B values were reduced from **373** to 94 and **442** to 95. This indicates that the 5000 nmwl filtration step is effective in removal of pigments and other substances that can confound results of the PPIA. Filtration of GL AT 1 and 2 to 5000 nmwl only slightly changed the % B values (95 to 91 and 92 to 91) and resulting levels of microcystins. Control values for these samples were all very low (-0.1 to -1.0).

1-10-02
Samples were green following filtration to 0.45 um only. Subtraction of the control (without PP) from the assay (with PP) yields values (mean = 102) that are close to the negative control (with PP) indicating little or no endogenous PP activity in these samples.

GL SE 1: **391** – **285** = 106 A % B value of ≈ 100 indicates a sample reading ≈ negative control

355 – **257** = 98

GL SE 2: **489** – **376** = 113

421 – **332** = 89

Centrifugation alone yielded a small white button for both samples. However, the high %B values were only reduced by 9 to 14 %, indicating that only a small amount of the high OD is due to particulates.

GL SE 1: **391** – **355** = 36 or 9% of %B is removable by centrifugation

GL SE 2: **489** – **421** = 68 or 14% of %B is removable by centrifugation

Conclusions

1. Green pigments and associated substances in 0.45 um filtrate give artificially high %B values that can mask the presence of microcystins.

2. Filtration to 0.45 um and then to 5000 nmwl removed these pigments and associated substances that, in this case, gave high %B values that could mask otherwise detectable levels of microcystins.

3. It is useful to run Controls to check for apparent endogenous protein phosphatase activity in samples. In the present samples, there was no apparent protein phosphatase activity.

4. Centrifugation only of samples previously filtered to 0.45 um showed that 9 to 14 % of the high %B values was due to particulates amenable to removable by filtration.

5. For the Green Lake SE samples, the yield from the ultrafiltration system was 120 uL each which was adequate for the PPIA which requires 20 uL per replicate. To be useful for the ELISA which requires 100 uL per replicate, a scaled up version of the ultrafiltration system, perhaps including centrifuge, would be most efficient.