Marine Phytoplankton Monitoring Program Sampling and Analysis Plan

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EXECUTIVE SUMMARY

King County's (KC) marine offshore water quality monitoring program collects data on the physical, chemical, and biological characteristics of marine waters within the boundaries of King County. These data are used to improve our understanding of ecosystem structure and function and for assessing potential impacts of human activities, such as from wastewater treatment plant, combined sewer overflow, and stormwater discharges. The phytoplankton component of this program collects information on phytoplankton community composition, abundance, and biomass. The paucity of phytoplankton abundance and community composition data in Puget Sound was identified by the Puget Sound Ecosystem Monitoring Program (PSEMP) as a substantial gap in long-term monitoring (Dzinbal et al., 2014). This program participates in a regional collaboration to increase biological monitoring and information synthesis by sharing data and results with the University of Washington, the National Oceanic and Atmospheric Administration, the Washington Department of Ecology, and other partners.

This technical document provides the plan for implementing the marine phytoplankton monitoring program, which is standard practice for monitoring programs. This document follows a standardized format and includes background information, program objectives, sampling design, sample collection and analysis protocols, and data management procedures for this program.

Phytoplankton are a group of tiny, free-floating algae that form the base of marine food webs as the dominant producers. There is a high level of diversity within this group, and different types of phytoplankton play complex and specific ecological roles. Detailed information on phytoplankton community composition is necessary to understand marine food webs and to sensitively assess biological responses to environmental factors and change. Some species of phytoplankton are also of particular interest for monitoring because they produce toxins and form blooms that can be harmful to humans and/or wildlife. The ecology of harmful algal species is a high priority for natural resource managers and commercial shellfish growers in Puget Sound.

King County's phytoplankton monitoring program began in 2008 with assessment of relative abundance metrics for phytoplankton taxa at three sites using microscopy. In May 2014, analysis with a new particle imaging analyzer (FlowCAM®, Fluid Imaging Technologies) was added to the program to analyze samples from eight sites (see map on page 10). This instrument partially automates identification, and the method quantifies both abundance and a proxy of phytoplankton biomass.

Phytoplankton sampling occurs twice monthly from February through November and once monthly in December and January (22 sampling events per year). Eight sampling locations provide broad spatial coverage in County offshore waters. Samples are collected from the surface at all sites and at a deeper depth (the chlorophyll maximum layer) at two of the sites (10 samples × 22 events = 220 samples per year). Three of these locations are also sampled for community composition, abundance, and biomass of zooplankton, important

consumers of phytoplankton. All locations are also sampled for water quality parameters such as salinity, nutrients, chlorophyll, and dissolved oxygen, all of which may impact or be impacted by phytoplankton.

At a minimum, data results are reported annually in summaries available on the web. Phytoplankton data are stored in the King County Environmental Laboratory's LIMS database. A more robust database to archive phytoplankton and zooplankton data began development in 2015 and will include a public web portal for data viewing and downloading.

1.0 INTRODUCTION

King County's (KC) marine offshore water quality monitoring program collects data on physical, chemical, and biological parameters in marine waters of central Puget Sound within the boundaries of King County. These data are used to provide an understanding of water quality near KC's wastewater plant outfalls and to assess if discharges are affecting water quality. This monitoring effort involves collecting environmental data from sites near discharge locations (outfall pipes) as well as data from "ambient" sites outside the immediate vicinity of known discharges.

The current lack of phytoplankton abundance and community composition data in Puget Sound was identified as a substantial gap in long-term monitoring by the Puget Sound Ecosystem Monitoring Program (PSEMP), an interagency effort tasked with monitoring the health of the Puget Sound environment on a regional basis (Dzinbal et al., 2014). KC's phytoplankton program participates in a regional collaboration to increase biological monitoring and information synthesis. Yearly results and analysis are included the PSEMP Marine Waters Work Group Annual Overview Report. Data are also shared with other monitoring groups via the Washington Department of Ecology's monthly Marine Condition Update seminars and Eyes Over Puget Sound reports, with researchers at the University of Washington Seattle and Tacoma branches, and with the National Oceanic and Atmospheric Administration SoundToxins harmful algal bloom monitoring program.

This sampling and analysis plan (SAP) describes the phytoplankton component of KC's marine monitoring program, which involves sampling and taxonomic analysis of phytoplankton in the Central Basin of Puget Sound. This program began in April 2008 with a semi-quantitative microscopy method of analysis, which assessed relative abundance of phytoplankton taxa. In May 2014, sample analysis with a particle imaging analyzer (FlowCAM®, Fluid Imaging Technologies) was added to the program. This method partially automates identification and quantifies both abundance and biovolume (a proxy for biomass) of identified and unidentified taxa. The semi-quantitative method was retired at the end of 2014 and superseded by qualitative microscopy, which provides detailed taxonomy to complement the quantitative FlowCAM method.

This document includes background information, program objectives, sampling design, sample collection and analysis protocols, and data management procedures for this program. Details of other KC marine monitoring activities and general field and laboratory procedures can be found in separate program component SAPs or in the general Marine Monitoring SAP (King County, *in prep.*).

2.0 BACKGROUND INFORMATION

Marine phytoplankton are a group of diverse, single-celled or colonial algae that form the base of marine food webs as the dominant primary producers. They range in size from around 1 μm to over 300 μm. Phytoplankton are generally photosynthetic (autotrophic), meaning they use solar energy to incorporate carbon dioxide into energy-storing carbon molecules, which can then be used (via respiration) for growth and reproduction. However, there are also many species of mixotrophic phytoplankton, which are able to take up dissolved organic matter or ingest other organisms in addition to photosynthesizing. Some types of algae often grouped with the phytoplankton are even fully heterotrophic (non-photosynthetic), ingesting other organisms as their sole source of energy and carbon.

Within the phytoplankton, there are several major, ecologically-distinct groups in coastal marine systems. The largest and most easily identified taxa are generally diatoms, which are predominantly autotrophic, and the dinoflagellates, which may be auto-, mixo-, or heterotrophic. There are a variety of smaller taxa as well, including the nanoflagellates (a group defined primarily by their ~2-30 μm size range rather than taxonomy), which may be auto-, mixo-, or heterotrophic, and the cyanobacteria (< 2 μm), which are auto- or mixotrophic. Cyanobacteria are dominant primary producers in pelagic systems, but are relatively less important in coastal and estuarine systems. Due to their small size, they are not currently quantified in this program and will not be discussed further.

Diatoms, which exist as either single cells or long chains of cells (colonies), are generally large species that are adapted to high-nutrient environments with periodic mixing. Diatoms can divide rapidly and accumulate high biomass under the right conditions, such as when deep winter mixing eases in coastal and estuarine environments (a phenomenon called the "spring bloom"). Diatoms are a major food source for other organisms in coastal and estuarine systems, including a wide variety of zooplankton and mixo-/heterotrophic dinoflagellates. These taxa are also prone to sinking out of the euphotic zone and onto the sea floor due to their heavy silica outer coverings and lack of motility, impacting dissolved oxygen levels as they decompose.

The photosynthetic dinoflagellates and nanoflagellates are generally more competitive under lower nutrient conditions than diatoms, tending to be smaller, slower growing, motile, and less efficient at nitrogen uptake (but often able to supplement their nutrient supply by ingestion of nitrogen-rich organic compounds or particles). The succession from heavily diatom-dominated communities in the spring to more diverse, small cell-dominated communities in the summer is a general pattern that is frequently observed in coastal and estuarine systems. Much of the autotrophic production in these communities is recycled within a complex "microbial loop" of small heterotroph ("microzooplankton") and bacterial interactions. This is in contrast to diatom blooms, of which a greater proportion of production is generally exported either to higher trophic levels via consumption by the larger "mesozooplankton" and fish, or to the sea floor.

Some species of phytoplankton are of particular interest for monitoring because they produce toxins and form blooms that can be harmful to humans and/or wildlife. An important example of a "harmful algal bloom" (HAB)-forming species in Puget Sound is the dinoflagellate *Alexandrium catenella*, which produces a suite of potent neurotoxins. Human ingestion of shellfish that have filter-fed on *Alexandrium catenella* can cause paralytic shellfish poisoning, which is potentially life-threatening. Understanding the ecology of this and other HAB species and their responses to environmental change is a high priority for natural resource managers and commercial shellfish growers in Puget Sound.

The diversity in size, physiology, toxicity, and nutritional modes within the phytoplankton community corresponds to a variety of complex and specific ecological roles played by the different taxa. Thus, detailed information on community composition is necessary for understanding marine food webs. Phytoplankton community composition and biomass at any one point in time is a product of various interacting bottom-up (e.g., nutrient concentration, temperature, light intensity, physical mixing) and top-down (i.e., consumption by microzooplankton) controls. This makes phytoplankton community composition both a valuable metric of biological response to environmental change and a key explanatory variable for the dynamics of higher trophic levels in the marine food web.

3.0 GOALS AND OBJECTIVES

The primary goal of this program is to collect data on marine phytoplankton data in order to assess biological response with respect to environmental variables and change. These data may also be used to further understanding of the Puget Sound marine food web and the assessment of human and climate impacts. In order to meet this goal, the following objectives were developed:

1. Catalog and quantify the phytoplankton present in the Central Basin of Puget Sound throughout the year.

2. Measure variability in community composition, size distribution, abundance, and biovolume over seasonal and inter-annual timescales.

3. Establish current baseline phytoplankton community patterns and variability in composition, abundance, and biovolume, and monitor for long-term changes.

4. Assess ecosystem and food web dynamics in the Central Basin of Puget Sound by analyzing data in conjunction with other KC monitoring datasets (chemical, physical, and biological parameters). Interpret data in context of historical phytoplankton datasets as available for Puget Sound.

5. Provide a yearly summary of the phytoplankton assemblage and seasonal dynamics for resource managers, researchers, and other interested parties.

4.0 PROGRAM ORGANIZATION AND RESPONSIBILITIES

The tasks involved in conducting the marine phytoplankton monitoring program and the personnel primarily responsible for those tasks are listed below.

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5.0 SAMPLING DESIGN

5.1   Temporal Aspects

Phytoplankton samples are collected during routine marine monitoring sampling events from a King County Environmental Laboratory (KCEL) research vessel or from a dock. These sampling events occur twice monthly from February through November, generally on the first and third weeks of each month. Sampling occurs only once monthly in December and January to allow time for vessel and instrument maintenance. All sampling occurs during daylight hours. A single sampling event generally encompasses a three-day period, with the northern (JSUR01-Brightwater Treatment Plant Outfall to LTED04-Elliott Bay), southern (LSEP01-South Treatment Plant Outfall to NSEX01-East Passage), and Vashon Island (NSAJ02-Outer Quartermaster Harbor) sites sampled on days 1, 2 and 3, respectively (Table 1).

5.2   Spatial Aspects

Of the 14 sites at which KCEL collects water samples for the marine offshore monitoring program, 8 are currently sampled for phytoplankton (Table 1, Figure 1). These sites were chosen to provide broad spatial coverage within King County waters in the Central Basin of Puget Sound and include both ambient sites and sites near wastewater treatment plant outfall pipes. Samples are collected within Elliott Bay (LTED04), a major shipping port with extensive shoreline development and freshwater influence from the industrialized Duwamish River, and outer Quartermaster Harbor (NSAJ02), an enclosed, poorly flushed embayment that experiences periods of low dissolved oxygen. Additionally, NSAJ02 was chosen because it is the location of an instrument mooring with high-frequency water quality data.

Samples are collected at the surface at all sites. Samples are also collected at the estimated chlorophyll maximum depth ("chl max") at two sites, KSBP01-Point Jefferson and NSEX01-East Passage. The chl max depth is determined upon inspection of the *in vivo* chlorophyll fluorescence depth profile (see 5.3 Measured Parameters and 7.2 Sample Collection).

5.3   Measured Parameters

Phytoplankton samples are analyzed quantitatively with the FlowCAM and qualitatively via microscopy (see 8.0 Sample Analysis). Abundance (particles/mL) and biovolume (mm^3/L) (FlowCAM) or presence/absence (qualitative microscopy) are currently assessed for all organisms in the 5-300 μm size range, which are identified to the lowest practical level or grouped into various unidentified categories. Protists, such as ciliates, and larval crustaceans, such as copepod nauplii (members of the microzooplankton), are also quantified to the extent feasible. Because it is frequently not possible to determine which organisms are photosynthetic, all taxa and unidentified categories are included in total abundance and biovolume estimates, which therefore reflect a combination of photosynthetic and heterotrophic microplankton.

In addition to phytoplankton collection, concurrent sampling of other parameters relevant to the analysis and interpretation of phytoplankton data is described below (at sites specified in Table 1). Details of these activities can be found in separate program component SAPs or the Marine Monitoring SAP (King County, *in prep*.).

Water column characterization: A suite of sensors mounted to a depth profiler and bottle rosette (known as a "CTD") measure conductivity (salinity), temperature, pressure (depth), dissolved oxygen, chlorophyll fluorescence, photosynthetically active radiation (PAR), and transmissivity. At all sites, profiles binned at 0.5 m intervals are taken of the entire water column down to 5 meters (m) above the bottom depth.

Conventionals and fecal indicator bacteria: Discrete water samples are taken from Niskin bottles on the CTD rosette for conventional water quality parameters at standard depths for all sites. Nutrients (ammonia nitrogen, nitrite + nitrate nitrogen, orthophosphate phosphorus, and silica) and total suspended solids are analyzed from samples taken at all depths, while total dissolved nitrogen is analyzed from samples at the 1 m depth only and at a subset of sites. Fecal indicator bacteria (fecal coliform and enterococci) are analyzed in samples from the 1-m depth at all sites. Chlorophyll-*a* and phaeophytin are extracted and analyzed from samples taken at the 1-, 15-, 25-, and 35-m depths. At KSBP01 (Point Jefferson) and NSEX01 (East Passage), samples are also taken at the estimated chlorophyll maximum depth, which is typically between 2 and 8 m.

Zooplankton: Three sites (KSBP01-Point Jefferson, NSEX01-East Passage, and LSNT01-Point Williams/Fauntleroy) are sampled for zooplankton taxonomy, biovolume, and abundance estimates. Vertical plankton net (200-μm mesh) tows capable of collecting smaller zooplankton species from the water column down to 200 m are performed at all three sites. At two depths offshore from LSNT01 (Point Williams), a "bongo" double plankton net (335-μm mesh) is also towed obliquely through the top 30 m of the water column to collect larger, faster-swimming zooplankton utilized as prey by salmonids. Samples are analyzed by Dr. Julie Keister's group at the University of Washington (Marine Zooplankton Monitoring SAP, King County, 2015).

Autonomous Moorings: Three autonomous *in situ* instrument moorings provide water quality data with high temporal resolution. These include dock-based moorings at NSAJ02 (Outer Quartermaster Harbor, depth from surface variable with tide) and SEAQYSI (Seattle Aquarium in Elliott Bay, 1- and 10-m depths), and a buoy-mounted system at PTWILLBUOY (Point Williams, 1-m depth). Moorings are equipped with YSI datasondes that measure the following parameters every 15 min: temperature, salinity, dissolved oxygen, chlorophyll fluorescence, turbidity, and pH (no YSI pH at PTWILLBUOY). The Point Williams buoy is also equipped with a Satlantic optical nitrate and high precision pH sensors (the latter as of summer 2015). The YSI datasondes are replaced monthly with cleaned and calibrated datasondes (less frequently in winter), at which time water samples for sensor validation are collected (salinity, dissolved oxygen, and temperature, plus nitrate and dissolved

inorganic carbon/total alkalinity for precise pH determination at PTWILLBUOY) (Marine Monitoring Moorings SAP, King County, *in prep*.).

Assessment of FlowCAM Particle Biovolume for Estimation of Particulate Organic Carbon (2015-2016): Samples for particulate organic carbon and nitrogen are collected from all sites at the surface depth and analyzed at the University of California Davis Stable Isotope Laboratory as part of a special study running from mid-2015 to mid-2016. The objective of this study is to assess the use of FlowCAM biovolume measurements for estimating particulate organic carbon (Appendix E).

Table 1.   Sites and depths sampled for phytoplankton. Other relevant parameters collected at these sites are indicated.

Sampling Run	Site Locator	Site Description	Phytoplankton Depth	Zooplankton	Nearby Moorings	CTD Profile	Conventionals and Nutrients
North	JSUR01	Brightwater Treatment Plant Outfall	Surface			×	×
North	KSBP01	Point Jefferson	Surface	Vertical tow		×	×
North	KSBP01	Point Jefferson	Chl max	Vertical tow		×	×
North	KSSK02	West Point Treatment Plant Outfall	Surface			×	×
North	LTED04	Elliott Bay	Surface		SEAQYSI	×	×
South	LSEP01	South Treatment Plant Outfall	Surface			×	×
South	LSNT01	Point Williams /Fauntleroy	Surface	Vertical & oblique tows	PTWILLBUOY	×	×
South	NSEX01	East Passage	Surface	Vertical tow		×	×
South	NSEX01	East Passage	Chl max	Vertical tow		×	×
Vashon	NSAJ02	Outer Quartermaster Harbor	Surface		NSAJ02	×	×

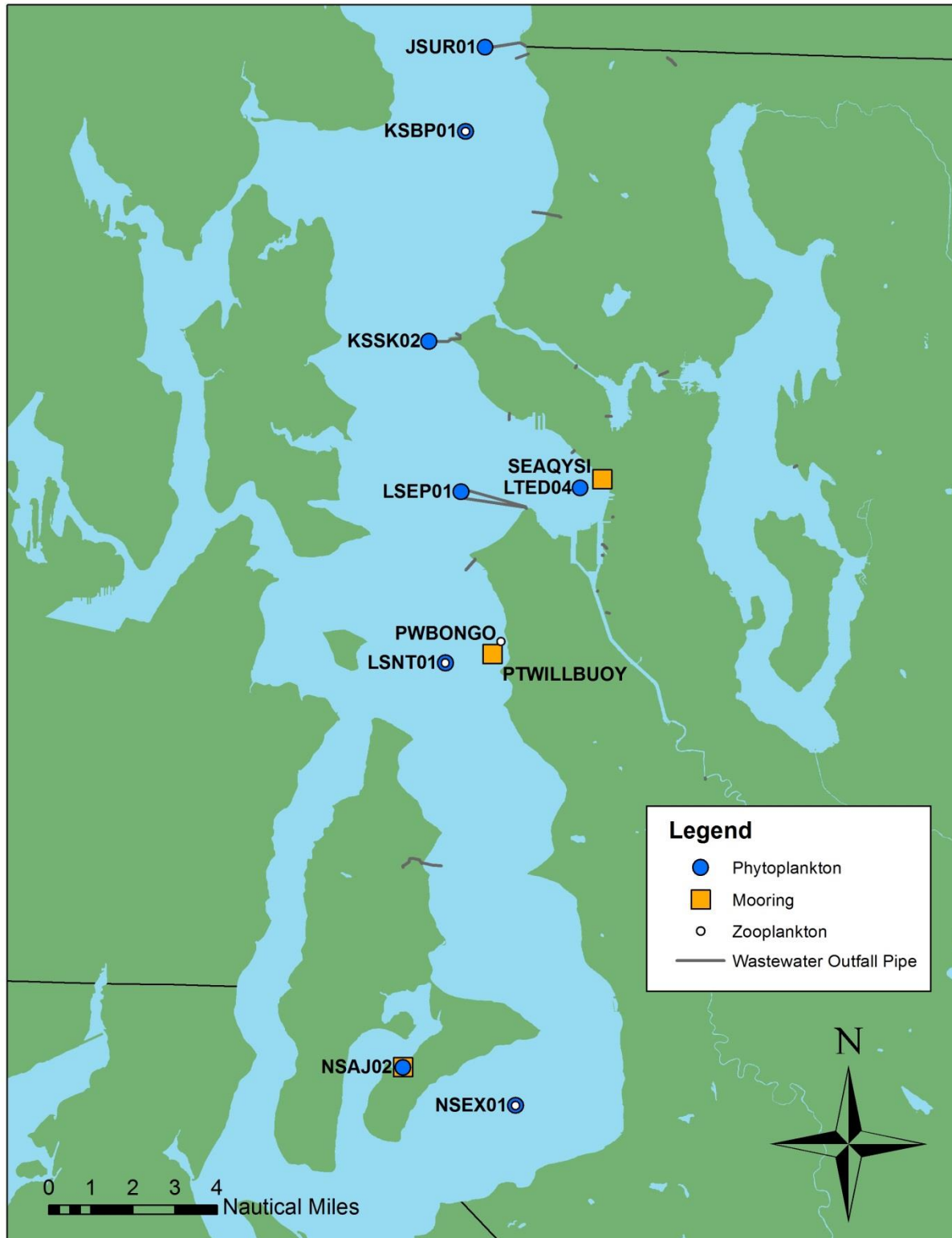**Figure 1.** **Sites sampled for phytoplankton and zooplankton, with locations of autonomous moorings and wastewater treatment outfall pipes.**

6.0 PREVIOUS ANALYTICAL METHODS AND HISTORICAL DATA

The phytoplankton component to the Marine Monitoring Program was added in 2008. Seasonal, twice monthly sampling was conducted at two deep water sites, KSBP01 (Point Jefferson), NSEX01 (East Passage), and NSAJ02 (Quartermaster Harbor) (Table 2), for analysis by semi-quantitative microscopy. For details, refer to KCEL Standard Operating Procedure (SOP) 459v0 (King County, 2011). Concentrated live samples were examined in order to generate a taxon list. In addition, concentrated preserved samples were used to assess relative abundance by grouping taxa into one of three categories (Present, Subdominant, Dominant).

Samples for live identification were concentrated 10-20x by reverse filtration (Dodson and Thomas, 1978) using filter assemblies made with 1 μm or 5 μm nitex screen sealed to the end of acrylic tubes. Samples collected for assessment of relative abundance were transferred to 500 mL conical centrifuge tubes, preserved by addition of 5 mL formaldehyde (final concentration 0.4% v/v) and allowed to settle at room temperature for at least 48 h. The overlying water was removed and the concentrated sample (ca. 10 mL) transferred to a glass scintillation vial.

Concentrated samples were transferred to a 0.06 mL PhycoTech nanoplankton chamber for observation. The semi-quantitative relative abundance analysis was based on cell counts and category was determined by tallying at least 9 different microscope fields. A genus (or higher taxonomic category if appropriate) was recorded as (1) Dominant (D) in the sample when it was dominant by count in >50% of the observed microscope fields (dominance by count was limited to fields that contained at least 3 cells of the same genus/category); (2) Subdominant (S) in the sample when it was dominant by count in less than 50% and more than 25% of the observed microscope fields; or (3) Present (P) if recorded live or preserved, but not dominant or subdominant. If necessary, preserved samples were further concentrated prior to viewing to ensure sufficient cell numbers across all fields. It is important to note that this method is biased towards (a) taxa that preserve well in unbuffered formalin (typically diatoms), (b) small-celled taxa, (c) nonmotile cells (flagellated taxa are difficult to identify when not active), and possibly (d) colonial taxa (may not be evenly distributed across microscope fields). An added difficulty and source of uncertainty with this method is clumping.

Modifications to the sampling program in subsequent years included a site switch at Quartermaster Harbor from inner (MSWH01) to outer harbor (NSAJ02), and changes in the number of months sampled (see Table 2). In addition, a fourth data category, Present-and-Common (PC), was added in 2010 to capture taxa that appeared common, large, or numerous only in the live sample, but did not meet criteria for the dominant or subdominant relative abundance categories. Five sites were added in May of 2014 for analysis by FlowCAM and qualitative microscopy as described in this SAP.

Table 2.   Historical overview of the Marine Phytoplankton Monitoring Program.

| Year | Frequency | Site/Depths Sampled | | | | | | | | | Method |
		KSBP01	NSEX01	MSWH01	NSAJ02	JSUR01	KSSK02	LTED04	LSEP01	LSNT01	
2008	2/month Apr-Oct	1 m, Chl max	1 m, Chl max	1 m							Semi-quant
2009 - 2010	2/month Apr-Sept	1 m, Chl max	1 m, Chl max		1 m						Semi-quant
2011 – Dec 2014	2/month Apr-Oct	1 m, Chl max	1 m, Chl max		1 m						Semi-quant
May 2014 - Present	2/month Feb-Nov, 1/month Dec-Jan	1 m, Chl max	1 m, Chl max		1 m	1 m	1 m	1 m	1 m	1 m	Qualitative, FlowCAM

7.0 SAMPLING PROCEDURES

All sampling is performed from a King County research vessel or from a dock by KCEL Field Science Unit (FSU) personnel. Details on procedures for vessel site positioning, shipboard health and safety considerations, and other general vessel operations can be found in the Marine Monitoring SAP (King County, *in prep.*).

7.1 Equipment

A Sea-Bird SBE 25 Sealogger CTD with a rosette of twelve 5-L Niskin bottles is used to collect water from the surface (1 m) and chlorophyll maximum depths. See KCEL SOP 220v3 (King County, 2006)for more complete information about operation, calibration and maintenance of the SeaBird CTD.

7.2 Sample Collection

The CTD is prepared for sampling and lowered at a rate of 0.5 m/s for the first 20 m and 1.5 m/s below 20 m to the appropriate depth in the water column. Profile data are binned at 0.5 m intervals on the downcast. Discrete water samples are collected on the upcast as the CTD is raised at a rate of 1.5 m/s until 20 m depth, then 0.5 m/s to the surface; the Niskin bottles capture water at pre-programmed depths. Once at the surface, the CTD is brought back on board. The CTD operator uploads the cast data and the deckhand(s) fill the laboratory containers from the appropriate Niskin bottles. The samples are collected and stored on ice in 1 L clear polyethylene bottles.

Typically, the samples for phytoplankton analysis are taken from the 1-m depth Niskin. At two offshore sites (KSBP01 and NSEX01) phytoplankton samples are also collected from the chl max depth Niskin. At these two locations, the CTD operator programs several additional Niskin bottles to trip at 2.5, 3.5, 5.5, 8, and 10 m depths in the water column. After the CTD data is uploaded, the operator examines the chlorophyll profile and determines which of the additional bottles was collected from the depth closest to the highest chlorophyll concentration. This information is communicated to the deckhand. The deckhand then fills the chl max containers from that Niskin bottle.

One additional bottle is filled for elemental particulate organic carbon and particulate organic nitrogen analysis at all sites, surface depth only (2015-2016, Appendix E).

7.3 Sample Documentation and Field Sheets

All laboratory containers are pre-labeled with LIMS-generated adhesive labels which have unique identifying information such as LIMS sample number, site name, depth, collect date and lab products. Associated fieldsheets have individual columns for each sample in the pre-logged batch. Field information is recorded for each sample as appropriate, typically including collect date and time, initials of field personnel, sample depth (if determined in the field), etc. A "field observations" form is completed by field staff and is stored in LIMS as

a document associated with the sample batch. Most field data is entered into LIMS after completion of field activities. Hardcopies of the field sheets are kept at KCEL for five years and all field and sampling records are archived according to KCEL policy for a period of 10 years from the date the samples were collected.

7.4   Sample Chain of Custody

Once collected, samples remain in the custody of KCEL staff. Upon return to the laboratory, samples are delivered directly to the sample login area. The samples are then "logged in" according to KCEL standard procedures. FSU is required to relinquish the samples to the login personnel, fill out the Chain of Custody Stamp and the Sample Delivery Log Book (including signatures) and inform the login personnel of any special circumstances associated with the sample collection or analysis.

Standard KCEL procedures for sample chain of custody are followed from the time at which each sample is collected. While in the field, all samples are under direct possession and control of the KC Field Sciences Unit staff. For chain of custody purposes, the research vessel is considered a "controlled area." All sample information is recorded on a chain of custody form (Appendix A). This form is completed in the field and accompanies all samples during transport and delivery to KCEL each day. The samples are stored in a secure location at KCEL until analysis and disposal. Original copies of chain of custody forms are archived in KC's program file for a period of 10 years from the date the samples were collected.

7.5   Sample Storage and Disposal

The sample bottles are held at 12°C under low light (12:12 light:dark) with the caps slightly loosened. Analysis of the samples by FlowCAM must be accomplished within 3 days of collection. Excess sample is disposed of by pouring down the drain in a laboratory sink.

8.0 SAMPLE ANALYSIS

8.1   FlowCAM Analysis

The FlowCAM combines elements of flow cytometry and microscopy to image, enumerate and analyze particles of interest in a stream of moving fluid. As the sample is pumped through a flow cell, images are captured by the camera at a high frequency. The current procedure uses the Autoimage mode, where images of the camera's field of view are captured at a user set rate. These images are then analyzed and particle-containing portions are segmented out and added to a collage of particle images for that sample. These particle images will then be used to calculate particle abundance and biovolume in the sample.

The system can record up to 64 image attributes (e.g., color, shape, size, texture) per particle; these are used by pattern recognition software (Visual Spreadsheet® v. 3.7.5) to partially automate the classification of particles into user defined categories, such as size class or taxonomic group (FlowCAM Manual, 2012).

8.1.1   Sample Preparation

Laboratory filtered seawater (0.45 µm) is used as a method blank and dilution water for size-fractionated samples. Filtered seawater is prepared as needed, microscopically examined for gross contamination and tested as a method blank with each batch of samples. Refer to section 8.1.5.2 for method blank limits and corrective actions.

The sample is separated into two fractions for analysis by FlowCAM: a <100 µm particle size-fraction and a 100-300 µm particle size-fraction for imaging with 10x and 4x objectives, respectively. The >300 µm fraction is saved for observation under a dissecting microscope.

A 200 mL volume of sample is poured through a 300 µm nitex mesh filter into a tall 200 mL beaker. The mesh is rinsed with laboratory filtered sea water into a 50 mL beaker; this the >300 µm fraction for observation with the dissection microscope. The volume in the tall beaker is then poured through the 100 µm nitex mesh into a second tall beaker, the mesh is rinsed over the first beaker, and the sample is made up to volume with filtered seawater; this is the 100-300 (4x objective) fraction. The volume remaining in the second tall beaker is the <100 µm (10x objective) fraction. Prepared samples awaiting analysis are stored in the beakers sealed with parafilm under the same light and temperature conditions as the sample bottles.

8.1.2   Sample Analysis

Acquisition or context templates have been developed in-house for the 4x and 10x magnifications in Autoimage mode. These templates specify instrument and software acquisition parameters such as flow rate, imaging rate, stop conditions, and camera

settings, all of which have been optimized for each magnification. A specific flow cell is used for each magnification: a 100 μm deep cell for the 10x objective and a 300 μm deep cell for the 4x objective. Objective, collimator (a lens designed to narrow the beam of light directed at the flow cell), and flow cell are exchanged together when switching between magnifications. A table of currently used context setting values as well as flow cell specifications can be found in KCEL SOP 467v0 (King County, 2015b).

To begin analysis of a sample, an appropriate context template is loaded, the system is primed and focused, and an Autoimage mode analysis is initiated. The system first images the background of the camera's field of view and then proceeds to image the particles present in the fluid as it is drawn through the flow cell. Current context settings specify collection of 2000 particle images (>10 μm) for the 4x objective and 3000 particle images (>5 μm) for the 10x objective, with a maximum of 45 minutes per run. Depending on particle density, runs take anywhere between a few minutes to 45 minutes. Once the analysis is completed, the files are saved and the instrument returns to standby. The sample temperature is kept between 10°C and 15°C during analysis via an ice bath.

The >300 μm fraction is observed under a dissecting scope and notes are made indicating approximate number and type of particles. A special effort is made to count *Noctiluca* cells.

8.1.3 Data Processing

The image data is processed using Visual Spreadsheet pattern recognition software to aid in the identification and sorting of the particle images. The collage of particle images is first scanned by the analyst for nonbiological materials, which are removed from the collage. The remaining images are sorted twofold: (1) by taxonomic category (Table C1) with the help of data filters developed in-house from libraries of example images collected from within the study area, and (2) by size into five size classes (Table C2), using an area-based diameter (ABD) estimation and simple data filters. Although pre-made templates that use data filters exist for taxonomic classification, additional image sorting is required by the analyst due to the limitations of the pattern recognition software. Particle images may be incorrectly labelled by the software because of poor image resolution, poor image focus, partial imaging, and other factors. Several "miscellaneous" categories exist in the taxonomic templates to group together biological, but unidentifiable particle images. Once particle images have been sorted by one analyst, an inter-analyst peer review of data is done by a second analyst to ensure accuracy. Representative images are saved in libraries for later use as taxonomic reference or to build taxonomic filters.

Taxonomic identification is based primarily on Horner (2002), Tomas (1997) and Hoppenrath, et al. (2009). FlowCAM libraries and archived microscope (http://green.kingcounty.gov/marine/photos.aspx) images are also used as a guide to make certain that identification is consistent.

8.1.4 Data End-Points

The analysis of live samples with the FlowCAM leads to the following major endpoints:

1. Abundance (Taxonomic): The total concentration of biological particles by taxon in the sample, as determined by pattern recognition software and confirmed by taxonomists. Based on images captured using 10x and 4x objectives (5-100 μm and 100-300 μm fractions, respectively). Reported as particles/mL.

2. Biovolume (Taxonomic): The total biovolume of taxonomically classified biological particles in the sample, as determined using ABD. Based on images captured using 10x and 4x objectives (5-100 μm and 100-300 μm fractions). Reported as μm^3/L.

3. Abundance (Size Distribution): The total concentration of biological particles in the sample, as determined using ABD; the particles are sorted into predetermined size classes. Based on images captured using 10x and 4x objectives (5-100 μm and 100-300 μm fractions). Reported as particles/mL.

4. Biovolume (Size Distribution): The total volume of biological particles in the sample, as determined using ABD; the particles are sorted into predetermined size classes. Based on images captured using 10x and 4x objectives (5-100 μm and 100-300 μm fractions). Reported as μm^3/L.

8.1.5   Quality Assurance and Method Assessment

8.1.5.1 Instrument Calibration

Preventative maintenance and instrument calibration are performed annually, or as necessary, at the Fluid Imaging Technologies facility. This service includes quality control procedures for both counting and sizing accuracy.

At KCEL, the instrument's counting and sizing calibration are verified every 20th sample or once per month, whichever occurs first. This procedure ensures that the measured and calculated values associated with particle images are accurate. To perform a calibration check, an Autoimage analysis is done using a certified 20 μm diameter bead standard solution (Fluid Imaging Technologies). The results for concentration and mean bead diameter are compared to the limits specified on the standard bottle. The calculated values must be within the specified limits; if not, a second calibration run must be done. If the instrument's calculations are still incorrect, the instrument needs to be serviced.

8.1.5.2 QC Samples

A method blank is run for each sampling event to test for sample carry over and background interference in the 100-300 μm fraction. The blank is freshly prepared 0.45 μm laboratory seawater that has been passed through the same sample fractionating preparation as the samples. In addition to a method blank, the laboratory seawater is also used as the 100-300 μm fraction dilution water. The method blank is run for 10 minutes at 10x following standard between-sample flushing; all particles >5 um are enumerated.

A laboratory duplicate sample is run for every sampling event to evaluate run to run variability. The duplicate site is on a rotating schedule and follows the sequence in LIMS number. For the purposes of this project, a laboratory duplicate is defined as two separate

subsamples, taken from the main field collected sample, with each of the two subsamples processed individually through the sample preparation and Flowcam analysis.

QC sample data processing is described in Section 10.2.1.

8.1.5.3 Method Validation

The current method used by KCEL for analysis of marine phytoplankton by FlowCAM is the result of extensive collaboration with Fluid Imaging Technologies technicians and input from other experienced FlowCAM users. According to Alvarez et al. (2011, 2012), the FlowCAM can generate results comparable to that of a trained taxonomist working through traditional methods. Our method, using 4x and 10x objectives, cannot attain the same level of resolution in taxonomic identification as traditional microscopy (compare Tables C1 and D1). However, enumeration of particle classes is more accurate with FlowCAM due to the larger volume of sample processed.

The current KCEL analytical method is documented in KCEL SOP 467v0 (King County, 2015b).

A special one-year study will evaluate the feasibility of using FlowCAM biovolume data to estimate carbon biomass (Appendix E). This study will also generate data on the proportion of plankton carbon outside the range of sizes currently analyzed by FlowCAM; i.e., <5 μm and >300 μm as captured on nitex screen.

8.2 Qualitative Microscopy

8.2.1 Sample Preparation and Analysis

Sample preparation and analysis follows King County SOP 459v1 (King County, 2015a). Samples are concentrated by reverse filtration (Dodson and Thomas 1978) using filter assemblies made with 5 μm nitex screen sealed to the end of acrylic tubes. 400 mL samples are concentrated to about 40 mL and examined immediately or as soon as possible. The live concentrate can be saved at 10-12 °C for up to 2 days, but it is preferable to refrigerate the whole water sample until the cells can be concentrated and viewed. One duplicate is analyzed with each batch on a rotating basis.

A drop of well mixed concentrate is placed on the chamber with a transfer pipet and covered with a cover slip. The chamber (about 0.06 mL PhycoTech nanoplankton chamber) is scanned in its entirety using a Nikon 80i compound microscope with 10x ocular and 10x – 60x objectives, and all taxa are recorded on the data sheet. Given a 10x concentrate, this corresponds to the taxa present in 0.6 mL of original sample. Taxonomic identification is based primarily on Horner (2002), Tomas (1997) and Hoppenrath et al. (2009). Archived images or images loaded to the King County Puget Sound Marine Life Photos website (http://green2.kingcounty.gov/marine/Photo) may also be used as a guide.

8.2.2   Data End-Points

The endpoint is a list of taxa or taxonomic categories for the sample (Appendix D). New taxa may be added to the list as needed.

9.0 DATA QUALITY OBJECTIVES

The data quality objectives for the phytoplankton monitoring program are to collect data that are sufficiently precise, accurate, representative, complete, and comparable to meet the program objectives.

9.1 Precision

Precision, or the repeatability of a measurement dependent upon random error, can be determined from analysis of duplicate subsamples taken from a single sample and analysis of multiple samples collected at the same time and place. To determine the precision associated with the FlowCAM analytical method, duplicate FlowCAM runs are performed on one sample from each sampling event, chosen on a rotating basis from all 8 sites. Field and laboratory time constraints generally do not allow replicate samples to be taken from separate CTD casts for the purpose of estimating sampling precision.

9.2 Accuracy and Bias

Accuracy, or the closeness of a sample mean to the true population mean, is affected by both systematic and random errors. Bias is a measure of the difference, due to systematic error, between a sample mean and the true population mean.

Care is taken to reduce individual taxonomist bias by maintaining and referring to an extensive library of microscopic and FlowCAM photos of identified phytoplankton taxa and by having taxonomists confirm each other's classification of each sample run. Bias is also minimized by following standard operating procedures established for sample processing and analytical methods.

Systematic bias and background interferences will be checked using a laboratory filtered seawater method blank. Method blanks exceeding the current limit of 100 particles/mL will be qualified with a data anomaly form (DAF) indicating the size distribution of particles in the method blank and possible biases to specific size classes. Corrective actions to resolve unacceptable background interferences will also be documented in the DAF.

9.3 Representativeness

Representativeness is the degree to which sample data accurately and precisely estimate the value of a parameter for the population of interest (in this case, the phytoplankton of the Central Basin of Puget Sound). The number of samples that are collected each year is limited by personnel time and the program budget. Phytoplankton populations are known to be spatially patchy, which must be a consideration when interpreting data from only one or two water depths and a relatively small number of sites. However, this program's sampling plan will achieve at least coarse-resolution estimates of the phytoplankton community and its spatial variability in the Central Basin. Phytoplankton community composition and abundance changes occur on the order of days; therefore the sampling

frequency used in this program (twice monthly) cannot fully characterize phytoplankton temporal dynamics, but will give a good estimate of broad seasonal patterns with consistent sampling over multiple years. Samples are well mixed prior to processing and analysis to increase representativeness.

The level of particle count resolution of the FlowCAM method varies with a number of factors, primarily the density of the particles of interest. A typical run collects 2000 (4x objective) or 3000 (10x objective) images, but fewer images may be collected for samples with low particle density. The sample volume processed for imaging is thus variable, ranging from approximately 5 to 75 mL. Because suitable images are then classified into categories, a theoretical reporting limit is a single particle divided by the volume processed (i.e., approximately 0.01 – 0.2 particles/mL). This reporting limit is deemed sufficient to collect a representative sample of relatively abundant phytoplankton taxa, but rarer taxa are likely to be missed or enumerated with high uncertainty.

Based on the volume of sample analyzed in the qualitative microscopy method (0.6 mL), the reporting limit of the method is approximately 1.7 cells/mL.

The FlowCAM method, using 4x and 10x objectives, cannot attain the same level of resolution or certainty in taxonomic identification as the qualitative microscopy method. However, the FlowCAM achieves greater representativeness due to the larger volume of sample processed. For this reason both methods are used in conjunction with each other.

9.4   Completeness

Completeness is the total number of samples for which acceptable data are generated compared to the total number of samples submitted for analysis. Adhering to standardized sampling and analytical protocols will aid in providing a complete set of data for each sampling year. If 100% completeness is not achieved, the program team will evaluate whether additional samples can be collected and analyzed within time and budget constraints. However, due to the seasonal nature of this sampling effort, sampling at a later date cannot directly replace lost data.

9.5   Comparability

Comparability is the confidence with which one data set can be compared to another, either over time or between research groups. This can be improved by standardizing protocols for collecting and analyzing samples and for validating and reporting data. Changes over time to the standardized protocols referred to in this SAP will be minimized to ensure comparability over the entire time series.

10.0 DATA MANAGEMENT AND ANALYSIS

10.1 FlowCAM File Management

For each run, Visual Spreadsheet generates a folder with a number of files. This folder is named with LIMS sample number, magnification and run number, and stored in the FlowCAM hard drive organized by sampling event. Data processing generates additional files that are stored in the same file directory along with the raw data files. Files on the FlowCAM hard drive may be accessed remotely from desk PCs for data processing and management. The FlowCAM hard drive is backed up weekly to an external drive.

10.2 Laboratory Information Management System and QA/QC

10.2.1 FlowCAM

FlowCAM data are entered to LIMS as a workgroup (i.e., LIMS batch) via a customized Excel interface that processes and transfers information from Visual Spreadsheet generated export files.

Image data processing generates two classifications for each FlowCAM run, one by taxonomic category and one by size-class (Appendix C). The groupings in a Visual Spreadsheet classification are called Classes (i.e., taxonomic categories or size-classes). A summary .csv export file is generated for each classification. The export file is formatted to contain the following parameters, organized by Class: Count, Particles/mL, Mean Diameter (from ABD), and Mean Volume (calculated by ABD). The following calculations are performed by the Excel interface prior to transferring the data to LIMS: (1) Total biovolume for each Class is calculated from Particles/mL and Mean Volume (ABD); (2) Volume units are expressed as mm^3/L, and 3) 4x and 10x data are summed for each overlapping Class.

Each Class contains both Abundance (Particles/mL) and Biovolume (mm^3/L) data. The respective parameter names in LIMS are Class-Abund and Class-Biovol (e.g., Chaetoceros-Abund, Chaetoceros-Biovol, Size-Class 10-25 um-Abund, Size-Class 10-25 um-Biovol).

QC data are loaded simultaneously as part of the workgroup using appropriate QC sample numbering and include MB (method blank) and LD (lab duplicate). Only Abundance is loaded for the method blank. QC reports include pass/fail for the method blank (threshold is 100 particles/mL), and the relative percent deviation (RPD) in abundance and biovolume for the lab duplicate (size class data only). RPD acceptable lab limits are set at 0-150%.

Data may be retrieved via LimsView using the AQ (Aquatox Parameters) or custom parameter lists. Current custom lists include FLOWCAM_ABUND, FLOWCAM_BIOVOL and FLOWCAM_ALL. Whereas AQ lists are automatically updated by IT staff, custom lists are

updated by the user when changes are made to FlowCAM parameter names, such as when a new taxon is added.

10.2.2 Qualitative Microscopy

All taxa present are entered to LIMS via a customized Excel interface using the qualifier "P" (Present). The LIMS product name is Marine Phyto-Qual (as for historical semi-quantitative data) and each taxonomic category represents a parameter name (Table D1). The lab duplicate is loaded simultaneously as part of the workgroup but there is no QC calculation associated with it.

Data may be retrieved via LimsView using the AQ (Aquatox Parameters) or custom parameter lists, such as the list MARINE PHYTO. Whereas AQ lists are automatically updated by IT staff, custom lists are updated by the user when changes are made to parameter names, such as when a new taxon is added.

10.3 Science Section Marine Portal

While summary taxonomic and size class abundance and biovolume data will be stored in LIMS, image analysis results (particle attributes) for each classified particle will be imported into the phytoplankton component of the Science Section's "Marine Portal," a Microsoft SQL Server relational database and data management system (DMS) that began development in 2015. Data from qualitative microscopy and the previous semi-quantitative microscopy method will also be included in the Marine Portal. This database will ultimately contain all routinely monitored discrete marine offshore parameters. The Marine Portal will allow additional functionality for data management that is not possible with LIMS:

1. Allowing additional data review and qualification by Science Section program staff.
2. Storing data in a structure that is more flexible for community composition/taxonomic data than LIMS.
3. Making quality-controlled and reviewed data available via web portal to program staff, internal KC clients, external researchers, resource managers, and the general public.

For the FlowCAM method, parameters included in the Marine Portal database will include all particle properties and their taxonomic classifications (see Appendix B). These data will be uploaded to the database by Science Section staff from files exported from the Visual Spreadsheet FlowCAM software by KCEL staff. All other data (including microscopy data) will be downloaded from LIMS in DMPA format and imported into the Marine Portal by Science Section staff. Entire LIMS records will be saved in tables visible to data stewards in order to preserve data integrity; however, many fields relevant only to KCEL data management will be hidden from public viewing and downloads. Data imports will occur approximately twice monthly, as data become available. In addition to particle properties and microscopy results, metadata for each sample (such as collection date, locator, laboratory sample number, etc.) or FlowCAM run (two runs per sample; includes run

duration, volume imaged, magnification, etc.) and a variety of useful parameters calculated dynamically by the DMS will also be made available for viewing and download. Calculated parameters will include:

1. Abundance (particles/mL) and total and mean ± standard deviation biovolume (mm^3/L) for each taxonomic class or any higher taxonomic level or functional group (see Appendix B)

2. Abundance (particles/mL) and total and mean ± standard deviation biovolume (mm^3/L) for each particle size class

3. Total particle abundance (particles/mL) and biovolume (mm^3/L) for each sample

4. Taxonomic richness (number of taxonomic groups identified) for each sample

5. Taxonomic diversity (a measure combining taxonomic richness and evenness) for each sample

Data and dynamic plots of many of the above calculated parameters over time and by site will be published online for public viewing and download with a free account. Details on other aspects of the Marine Portal can be found in the Marine Monitoring SAP (King County, *in prep*).

10.4 Data Review, Analysis, and Reporting

Data will be reviewed approximately twice per year for the purpose of ongoing method assessment and validation as a longer time-series is obtained. The review process will include comparing duplicate FlowCAM runs to estimate precision for different taxa or size classes and to spot check for inconsistencies. Review will also involve comparison of the taxa detected by FlowCAM vs. qualitative microscopy (with the caveat that identification is possible to a lower taxonomic level with microscopy) to determine what taxa might be missed/underrepresented by either method and to check for inconsistencies. Abundance and biovolume for functional groups and for select commonly observed or frequently abundant/high biovolume taxa will be plotted over time to check for outliers or unreasonable values (compared to historical data as they are accumulated). Appropriate additional qualifying flags summarizing problems with data found during this review process or specified by KCEL staff in the comments field will be appended to LIMS qualifier fields as necessary via the Marine Portal data steward interface. In cases where sample data are deemed unusable due to sampling, analytical, or other errors (e.g., flagged with qualifier "R" in LIMS), data will be filtered and hidden from public users.

Data will be statistically analyzed using univariate (e.g., trend analysis) and multivariate (e.g., nonmetric multidimensional scaling) techniques to investigate variability between sites and over seasonal, annual, and multiannual time scales. KC water quality, weather, oceanographic, and zooplankton data will be considered alongside the phytoplankton data and used to generate testable hypotheses, interpret results, and inform an improved understanding of lower trophic-level ecology in the Central Basin of Puget Sound.

Data and analysis will be published in short written summaries for the KC website (yearly) and water quality reports (every five years).

11.0 HEALTH AND SAFETY

11.1 Field Health and Safety

Details on general health and safety considerations for field work and vessel operations can be found in the Marine Monitoring SAP (King County, *in prep.*). There are no field health and safety considerations specific to this program.

11.2 Laboratory Health and Safety

General laboratory health and safety considerations are listed in method specific SOP's and the Environmental Laboratory Health and Safety Plan. There are no laboratory health and safety considerations specific to this program.

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Appendix A: Forms

CHAIN OF CUSTODY RECORD

King County Zooplankton   Page _____ of _____
Contact: Kim Stark (206) 477-4829
201 S. Jackson St., Suite 600
Seattle, WA 98104

Sample ID	Collect Date	No. of Containers	Replicate	Comments

	total containers this page
	total all pages

RELINQUISHED BY	Date
Signature	
Printed Name	Time
Organization	

RECEIVED BY	Date
Signature	
Printed Name	Time
Organization	

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Appendix B: FlowCAM Particle Properties

List of Visual Spreadsheet particle properties used in classifications. All values are calculated from a saved image and recorded during the run.

Area (ABD)	Compactness	Scatter Area
Area (Filled)	Convex Perimeter	Scatter Peak
Aspect Ratio	Convexity	Scatter Width
Average Blue	Diameter (ABD)	Sigma Intensity
Average Green	Diameter (ESD)	Sphere Complement
Average Red	Edge Gradient	Sphere Count
Biovolume (Cylinder)	Elongation	Sphere Unknown
Biovolume (P. Spheroid)	Feret Angle Max	Sphere Volume
Biovolume (Sphere)	Feret Angle Min	Sum Intensity
Ch1 Area	Fiber Curl	Symmetry
Ch1 Peak	Fiber Straightness	Transparency
Ch1 Width	Geodesic Aspect Ratio	Volume (ABD)
Ch2 Area	Geodesic Length	Volume (ESD)
Ch2 Peak	Geodesic Thickness	Width
Ch2 Width	Intensity	
Ch2/Ch1 Ratio	Length	
Circle fit	Perimeter	
Circularity	Ratio Blue/Green	
Circularity (Hu)	Roughness	

Selected Definitions:

Area Based Diameter (ABD): The diameter of a circle with an area equal to that of a given particle. The ABD is calculated by arranging all of the pixels deemed part of the particle into a solid circle and then measuring that circle's diameter.

Ch1/Ch2: Two channels that utilize photomultiplier tubes (PMT) to detect fluorescence signals from particles in the sample (not currently used in this program) Ch1 is the >650 nm PMT; Ch2 is the PMT for the 575±15 nm filter.

Circularity: A shape parameter computed from the perimeter and the (filled) area. A circle has a value of 1.0. Formula: $(4 \times \pi \times Area)/Perimeter^2$. (real [0,1])

Convexity: A shape parameter that is computed as the ratio of filled area to the area of the convex hull of the particle. This property is sometimes called "Solidity." A circle has a value of 1.0. (real [0,1])

Equivalent Spherical Diameter (ESD): The diameter of a sphere with an equivalent volume to a given particle. The ESD is calculated as the mean ferret measurement of the particle based on 36 sample measurements conducted every 5°.

Feret angle: The longest distance between any two points along the particle boundary, also known as feret diameter.

Fiber: A thread or filament-like particle.

Geodesic: Of, relating to, or denoting the shortest possible line between two points on a sphere or other curved surface.

Intensity: The average grayscale value of the pixels making up a particle (grayscale sum / number of pixels making up the particle). (real [0, 255]; 255 is most intense)

Scatter: The multidirectional reflection of the light used to illuminate an object.

Appendix C: Identified Taxa/Categories (FlowCAM Analysis)

Table C1. Taxonomic categories (prefixes for LIMS parameter names) used in FlowCAM analysis. Samples are run and processed according to FlowCAM protocol and images classified to the lowest possible category listed.

Class	Common Group Name	Family	Taxon/Category (LIMS Parameter Name Prefix) [1]	Morphology
Bacillariophyceae	Centric Diatoms	Asterolampraceae	*Asteromphalus*	Solitary
		Chaetocerotaceae	*Chaetoceros*	Chain
		Coscinodiscaceae	*Coscinodiscus*	Solitary
		Heliopeltaceae	*Actinoptychus*	Solitary/colony
		Hemiaulaceae	*Cerataulina*	Chain
			Eucampia	Chain
			Hemiaulus	Chain
		Lauderiaceae/Skeletonemataceae	*Lauderia/Detonula*	Chain
		Lithodesmiaceae	*Ditylum*	Chain
		Paraliaceae	*Paralia sulcata*	Chain
		Rhizosoleniaceae	*Guinardia* et al [2]	Chain
			Rhizosolenia	Chain
		Skeletonemaceae	*Skeletonema*	Chain
		Stephanopyxidaceae	*Stephanopyxis*	Chain
		Thalassiosiraceae	*Thalassiosira*	Chain
		Triceratiaceae	*Odontella*	Chain
	Pennate Diatoms	Bacillariaceae	*Nitzschia*	Solitary glider
			Pseudo-nitzschia	Chain glider
		Fragilariaceae	*Asterionellopsis*	Chain/colony
			Cylindrotheca	Solitary glider
		Naviculaceae	*Tropidoneis*	Solitary

Class	Common Group Name	Family	Taxon/Category (LIMS Parameter Name Prefix) [1]	Morphology
Bacillar.	Pennate Diatoms	Pleurosigmataceae	Pleurosigma	Solitary glider
		Thalassionemataceae	Thalassionema	Chain
		Other	misc diatoms[3]	Unknown
Dinophyceae	Dinoflagellates	Ceratiaceae	Ceratium	Solitary flagellate
		Dinophysiaceae	Dinophysis	Solitary flagellate
			Oxyphysis	Solitary flagellate
		Gonyaulacaceae	Alexandrium	Chain/solitary flagellate
			Amylax	Solitary flagellate
			Gonyaulax	Solitary flagellate
			Protoceratium	Solitary flagellate
		Gymnodiniaceae	Akashiwo	Solitary flagellate
			Amphidinium	Solitary flagellate
			Cochlodinium	Solitary flagellate
			Katodinium	Solitary flagellate
			Torodinium	Solitary flagellate
			gymnodinioids	Solitary flagellate
		Kareniaceae	Karlodinium	Solitary flagellate
		Noctilucaceae	Noctiluca	Solitary flagellate
		Peridiniaceae	Scrippsiella	Solitary flagellate
			Heterocapsa	Solitary flagellate
		Polykrikaceae	Polykrikos	Solitary flagellate
		Protoperidiniaceae	Protoperidinium	Solitary flagellate
		Prorocentraceae	Prorocentrum	Solitary flagellate
		Warnowiaceae	Nematodinium	Solitary flagellate
		Other	misc small dinos[3]	Solitary flagellate
			misc med/large dinos[3]	Solitary flagellate

Class	Common Group Name	Family	Taxon/Category (LIMS Parameter Name Prefix)[1]	Morphology
Dictyochophyceae	Silicoflagellates	Dictyochaceae	Dictyocha	Solitary flagellate
Ebriophyceae	Ebrideans	Ebriaceae	Ebria	Solitary flagellate
Prymnesiophyceae	Prymnesiophytes	Phaeocystaceae	Phaeocystis	Colony
Raphidophyceae	Raphidophytes	Chattonellaceae	Heterosigma	Solitary flagellate
Xanthophyceae	Xanthophytes	Pleurochloridaceae	Meringosphaera	Solitary flagellate
Litostomatea	Ciliates	Mesodiniidae	Mesodinium	Solitary ciliate
Other	Ciliates	Other	misc ciliates[3]	Solitary ciliate
	Other zooplankton	Other	misc zoo[3]	Solitary
	Other	Other	all 5-10 um	Unknown
			misc 10-25 um	Unknown
			misc 25-100 um	Unknown
			misc >100 um	Unknown

[1] LIMS FlowCAM parameter names are "Taxon-Abund" or "Taxon-Biovol", where "Taxon" refers to the Taxon/Category listed above.

[2] The category "*Guinardia* et al" includes *Guinardia*, *Dactyliosolen*, *Leptocylindrus danicus*, and at times *Cerataulina*; all <25 μm diameter.

[3] These include living particles that can be grouped together into general taxonomic categories, but cannot be identified more specifically due to resolution, focus, or other imaging issues.

Table C2. Size Class categories and prefixes for LIMS parameter names used in FlowCAM analysis. Samples are run and processed according to FlowCAM protocol and images classified by size using Area Based Diameter.

Size Class	LIMS Parameter Name Prefix [1]	FlowCAM Magnification	
5-10 μm	Size-Class 5-10 μm		10x
10-25 μm	Size-Class 10-25 μm	4x	10x
25-100 μm	Size-Class 25-100 μm	4x	10x
100-300 μm	Size-Class 100-300 μm	4x	10x
>300 μm	Size-Class >300 μm	4x	

[1] LIMS FlowCAM parameter names are "Size-Class xxx-Abund" or "Size-Class xxx-Biovol", where "Size-Class xxx" refers to the parameter name prefixes listed above.

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Appendix D: Identified Taxa/Categories (Qualitative Analysis)

Table D1. Taxonomic categories (LIMS parameter names) used in qualitative analysis and semi-quantitative analysis for historical data. Samples are concentrated by reverse filtration, observed with a compound light microscope and identified to the lowest possible taxonomic level.

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Bacillariophyceae	Centric Diatoms	Asterolampraceae	*Asteromphalus heptactis*	Solitary
		Aulacodiscaceae	*Aulacodiscus kittonii*	Solitary
		Chaetocerotaceae	*Bacteriastrum delicatulum*	Chain
			Chaetoceros affinis	Chain
			Chaetoceros contortus	Chain
			Chaetoceros concavicornis	Chain
			Chaetoceros convolutus	Chain
			Chaetoceros crucifer	Chain
			Chaetoceros danicus	Chain
			Chaetoceros debilis	Chain
			Chaetoceros decipiens	Chain
			Chaetoceros diadema	Chain
			Chaetoceros didymus	Chain
			Chaetoceros eibenii	Chain
			Chaetoceros laciniosus	Chain
			Chaetoceros lorenzianus	Chain
			Chaetoceros radicans	Chain
			Chaetoceros similis	Chain
			Chaetoceros socialis	Chain
			Chaetoceros subtilis	Chain
			Chaetoceros teres	Chain
			Chaetoceros vanheurckii	Chain

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Bacillariophyceae	Centric Diatoms	Chaetocerotaceae	*Chaetoceros* (Hyalochaete) sp.	Chain
			Chaetoceros (Phaeocerus) sp.	Chain
		Corethraceae	*Corethron hystrix*	Solitary
		Coscinodiscaceae	*Coscinodiscus centralis*	Solitary
			Coscinodiscus concinnus	Solitary
			Coscinodiscus curvatulus	Solitary
			Coscinodiscus granii	Solitary
			Coscinodiscus marginatus	Solitary
			Coscinodiscus oculus-iridis	Solitary
			Coscinodiscus wailesii	Solitary
			Coscinodiscus sp.	Solitary
		Heliopeltaceae	*Actinoptychus senarius*	Solitary/colony
		Hemiaulaceae	*Cerataulina pelagica*	Chain
			Eucampia zodiacus	Chain
			Hemiaulus hauckii	Chain
		Lauderiaceae	*Lauderia annulata*	Chain
		Leptocylindraceae	*Leptocylindrus danicus*	Chain
			Leptocylindrus minimus	Chain
		Lithodesmiaceae	*Ditylum brightwellii*	Chain
		Melosiraceae	*Melosira moniliformis*	Chain
		Paraliaceae	*Paralia sulcata*	Chain
		Rhizosoleniaceae	*Dactyliosolen fragilissimus*	Chain
			Guinardia delicatula	Chain
			Guinardia striata	Chain
			Rhizosolenia setigera	Chain
		Skeletonemaceae	*Detonula pumila*	Chain
			Skeletonema costatum	Chain

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Bacillariophyceae	Centric Diatoms	Stephanopyxidaceae	*Stephanopyxis nipponica*	Chain
			Stephanopyxis palmeriana	Chain
		Thalassiosiraceae	*Thalassiosira anguste-lineata*	Chain
			Thalassiosira eccentrica	Chain
			Thalassiosira nordenskioeldii	Chain
			Thalassiosira punctigera	Chain
			Thalassiosira rotula	Chain
			Thalassiosira pacifica/aestivalis	Chain
			Thalassiosira sp.	Chain
		Triceratiaceae	*Odontella longicruris*	Chain
		Other	unidentified centric	Unknown
	Pennate Diatoms	Bacillariaceae	*Nitzschia acicularis*	Solitary glider
			Pseudo-nitzschia americana	Solitary sessile
			Pseudo-nitzschia sp. (large)	Chain glider
			Pseudo-nitzschia sp. (small)	Chain glider
		Fragilariaceae	*Asterionellopsis glacialis*	Chain/colony
			Cylindrotheca closterium	Solitary glider
		Naviculaceae	*Tropidoneis antarctica*	Solitary
			Navicula sp.	Solitary glider
		Pleurosigmataceae	*Pleurosigma* sp.	Solitary glider
		Striatellaceae	*Striatella unipunctata*	Chain
		Thalassionemataceae	*Thalassionema nitzschioides*	Chain
		Other	unidentified pennate	Unknown
Dinophyceae	Dinoflagellates	Ceratiaceae	*Ceratium* sp.	Solitary flagellate
			Ceratium furca	Solitary flagellate
			Ceratium fusus	Solitary flagellate
		Dinophysiaceae	*Dinophysis acuminata*	Solitary flagellate

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Dinophyceae	Dinoflagellates	Dinophysiaceae	*Dinophysis acuta/norvegica*	Solitary flagellate
			Dinophysis fortii	Solitary flagellate
			Dinophysis parva	Solitary flagellate
			Dinophysis rotundata	Solitary flagellate
			Dinophysis sp.	Solitary flagellate
			Oxyphysis oxytoxoides	Solitary flagellate
		Gonyaulacaceae	*Alexandrium catenella*	Chain flagellate
			Alexandrium sp.	Chain/solitary flagellate
			Amylax triacantha	Solitary flagellate
			Gonyaulax digitale	Solitary flagellate
			Gonyaulax sp.	Solitary flagellate
			Protoceratium reticulatum	Solitary flagellate
		Gymnodiniaceae	*Akashiwo sanguinea*	Solitary flagellate
			Amphidinium sphenoides	Solitary flagellate
			Cochlodinium sp.	Solitary flagellate
			Gymnodinium gracile	Solitary flagellate
			Gymnodinium sp.	Solitary flagellate
			Gyrodinium spirale	Solitary flagellate
			Gyrodinium sp.	Solitary flagellate
			Katodinium sp.	Solitary flagellate
			Torodinium sp.	Solitary flagellate
			gymnodinioid dinoflagellate	Solitary flagellate
		Kareniaceae	*Karlodinium* sp.	Solitary flagellate
		Noctilucaceae	*Noctiluca scintillans*	Solitary flagellate
		Oxytoxaceae	*Oxytoxum* sp.	Solitary flagellate
		Peridiniaceae	*Scrippsiella trochoidea*	Solitary flagellate
		Peridiniida incertae sedis	*Heterocapsa triquetra*	Solitary flagellate

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Dinophyceae	Dinoflagellates	Polykrikaceae	*Polykrikos schwartzii*	Solitary flagellate
		Protoperidiniaceae	diplopsalid	Solitary flagellate
			Minuscula bipes	Solitary flagellate
			Protoperidinium brevipes	Solitary flagellate
			Protoperidinium conicum	Solitary flagellate
			Protoperidinium depressum	Solitary flagellate
			Protoperidinium excentricum	Solitary flagellate
			Protoperidinium leonis	Solitary flagellate
			Protoperidinium oblongum	Solitary flagellate
			Protoperidinium oceanicum	Solitary flagellate
			Protoperidinium pellucidum	Solitary flagellate
			Protoperidinium steinii	Solitary flagellate
			Protoperidinium sp.	Solitary flagellate
		Prorocentraceae	*Prorocentrum gracile*	Solitary flagellate
			Prorocentrum micans	Solitary flagellate
		Pyrocystaceae	*Dissodinium pseudolunula*	Solitary flagellate
		Pyrophacaceae	*Pyrophacus horologium*	Solitary flagellate
		Warnowiaceae	*Nematodinium armatum*	Solitary flagellate
		Other	unidentified dinoflagellate (<25 um)	Solitary flagellate
			unidentified dinoflagellate (>25 um)	Solitary flagellate
Chrysophyceae	Chrysophytes	Dinobryaceae	*Dinobryon* sp.	Colony
Cryptophyceae	Cryptophytes	Cryptomonadaceae	cryptomonad	Solitary flagellate
Dictyochophyceae	Silicoflagellates	Dictyochaceae	*Dictyocha fibula*	Solitary flagellate
			Dictyocha speculum	Solitary flagellate
		Pedinellaceae	*Apedinella spinifera*	Solitary flagellate
Ebriophyceae	Ebrideans	Ebriaceae	*Ebria tripartita*	Solitary flagellate
Euglenoidea	Euglenoids		euglenoid	Solitary flagellate

Class	Common group name	Family	Taxon/Category (LIMS Parameter Name)	Morphology
Prymnesiophyceae	Prymnesiophytes	Phaeocystaceae	*Phaeocystis* sp.	Colony
		Chrysochromulinaceae	*Chrysochromulina* sp.	Solitary flagellate
Raphidophyceae	Raphidophytes	Chattonellaceae	*Heterosigma akashiwo*	Solitary flagellate
Xanthophyceae	Xanthophytes	Pleurochloridaceae	*Meringosphaera mediterranea*	Solitary flagellate
	Copepods		copepod	Solitary
			nauplius	Solitary
Oligotrichea	Oligotrich ciliates	Strobilidiidae	*Strobilidium* sp.	Solitary ciliate
		Xystonellidae	*Parafavella* sp.	Solitary ciliate
	Tintinnid ciliates	Metacylididae	*Heliocostomella* sp.	Solitary ciliate
		Ptychocylididae	*Favella* sp.	Solitary ciliate
		Other	unidentified tintinnid	Solitary ciliate
Litostomatea	Other ciliates	Mesodiniidae	*Mesodinium rubrum*	Solitary ciliate
Prostomatea	Other ciliates	Colepidae	*Tiarina fusus*	Solitary ciliate
Other	Other ciliates	Other	unidentified ciliate	Solitary ciliate
	Nanoflagellates	Other	unidentified nanoflagellates	Solitary flagellate
	Other zooplankton	Other	unidentified zooplankton	Solitary

Appendix E: Assessment of FlowCAM Particle Biovolume for Estimation of Particulate Organic Carbon and Nitrogen (2015-2016)

Background

This appendix describes a special study aimed at assessing the feasibility of using FlowCAM biovolume data for providing estimates of phytoplankton biomass in terms of a universal currency, such as µg of carbon per L of water, which is critical information for accurate characterization and modeling of primary production, food webs, and carbon cycling. This study will also result in information on carbon to nitrogen ratios in phytoplankton, which will be informative of the quality of phytoplankton as food sources and of nitrogen dynamics in the study area. Results from this study will also be used to estimate the proportion of phytoplankton biomass outside the range of the FlowCAM in the largest (>300 µm) size fraction, which it is not currently within the scope of this program to quantify.

The biovolume of phytoplankton is often measured as a proxy for estimating carbon (e.g., Menden-Deuer and Lessard, 2000). However, the FlowCAM method estimates biovolume of whole particles, often consisting of many cells. This is an approach that is very different from studies that have been previously published, which determine carbon content corresponding to biovolume measured on a per cell basis. In addition, the type of phytoplankton and the environmental conditions (light, nutrients) under which the phytoplankton are growing both affect their carbon to biovolume ratios, making validation of any method of estimating carbon biomass from biovolume in a specific study area and season necessary.

At the time of this writing, this sampling effort will occur approximately July 2015 – July 2016. Because analysis of total particulate organic carbon and nitrogen is not currently performed by KCEL, samples will be analyzed by combustion with an elemental analyzer at the UC Davis Stable Isotope Facility.

Objectives

- Determine the relationship between phytoplankton (5-300 µm) biovolume in water samples as measured by the FlowCAM and particulate organic carbon (POC, 1.5-300 µm) determined by combustion.

- Determine the relationship between phytoplankton (5-300 µm) carbon calculated from FlowCAM biovolume using literature values for various taxa and POC (1.5-300 µm) determined by combustion.

- Use data to generate and assess an equation (or mathematical model) for predicting POC and potentially particulate organic nitrogen (PON) from future FlowCAM biovolume measurements and environmental conditions.

- Determine the proportion of carbon in large particulates not analyzed by the FlowCAM and how this changes seasonally at a single location (LSNT01, chosen as one of the most comprehensively sampled of the marine ambient sites and due to its mid-Central Basin location).

- Determine the seasonal cycle of carbon to nitrogen (C:N) ratios in two size-fractions of microplankton, and determine if differences exist among sites over time.

Sample Collection

Samples must be collected from diverse locations and times for at least one year in order to have sufficient data to develop a predictive model, if a relationship between FlowCAM biovolume measurements and POC/PON exists. Samples will be collected from the same sites sampled for phytoplankton and with the same frequency, but from the surface depth only. Samples will be collected using the same method as phytoplankton samples and from the same Niskin/Scott bottle grab. The water volume required will depend on the density of particles in water and their carbon content (with more volume required in winter and less in spring), but should range from 200-1000 mL for each sample. Samples will be collected into separate bottles from the FlowCAM samples (500-1000 mL capacity). This effort will result in a total of 176 water samples collected over the course of 12 months of sampling (8 sites/event × 22 events = 176 water samples).

Sample Processing

Water samples will be split for fractionation, duplicates, whole water, and blanks as detailed in Table E1.

Following fractionation, cells are filtered onto pre-weighed 934-AH, 25 mm glass fiber filters (nominal 1.5 µm retention) using a vacuum pump and manifold. The filters are loaded with the necessary volume to yield some coloration – approximately 250 mL during the bloom season, more at other times. For any given sample, the same volume is filtered for the two fractions. For the LSNT01 whole water sample, the volume filtered is one half of the volume filtered for the fractionated LSNT01 sample. The blank is prepared by filtering 250 mL seawater that has been pre-filtered (0.45 µm) for FlowCAM use. Using forceps and a sample crimper plate, the filter is rolled, folded and transferred to a standard weight 10.5 x 9 mm tin capsule (Elemental Microanalysis), loosely crimped, and dried overnight at 50 °C. Capsules are crimped to seal, shaped to 8 mm diameter, weighed, organized in 48-well plates and stored with desiccant until shipping. The total weight of the prepared sample (including tin) should not exceed 200 mg.

Table E1. Details of how water samples will be split and processed prior to filtering for analysis, and the total number of filters to be sent for analysis at UC Davis Stable Isotope Facility.

Sample splits	Processed particle size	Frequency	Site	Number of filters/event
Small fraction	<100 μm	All events	All sites	8
Large fraction	100-300 μm	All events	All sites	8
Small fraction duplicate	<100 μm	Every other event	Same site as FlowCAM duplicate	0.5
Large fraction duplicate	100-300 μm	Every other event	Same site as FlowCAM duplicate	0.5
Whole seawater	All particles	All events	LSNT01 only	1
Method blank	<0.45 μm	Every other event		0.5
Total =				18.5 filters/event
× 22 events =				407 filters/year

Sample Analysis

Samples are analyzed at the UC Davis Stable Isotope facility by combustion using an Elementar Vario EL Cube or Micro Cube elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (http://stableisotopefacility.ucdavis.edu). Samples are combusted at 1000°C in a reactor packed with copper oxide and lead chromate. Following combustion, oxides are removed in a reduction reactor. N_2 and CO_2 are separated using a molecular sieve adsorption trap before entering the IRMS. During analysis, samples are interspersed with several replicates of at least two different laboratory standards which are selected to be compositionally similar to the samples being analyzed. The C and N analysis data are reported as elemental total C and N in micrograms. The MDLs for a 200 mg sample are approximately 100 μg C and 20 μg N (Emily Schick, pers. comm.).

Data Analysis

Data will be analyzed to reach the objectives outlined above. Graphical inspection and preliminary assessment of the full dataset may dictate more complex nonlinear modeling methods if nonlinear relationships are found, but first steps may include:

▪ Determining whether FlowCAM biovolume in each size fraction is significantly and strongly correlated to POC in each size fraction, or total FlowCAM biovolume to whole water POC at LSNT01. Determining if the relationship between biovolume fraction and POC is consistent so that a correction factor can be applied, or if this varies seasonally and spatially.

- Determining whether calculated FlowCAM carbon in each size fraction is significantly and strongly correlated to POC in each size fraction, or total calculated FlowCAM carbon to whole water POC at LSNT01. This is likely to vary seasonally and spatially.

- Running step-wise multiple regression to determine FlowCAM-derived characteristics of samples (and, potentially, water quality parameters) that may be used to predict POC (and potentially, PON) in each size fraction, and what percentage of variability in POC values the chosen characteristics explain. Examples of chosen characteristics may include total FlowCAM biovolume or calculated carbon, percent diatom taxa, ratio of diatom to dinoflagellate taxa, and other measures of community size distribution or composition. However, these potential explanatory variables are likely to be highly collinear with each other, violating the assumption of multiple regression. In this case, new and orthogonal variables for the regression model representing community size distribution or composition may be created by first running principle components analysis (or other multivariate techniques) on the FlowCAM biovolume or calculated carbon data. An iterative partial dataset or jack-knifing technique may be used to create a model for predicting POC, which can then be tested on data not included in the model.

- Comparison of paired size-fractionated and whole water POC samples at LSNT01 to determine the proportion of carbon missed with the size fractionation technique and FlowCAM analysis, and whether this proportion is significant or varies seasonally or between sites (randomized block, paired comparison ANOVA).

- Graphical inspection of seasonal cycles in C:N ratio at all sampled sites. Data will be interpreted in context of nutrient (nitrate + nitrite and ammonia) concentrations and seasonal community succession and bloom development.