FINAL PROJECT REPORT

PROJECT TERM: JULY 1, 2007 – JUNE 30, 2011

PROJECT TITLE: USING FLOWCAM TECHNOLOGY TO MEASURE HIGH FREQUENCY SPATIAL AND TEMPORAL VARIATION IN PHYTOPLANKTON AND ZOOPLANKTON SPECIES COMPOSITION AND DEVELOP STATE-OF-THE-ART PLANKTON MONITORING

(CALFED AGREEMENT 1047)

AMOUNT FUNDED: $150,000

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PROJECT SUMMARY

This pilot study (CALFED Agreement 1047) evaluated the use of the FlowCAM, a state-of–the-art digital imaging flow cytometer, to rapidly identify, enumerate and estimate biomass for in situ and laboratory phytoplankton and zooplankton species biomass and composition for San Francisco Estuary (SFE). The FlowCAM successfully identified

phytoplankton genera in live samples and phytoplankton and zooplankton genera and taxa in preserved samples from SFE. Sampling procedures for live and laboratory sampling were developed and documented in an operation manual which includes a library of digital images. Comparisons with microscopy indicate the FlowCAM was a reliable tool and easier to use than standard inverted or dissecting microscopes for plankton counting. The FlowCAM was also more accurate than the Fluoroprobe phytoplankton pigment monitor for characterizing the variation of phytoplankton community composition in the delta. In field studies conducted between April and October 2010, the FlowCAM demonstrated the presence of high frequency phytoplankton community composition. This study further demonstrated that phytoplankton commuities in SFE are patchy, differ among rivers, vary on a temporal scale and change with water quality conditions. The field study suggests that the low frequency fixed station sampling currently conducted in SFE is inadequate to quantify the food resources at the base of the food web. We conclude that the FlowCAM digital imaging flow cytometer is an important new tool for quantifying plankton in SFE and should be included in monitoring and special study programs.

BUDGET SUMMARY

EXPENSE	AMOUNT INVOICED (2007-2008)	AMOUNT INVOICED (2008-2009)	AMOUNT INVOICED (2009-2010)	AMOUNT INVOICED (2010-2011)	AMOUNT INVOICED TO DATE (ALL YEARS)
DWR Labor				15333.08	
Benefits				6571.32	
Equipment and services			87024.29		
Subcontracts	6886.85		2792.00	31392.28	
Totals	6886.85		89816.29	53296.68	149999.8

LIST OF TASKS AND ACTIVITIES PERFORMED

Tasks were identified as part of the scope of work, and this section is a cummulative overview of the activities performed for each task. This section serves as the full historical record of all activities performed for this project.

Task 1. Management of contracts, subcontracts, accounting, personnel and reporting.

- The contract was implemented in July 2007 and completed in June 2011.
- The purchasing process began for the FlowCAM in July 2007 but was delayed by administrative issues.
- The FlowCAM was finally received in July 2008
- Operation and evaluation of the FlowCAM was initiated in July 2008 but was delayed by contract issues. The contract was frozen over many months each by CALFED twice and by the California legislature due to budget issues. These stoppages lead to a loss of personnel and subcontracts put in place for students to conduct the work, making start up difficult. Two UC Davis students were lost during this process and were finally replaced by a post-doctor at Bigelow Labs. Some of the work was also diverted to permanent staff in DWR due to time constraints.

Task 2-3. Conduct high frequency spatial and temporal phytoplankton field studies.

- Between April and October 2010, DWR staff conducted monthly longitudinal field transect surveys to quantify high frequency phytoplankton patches along longitudinal transects in the Sacramento and San Joaquin Rivers. Samples for phytoplankton species composition were analyzed in situ every 6-10 minutes, for a total of about 30 samples per river each sampling day. Simultaneous and continuous measurements of the water quality variables water temperature, pH, specific conductance, dissolved oxygen concentration, chlorophyll *a* fluorescene and turbidity with YSI water quality sondes were also collected to facilitate an evaluation of the influence of environmental conditions on phytoplankton species composition.

Task 4. Compare FlowCAM and Fluoroprobe for estimating phytoplankton community composition and biomass.

- Fluoroprobe measurements of phytoplankton pigment composition were conducted in conjunction with the July 2010 field transect survey. These estimates of phytoplankton community composition were compared with the FlowCAM estimates.

Task 5. Evaluate the accuracy and efficiency of using the FlowCAM for analyzing phytoplankton and zooplankton species composition compared with microscopy.

- Phytoplankton species counts were compared with inverted microscope cell counts and zooplankton biomass estimates were compared with dissecting microscope counts for a random suite of samples.

Task 6. Data analysis and preparation of operation manual.

- Operation of the FlowCAM for identificaiton of preserved phytoplankton and zooplankton in the laboratory and live phytoplankton in the field was docmented in an operations manual. The manual includes a digital image library for future phytoplankton identification (disk included).

Task 7. Reporting.

- Poster and oral presentations were made including a) posters at the Environmental Scientist Workshop 2009, the Environmental Scientist Workshop 2010, the Delta Science conference 2010 and the Interagency Ecological Annual Meeting 2010 and 2011 and b) oral presentations at the Delta Science Technical Workshop 2009, the Annual Meeting of the Society of Limnology and Oceanography 2011 and the Estuarine Research Federation Meeting 2011.
- Written and digital documents include a) Progress reports, b) Operations manual, c) Phytoplankton digital image library, d) Preliminary draft journal paper: High frequency variation of phytoplankton in the Sacramento and San Joaquin Rivers and their association with high frequency changes in water quality conditions

LIST THE ACHIEVED OBJECTIVES, FINDINGS, AND MAJOR CONTRIBUTIONS

1. The FlowCAM digital imaging flow cytometer was evaluated and determined to be an easy to use, fast, inexpensive and accurate method of quantifying phytoplankton and zooplankton in San Francisco Estuary.
2. High frequency measurements with the FlowCAM made during field surveys indicate phytoplankton community composition is very patchy along river transects, even at the 3 km scale, and accurate estimates of food resources at the base of the food web require more frequent sampling than is currently conducted in the estuary.
3. An operations manual for field and laboratory analysis of phytoplankton and zooplankton samples and a digital image library were developed for future application in monitoring and special studies programs.
4. Multiple oral and poster presentations at local and international meetings and a journal paper will distribute this information to the scientific community.

DISCUSS THE MANAGEMENT IMPLICATIONS OF PROJECT FINDINGS

The patchiness of the phytoplankton community at the 3 km spatial scale suggests high frequency measurements are needed to gain an accurate knowledge of the food resources available at the base of the food web in the estuary. Addition of FlowCAM phytoplankton counts to monitoring and special study programs would provide a simple, relatively fast and inexpensive way to gain this needed information on the phytoplankton and zooplankton food

resources at the base of the food web that supports fish production. In addition, because both phytoplankton and zooplankton counts can be quickly analyzed by agency staff compared with the long delays with contract laboratories, the FlowCAM can provide data for near real-time management decisions.

PROJECT DELIVERABLES

- List here any presentations given at the Bay-Delta Science Conference and at other events
    1. Oral presentation at Delta Science Technical Workshop October 2009: Using the flowCAM to Analyze Plankton Samples
    2. Poster presentation DWR Environmental Scientist Workshop May 2010: Using flowCAM Technology to Characterize Delta Plankton
    3. Poster presentation at Delta Science Conference Oct 2010: Using FlowCAM Technology to Quantify Real Time Changes in Phytoplankton Communities in the Delta
    4. Oral presentation American Society of Limnology and Oceanography Annual Meeting Feb 2011: Using FlowCAM to quantify Microcystis in San Francisco Estuary
    5. Poster presentation at Interagency Ecological Program Annual Meeting March 2011 : High Frequency Spatial Variation of Phytoplankton and Water Quality in the Sacramento and San Joaquin Delta

- List here and provide hardcopies and electronic files of all materials and published papers resulting from this grant

1. Appendix 1: Operations Manual for use of the FlowCAM to quantify phytoplankton and zooplankton species and biomass (Task 6 and 7)

2. Appendix 2: Evaluation of the FlowCAM for phytoplankton and zooplankton species composition and biomass in the Sacramento and San Joaquin River Delta (Task 5 and 7)

3. Appendix 3: Spatial and temporal variation of phytoplankton species distribution in the Sacramento and San Joaquin River Delta (Task 2, 3, 6 and 7)

4. Appendix 4: Comparison of the FlowCAM and Fluoroprobe for estimates of percent phytoplankton biomass (Task 4 and 7).

5. 5. Appendix 5: digital library files for live phytoplankton analysis (disk format).

Appendix 1: Operations Manual

DWR Lab Operation Manual

FlowCAM digital imaging flow cytometer

Model 1

by
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June 2011

This work was completed for the Bay-Delta Authority Science Program
Grant 1047

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Summary

The FlowCAM is a digital imaging flow cytometer made by Fluid Imaging Technologies, Inc. that measures the abundance and dimensions of phytoplankton and zooplankton species in a stream of water that passes through a small flow cell positioned in front of a microscope lens. Digital images of each particle as they pass by the microscope are recorded by a camera. The resolution of the particles is adjusted through the magnification and flow cell combination. The current camera can readily resolve images of 12 µm or greater, but is less reliable for smaller particles. The FlowCAM can be used to quantify live phytoplankton in the field or preserved phytoplankton and zooplankton samples in the laboratory. It is best used for live phytoplankton in the field where a fluorescence probe isolates the live phytoplankton from detrital material that often dominates the digital image files. Live zooplankton were not quantified live in the field because they swim against the current in the flow cell; this aspect of sampling requires further study.

I. Machine Operation

Machine setup

1. Open the FlowCAM
2. Plug in the power cord on the left side
3. Flip the “P” toggle up to power on the machine
4. At windows prompt logon
   User ID: esograph **Password**: DES123abc **Location**: Water
5. Install the objective, collimator and flow cell combination as needed (Table 1)
6. Using lens paper, gently wipe both sides of the flow cell.
7. Place the flow cell in the appropriate flow cell chamber (Table 1) and gently screw down the lid until it is just set.
   a. If the flow cell is still loose in the chamber, carefully adjust the ring on the inside of the chamber.
   b. The cells are very fragile, so be gentle or you will break the flow cell
8. Attach the funnel stand to the sliding ring and then the stand to the FlowCAM.
9. Thread the upper tube of the flow cell chamber through the funnel ring
10. Attach the appropriate size funnel and position so the tubing is taught
11. Attach the tubing on the bottom side of the flow cell to the appropriate size pump tubing (Table 1)
12. Stretch and secure the tubing around the pump wheel
13. Attach a second piece of tubing of the appropriate size to the end of the tubing around the pump wheel and thread through the hole on the left side of the case into a waste beaker

Table 1 Flow cell and objective combinations

Flow cell	Flow cell Chamber	Objective Lens	Collimator	Tubing
50µm	300µm	20x	20x	20x
100µm	300µm	10x	10x	4x/10x
300µm	300µm	4x/10x	None/10x	4x/10x
600µm	600µm	4x/2x	None/Diffuser	4x/2x
800µm	800µm	2x	Diffuser	2x
2 mm	2 mm	2x	Diffuser	2x

7. Open the Visual Spreadsheet software on the desktop which matches the objective.
8. On the main window select [File] → [Load Context]
   a. load the context file that matches the objective, flow cell, and sample type (e.g. 4x 300 lugols.ctx)
9. Select [Set-up] → [Context] to open the context window.

10. Verify the objective dependant settings in the context file Table 2.

Table 2 Objective Dependant Settings

Hardware								
Objective	2x	2x	2x	4x	4x	10x	10x	20x
Size Range (um)	36-600	101-600	301-600	36-600	36-300	36-100	6-100	6-35
Pump Tubing	2x	2x	2x	4x/10x	4x/10x	4x/10x	4x/10x	20x
Capture								
Distance to nearest neighbor (um)	20	50	75	20	20	20	15	4
Calibration								
Calibration factor (um/pixel)	3.2623	3.2623	3.2623	1.6642	1.6642	0.5813	0.5813	.2857
Flow cell Depth (um)	600	600	600	600	300	300	100	50
Camera								
AutoImage Rate	6	6	5	5	5	7	7	7
Flash Duration	6	6	100	28	30	40	50	50
Gain	783	783	400	384	425	540	607	648
Stop								
Recalibration Interval								
	20	20	20	12	12	12	12	6

12. Fill the funnel with deionized distilled water
13. Turn the pump on forward and PRIME
14. Wait until water passes through the flow cell tubing and exits into the beaker before focusing
15. Gently squeeze the tubing between the cell and the pump to flush all air out of the system.
16. Plug in the air pump, set to Whisper 10 and drape so that the tip of the pipette rests near the base of the funnel.

Focusing

1. From the main window, select: [Set-up] → [Set-up and Focus] to open the camera window.
2. Rough focus by sliding the base until you can see spots on the surface of the flow cell on the screen
3. Secure by screwing in the vertical screw.
4. Rotate the flow cell chamber with the side and upper screws until the striations in the glass are vertical.
5. Shift the flow cell chamber so you are near the center rather than the edge of the flow cell
6. Using the fine focus, find one plane and then the other plane of the flow cell and record the micrometer readings.
7. Subtract the micrometer readings of one plane from the other, divide by 2 and then scale the focus back so that the reading is at the center of the cell (add this amount to the lowest micrometer value)
8. go to main window layout
9. To check the focus,
    a. place test sample in the funnel and run pump on slow to move sample into flow cell
    b. select: [Set-up] → [auto image mode (no save)] to open the collage window
    c. If most of the images are in focus skip to step e
    d. If most of the images are not in focus return to the main window, select [Set-up and Focus] → [auto image mode (no save)] again and turn the micrometer slightly away from you to bring the focus towards the front of the cell until a majority of the particles are in focus. Return to select [Set-up and Focus] → [auto image mode (no save)] to re-check the focus
    e. You cannot change the focus once the has started

3. Running a Sample

1. Remove sample down to base of funnel with pipette
2. Add de-ionized distilled water to the funnel with a pipette and run the pump for about a minute on PRIME at forward setting to clear the old sample
3. Place air pump tube at base of funnel for very gentle mixing and not rapid bubbling
4. Remove deionied water from the funnel down to the base of the neck and stop pump
5. select [Imaging] → [auto image mode] to open stop window
    a. enter settings, date, sample etc then [Okay] to open save window
6. Open folder for sample files
7. Add file name at end of numeric file name (Julian time and date) *ex.* ***068-112636*** *AT 9-4-07N 4x.lst*

and do not save

8. Add sample water and set pump speed
    a. Pump speed should be fast enough so that each image is only captured once but the particle capture rate is about 1. Suggested speeds are listed in Table 6.
9. Set the pump to forward and wait for the sample to move into the flow cell. Suggested times are listed in Table 6.
10. click [save] to open the camera and collage window and start the run
11. Sample will run the length of time specified in the context file
12. Once the sample collage window closes, the sample will be finished
13. To help track the sample time use the timer on windows
14. For next sample, repeat from 1
15. Stop the sample prematurely by clicking [stop]

Table 3. Suggested pump speeds for flow cells and suggested delay time needed to clear old sample.

	50 µm	**100 µm**	**300 µm**	**600 µm**
Pump Speed	Slow 0	Slow 2 or 3	Slow 5	Fast 5
Wait Time (m:s)	3:00 (prime)**	2:00	1:20	0:30

4. Cleaning

1. Remove any remaining sample and fill the funnel with a 10% Simple Green® solution and allow it to run through the system. Once you are sure that the solution is inside the flow cell, repeatedly pinch the tubing between the cell and the pump to dislodge any cells which may be stuck to the inside of the glass.
2. Rinse the system 2x with de-ionized distilled water again and let the flow cell run completely dry.
3. Add a small amount of Ethanol or Acetone (0.1-0.5 ml) for drying and let that run completely through the system.
4. Remove the funnel and funnel stand. Place the funnel stand inside the lower right corner of the flow cell or with the funnel. Remove the tubing from around the wheel, leaving it connected.
5. Close any open windows and shut down the computer: [Start] → [Shut Down] → [Okay].
6. Replace the protective cover/bubble wrap on the monitor, close the lid and latch it.
7. Unplug the power cord, remove the battery from the mouse and one battery from the keyboard to preserve battery life.
8. Charge the mouse battery if necessary.

9. Secure the lock on the front of the FlowCAM. **Combination**: Middle Ground site # or 504.

4. Suggestions

1. When using a new flow cell, re-measure the wait time for the sample to reach the flow cell because it varies with the length of tubing at the top of the flow cell.

2. Prevent clogging of the 300 & 600µm cell by periodically gently twisting or flicking the tubing above the cell to dislodge any large colonies.

3. To keep the sample evenly mixed, occasionally mix the sample with the pipette by gently filling and emptying some water into the pipette

4. Be sure to monitor the screen for double images and adjust by increasing the pump speed, lowering the auto image rate, or using a larger pump tubing.

5. If the images become blurred during the run, the background image may be distorted. Fix this problem by recalibrating the background: select [Tools] → [Recalibrate] from the Main menu

5. Troubleshooting

a. Focusing with Starch
   1. Place a minute amount of starch solution in the funnel and from the main window select: [Set-up and Focus] → [auto image mode (no save)]; this will open up the view window where the images of the starch will be captured.
      Potato Starch = 300µm+ Flow cell Corn Starch = 50-100µm Flow cell

b. Leaky System
   After the pump is turned off, water may still siphon through the system. This is most noticeable at 2x (600). This problem is caused by the tubing around the pump wheel being too long. Shorten the tubing or replace the tubing so that the length is roughly 12 cm. This should fix the system so that it doesn't leak.

c. Accidental Dry Cell
   Once you fill the flow cell with water and remove the air bubbles, always keep the cell full. If the cell dries out, simply reverse the pump to bring water back into the cell. This should refill the cell without creating air

bubbles. It is very important that no air bubbles are in the system prior to running your sample.

d. Getting the Air Out
All the air must be out of the system prior to a run to ensure even flow through the cell. This is challenging and may take several minutes: Gently agitate the tubing between the funnel and the cell. Squeeze the tubing repeatedly between the cell and the pump. Changing the direction or stopping the flow is often helpful. The larger flow cells are more durable and can handle a bit of force, but the 50 and 100 um cannot and must be handled delicately.

e. Imaging
Occasionally, one image may be split into several images when captured. Increasing the Distance to Nearest Neighbor and lowering the Segmentation Threshold should fix this problem. If particles on the screen are being captured: select [Tools] → [Recalibrate] to recalibrate the background image. You may also need to clean or replace the cell if there are too many on-screen particles.

f. Particles Stuck to the Screen
During the run, particles may get stuck to the screen generating multiple images. When this happens, click [Stop] on the camera window and stop the pump. Gently and alternately squeeze the tubing above and then below the cell to flush the particles off the inside of the cell. Start the pump and let it run for a 10-20 seconds before clicking [Resume] on the camera window. Then select [Tools] → [Recalibrate] and finish processing the sample. Ultimately, you may have to flush the system entirely and repeat the run if the above methods prove ineffective.

II. Sample Preparation

1. **Microcystis Lugol's preserved samples**

1. Gently shake the brown amber bottle containing *Microcystis* preserved in Lugol's solution from dense net samples
2. Empty all of the sample into a small beaker
3. Collect a 1 ml subsample using a 1 ml Hensen-Stemple volumetric pipette
2. Depress the plunger while mixing the sample in a figure-8 pattern
3. Gently tap the sides of the beaker with the pipette to remove any air bubbles
4. Release the plunger to draw up the sample.

7. Filter the sample if appropriate for 3 colony diameter ranges
    i. Colony diameter 301 to 3000 µm
        1. Do not filter
            a. Dilute sample to less than 200 *Microcystis* images per ml using double distilled de-ionized water
    ii. Colony diameter 36 to 300 µm
        1. Filter 1 ml sample through a 300 µm sieve
            a. Fold sieve fabric to create a cone
            b. Place sieve in the top of a 10 ml fluted graduated cylinder
            c. Add 1 ml sample
                i. dilute filtered sample using double distilled de-ionized water to less than 200 *Microcystis* images per ml
    iii. Colony diameter 15 to 35 µm
        1. Filter 1 ml sample through a 300 µm sieve
            a. Fold sieve fabric to create a cone
            b. Place sieve in the top of a 10 ml fluted graduated cylinder
            c. Add 1 ml sample
        2. Filter sample again through a 36 µm sieve
        3. Dilute filtered sample using double distilled de-ionized water to less than 200 *Microcystis* images per ml

8. Run samples at three magnifications using the following flow cell sizes depending on colony diameter (Table 4)

Table 4. Flow cell size, suggested dilution, sieve pore size, colony diameter and magnification used for dense net samples

Flow cell size	**50µm**	**300µm**	**300µm**	**600µm**
Dilution (suggested)	1:4	1:40	1:10	1:40
Filter (µm pore size)	300 & 35	300 & 35	300	none
Cell diameter, µm	15-35	15-35	36-300	>300
Magnification	20X	4X	4X	2X

9. Apply machine settings for the sample runs at each size category according to Table 5 or as is appropriate.

Table 5. Context file settings used in FlowCAM software to quantify of *Microcystis* colonies in preserved water samples for three size categories.

Factor	Sub factor	Item	Colony diameter um	Colony diameterum	Colony diameterum
Cell diameter range			301-3000	36-300	15-35
Magnification			2X	4X	4X
Flow cell			600 um	300 um	300 um
Pump speed			Fast 2	Slow 5	Slow 5
Load			Yes	Yes	Yes
Capture	Particle capture	Distance to nearest neighbor	75	20	20
		Closed holes	0	2	2
		Convolution	None	None	None
	Images	Collage image border	2	4	4
		Save collage image	Yes	Yes	Yes
		Save binary image	Yes	Yes	Yes
	Particles defined by acceptable region	Both light and dark pixels	Yes	Yes	Yes
		Left	0	0	0
		Right	1022	1022	1022
		Top	0	0	0
		Down	766	766	766
Calibration		Calibration um per pixel	3.2623	1.6642	1.6642
		Calibration default	3.2771	1.6386	1.6386
Camera	Behavior	Auto image rate	5	5	5
		Flash	100	30	30
		Flash delay	0.10	.10	.10
	Settings	gain	400	425	425
		Shutter	45	45	45
Filter	Basic size	ABD	Yes	Yes	Yes
		Diameter min	301	36	15
		Diameter max	3000	300	35
Stop	Stop conditions	Stop when run exceeds	6 min 20 sec	6 min 10 sec	6 min 10 sec

	Recalibration		20 min	20 min	20 min
Reports	exports	Export list data when run terminates	Yes	Yes	Yes
		Export list summary when run terminates	yes	yes	yes

2. Non-*Microcystis* Lugol's preserved samples

    1. Gently shake the brown amber bottle containing Microcystis preserved in Lugol's solution from dense net samples
    2. Empty all of the sample into a small beaker
    3. Collect a 1 ml subsample using a 1 ml Hensen-Stemple volumetric pipette
        a. Depress the plunger while mixing the sample in a figure-8 pattern
        b. Gently tap the sides of the beaker with the pipette to remove any air bubbles
    4. Release the plunger to draw up the sample.
    5. Filter the sample if appropriate for desired diameter range
    6. Run FlowCAM according to Tables 1 through 5

7. Live samples (no preservative)

    1. Turn on fluorescence probe and let warm for 20 min before sample run
    2. Set up 10X objective, pump speed 2 and 100 µm flow cell
    3. Run sample in Trigger mode instead of auto image mode
    4. Rinse before and after each sample with distilled water
        a. Remove sample from funnel with pipette and fill with distilled water
        b. Let distilled water run through system on PRIME until it clears about 2 min
        c. Remove distilled water from funnel with pipette and fill well mixed sample water
        d. Run sample through the flow cell by putting on PRIME for 1 minute
        e. Run sample at pump speed slow 2 for as long as desired, at least 6 minutes
        f. Repeat from a for next sample

III. Computing abundance and biomass

1. *Microcystis* abundance

a. Determine the ABD volume of *Microcystis* for a single cell at each magnification and diameter range separately
b. Sum ABD volume of *Microcystis* images measured for each magnification and diameter range separately
c. Divide ABD volume by ABD volume of a single *Microcystis* cell at each magnification and diameter range separately to get cell abundance at each magnification
d. Sum cell abundance from all magnifications
e. Divide total abundance by the number of ml processed for the sample to get cells ml^{-1}
f. Adjust for dilution

2. Single cell abundance
    a. Sum cell abundance from all magnifications
    b. Divide total abundance by the number of ml processed for the sample to get cells ml^{-1}
    c. Adjust for dilution

3. Carbon biomass
    a. Sum the total ABD volume of single cells or colonies
    b. Compute carbon biomass using equations that convert cell volume to carbon
        i. Equations such as: Menden-Deuer, S. & E. J. Lessard. 2000. Carbon to volume relationships for dinoflagellates, diatoms and other protest plankton. Limnology and Oceanography 45:569-579.
        ii. Note: diatom equations differ from other phytoplankton and cyanobacteria

IV. Classification

1. Creating a library
    a. Open list file, view window and then the classification and library windows
    b. Select a few representative images of one species from the view file by left clicking
    c. Right click and save the selected species in a tab within the library file
        i. Note: this library file is unique to the sample
        ii. The names of the tabs are ones you create
    d. Run the library file to sort the remaining species in the view file based on your classification
        i. All of those images that look like the ones in the library tab you made will be removed from the view window and placed in a tab within a classification file that is unique to the sample
    e. Continue to create and run library files until all of the species in the view window are classified in the classification file (the view window will be empty)
    f. Export the information from the classification file to an excel spreadsheet to obtain the final count
    g. The library file of a single sample can be saved as a generic library for future use with other samples

2. Applying a generic library
    a. Open generic library, classification and view windows simultaneously
    b. Run generic library on view window file and create a classification file for the sample

3. Final count

a. export classification data into an excel spreadsheet to get abundance or total volume of each species in classification file
b. manually sum abundance of each species to get total abundance or total volume of sample

Appendix 2: Evaluation of FlowCAM for phytoplankton and zooplankton sampling

2a. Phytoplankton

Using digital flow cytometry to quantify *Microcystis* in San Francisco Estuary

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Summary
Abundance estimates for *Microcystis* spp. are difficult to obtain using traditional microscopic techniques because single cells aggregate into large colonies surrounded by a mucilaginous sheath that forms irregular folded masses, making counts difficult. Chemical and physical techniques and combinations of the two have been used to disaggregate the colonies with mixed results. This study evaluated the use of a digital imaging flow cytometer, the FlowCAM, to estimate *Microcystis* abundance in natural colonies preserved in Lugol's solution. Due to the flow environment and narrow shape of the flow cell chamber, the colonies unfolded and laid out flat, making it easy to quantify the diameter of each *Microcystis* colony and to see individual cells. Dense samples were diluted and fractionated into two magnification and flow cell combinations and three colony diameter ranges for analysis to avoid overloading the digital imaging system, simplify counting and reduce interference from suspended matter.; 4X and 300 µm cell for 15-36 µm diameter, 4X and 300 µm cell for 36-300 µm diameter, and 2X and 600 µm cell for greater than 300 µm diameter. These size ranges allowed measurement of over 98% of the *Microcystis* in the sample. Abundance estimates for each size range were combined to give the total abundance in the sample. Counts were variable for these natural samples due to the variable nature of the colonies and varied by 57% between samples. However, even this relatively high variation replication was still within the 95% confidence interval. Biomass estimates from the FlowCAM were accurate and linearly correlated with chlorophyll *a* concentration (r = 0.81). Abundance estimates with the FlowCAM were also significantly correlated with estimates made by microscope counts (r = 0.88). Digital imaging of the samples was uniform. Abundance estimates at

each minute of sampling were not significantly different for each size range and total abundance increased linearly with sampling time. Overall the FlowCAM was a rapid, comparatively accurate and easy to use technique for quantifying *Microcystis* colonies without time consuming and destructive sample processing.

Introduction

The cyanobacterium *Microcystis* is the most common cyanobacteria harmful algal bloom (CHAB) in freshwater lakes and reservoirs that are used for drinking water. *Microcystis* is considered a toxic CHAB because some species contain powerful hepatotoxins called microcystins that initiate cancer and promote tumor formation in the liver of humans and wildlife (Zegura et al., 2003; International Agency for Research on Cancer, 2006; Ibelings & Havens, 2008). Current guidelines established by the World Health Organization recommend cell abundance less 20,000 cells L^{-1} for recreational use (World Health Organization 1998) and State of California guidelines for the Klamath River recommend posting warning signs at cell abundance of 40,000 cells L^{-1} or greater (State Water Resources Control Board et al. 2010). Because regulations for use are often based on cell abundance, rapid and accurate quantification is necessary. However, accurately quantifying *Microcystis* abundance can be difficult. Individual cells are grouped into colonies surrounded by a mucilaginous sheath. Cells within this sheath aggregate and fold into amorphous masses, making individual cells difficult to see and lead to large variations in abundance estimates when conventional microscopic techniques are used (Reynolds and Jaworski 1978). The problem is compounded by the fact that colonies can be very large and contain millions of cells.

Considerable effort has been expended to develop techniques which disaggregate the colonies into individual cells for ease of microscope counting. Methods include heating, grinding, shaking, sonication, alkaline hydrolysis and combinations of these (Box 1981). All of these techniques have been determined to be good and bad at separation of the colonies into individual cells. For example, grinding was determined to be better than heating with vortexing by Bernard et al. (2004). Jounge et al. (2006) found heating to be

better than vortexing, sonication and titanium oxide treatment. Utilizing the right treatment is critical because these methods can cause cell loss. The percent recovery from sonication, heating, freeze thawing and stirring ranged from 40% to 93% (Garcia et al. 2010). Importantly, the effectiveness of the treatments vary for each sample and depend on the *Microcystis* strain in the sample, the environmental conditions to which the cyanobacterium was exposed and the preservation technique (Box 1981).

The use of the FlowCM digital imaging flow cytometer to identify and enumerate plankton was first described by Sieracki et al. (1998). This technique obtains a digital image of the plankton cells in a fluid stream as they pass by a microscope objective. The collected images are sorted, counted, sized and summed to give a final abundance or biomass based on cell dimensions. This technique was determined to be equally accurate, easier to use and more rapid for enumeration of natural plankton cells compared with microscopic techniques (Buskey 2006; Campbell et al. 2005). Use of this technique to quantify *Microcystis* colonies within blooms has not been formally evaluated.

Quantifying Microcystis blooms became important for SFE in 1999 when *Microcystis* blooms first appeared in the freshwater and brackish water regions of the estuary (Lehman et al. 2005). These blooms consist of wide diameter colonies reaching 25,000 um in diameter or greater and are a previously unidentified strain (Moisander et al. 2009). Cell abundance has reached levels that exceed WHO guidelines for recreation and drinking water and has required regulation (Lehman pers. com.). Rapid analysis of *Microcystis* cell abundance is therefore needed to establish of health guidelines and facilitate estuarine fishery management during the summer months when the bloom occurs. The purpose of this study was to evaluate the speed, ease and accuracy of using of digital imaging flow cytometry to quantify natural samples of *Microcystis* colonies in blooms within San Francisco Estuary (SFE).

Methods

Microcystis tissue samples were collected from various stations throughout the freshwater and brackish regions of the San Francisco Estuary using a surface tow of a 0.5 m diameter net with 75 µm mesh. Samples were immediately preserved with Lugol's

iodine solution; 1 ml Lugol's solution per 50 ml sample. *Microcystis* colonies in these concentrated samples were partitioned into three size classes for analysis based on the diameter of the colonies, 15 to 35 µm, 36 to 300 µum and 300 to 2000 µm (Fig. 1). These size fractions were obtained by filtering the samples through a 300 µm and then a 35 µum sieve for the 15 to 35 µm size fractions and a 300 µm sieve for the 36 to 300 µm size fraction. The greater than 300 µm size fraction was not filtered. Partitioning the sample into these size fractions reduced the number of colonies that passed by the sampling window, prevented overlap of large and small colonies and reduced the impact of the high suspended sediment in the samples. Colonies within each sample were subsampled by placing the entire sample in a 100 ml beaker, diluting the sample with deionized distilled water so that only about 200 cells ml^{-1} passed by the sample window at one time, mixing the sample with a figure eight stirring motion and withdrawing 1 m aliquots of sample with a Hensen-Stemple volumetric pipette until sufficient volume was obtained for the sample run. The Hensen-Stemple pipette was used so that there would be no bias created by sampling these large colonies by a narrow pipette tip. Samples were run using a flowCAM digital imaging flow cytometer (Sieracki et al. 1998) at 4X magnification using a 300 µm flow cell for the 300 µm or less size fractions and at 2X magnification using a 600 µm flow cell for the greater than 300 µm size fraction (Table 1).

Table 1. Flow cell size, sieve pore size and dilution used for *Microcystis* samples in San Francisco Estuary based on the diameter of the colony.

Flow cell size	**50µm**	**300µm**	**300µm**	**600µm**
Dilution (suggested)	1:4	1:40	1:10	1:40
Filter (µm pore size)	300 & 35	300 & 35	300	none
Colony diameter, µm	15-35	15-35	36-300	>300
Magnification	20X	4X	4X	2X

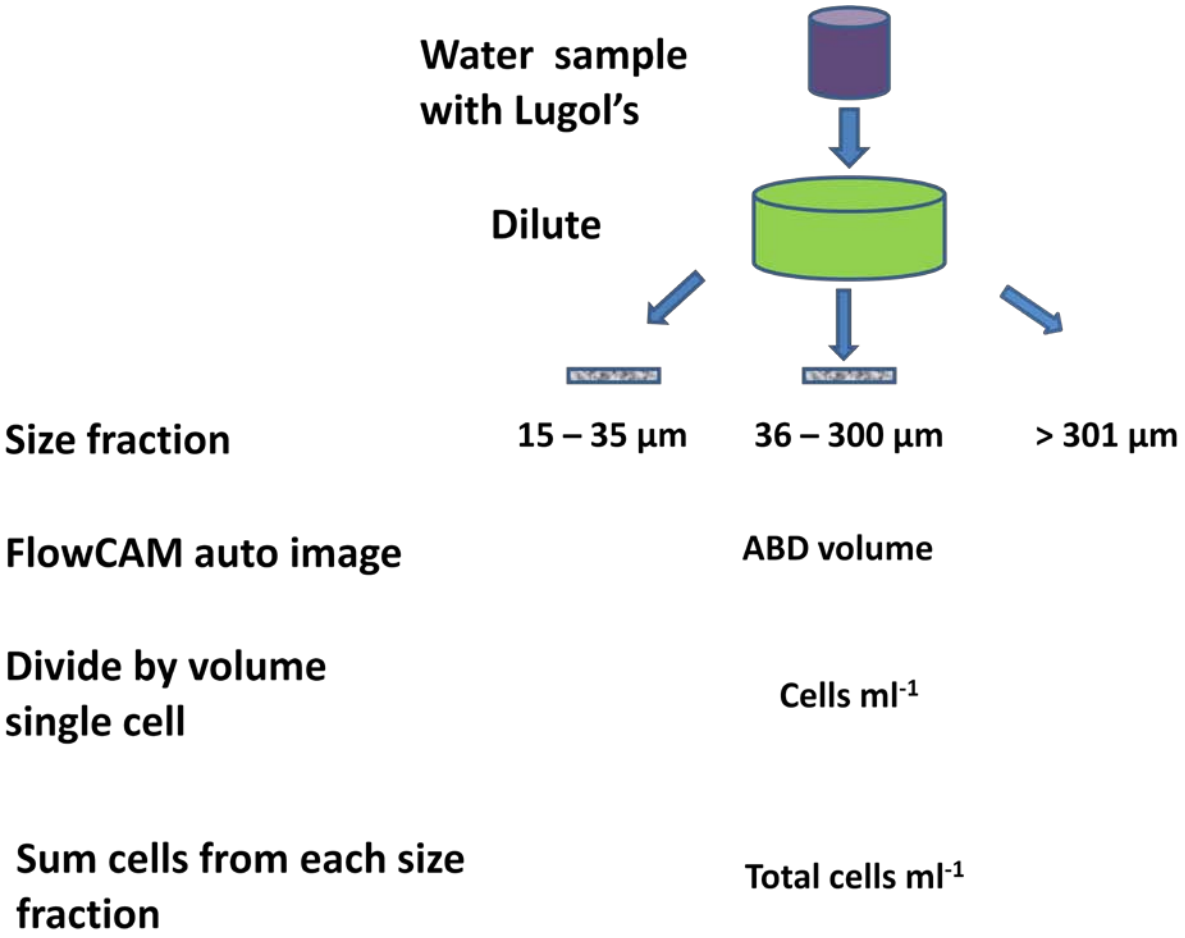

Fig. 1. Schematic of the *Microcystis* sample processing procedure for abundance estimates using the FlowCAM.

Results

Sample processing

The cell diameter of *Microcystis* colonies was easily measured in digital images (Fig. 2). As the *Microcystis* colony moved through the flow cell, it spread out into a single sheet that could easily be measured at all magnifications. Single cells were grouped into colonies that had an appearance similar to a bunch of grapes with loosely and non-linearly aggregated spheres. At high magnification, solitary cells were more difficult to identify because their appearance as a sphere could be confused with other small

spheres. However, the *Microcystis* single cells were characterized by a white plus sign at the center of the cell that aided with identification.

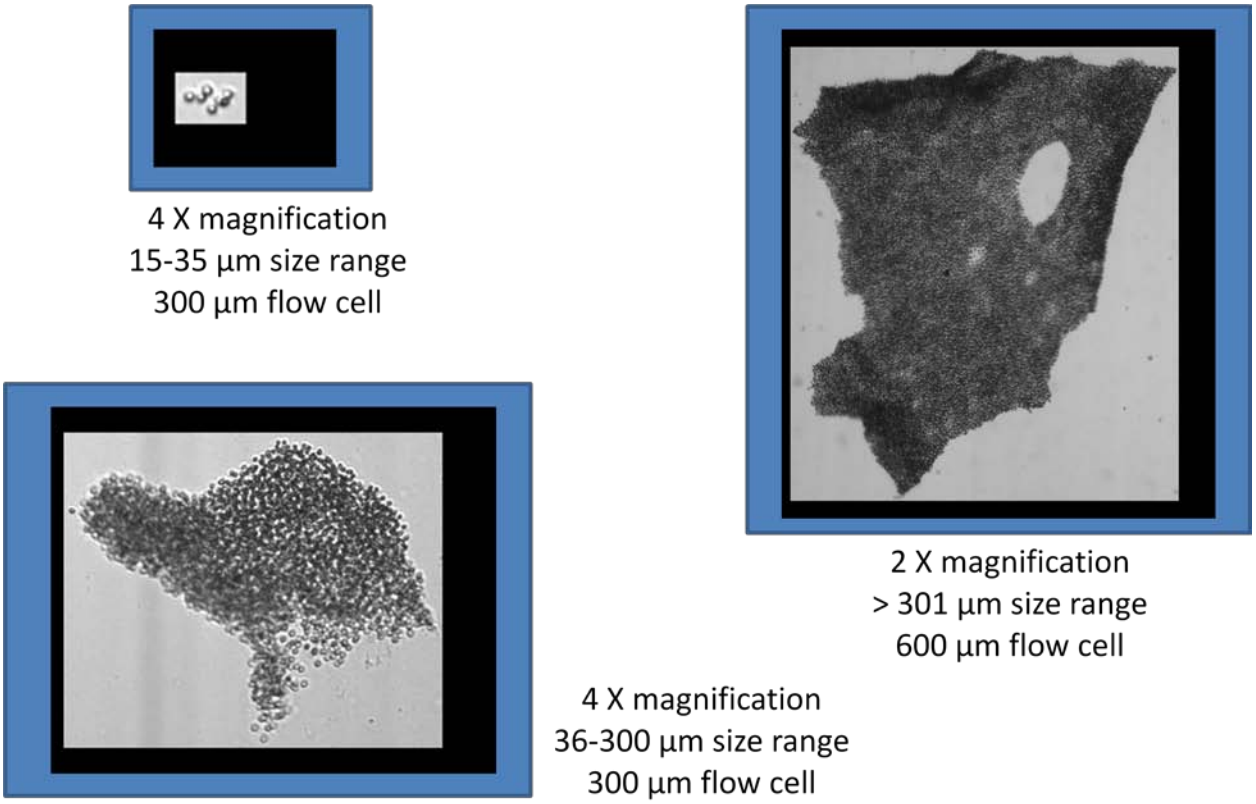

Fig. 2. Digital images of *Microcystis* colonies in the diameter range of 5 to > 300 µm diameter obtained using the FlowCAM set at different magnification and flow cell combinations.

Sample replication

Abundance estimates for replicate *Microcystis* samples collected at random from 10 stations in 2007 and 2008 between June and October varied by an average of 57%. This error is within the 95% confidence interval for these values (Fig. 3).

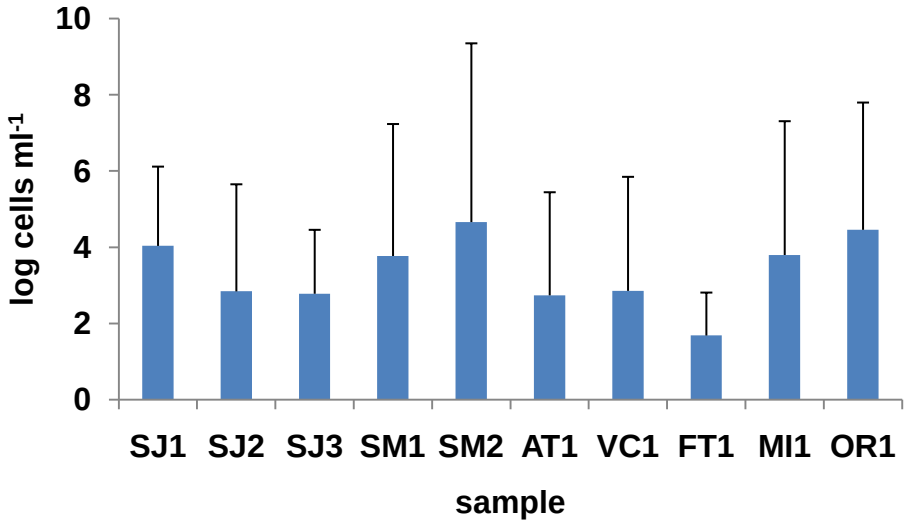

Fig. 3. Mean and standard deviation of *Microcystis* abundance in replicate plankton samples collected in the San Francisco Estuary between June and October for 2007 and 2008.

Accuracy

Microcystis single cell abundance and chlorophyll *a* concentration in concentrated net samples were significantly correlated ($r = 0.96$; $p < 0.01$) for a wide range of abundance samples up to 2 million cells ml^{-1} (Fig. 4). Separation of high and low abundance values suggests this correlation was only slightly lower for values less than 500,000 cells ml^{-1} ($r = 0.81$, $p < 0.01$).

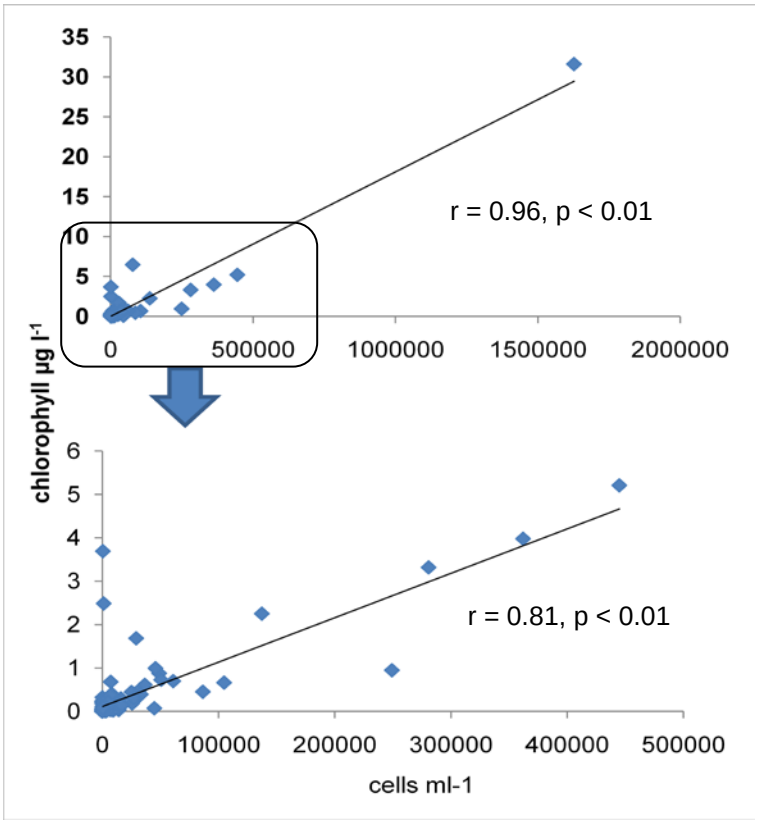

Fig. 4. Correlation between average chlorophyll *a* concentration and *Microcystis* abundance in randomly selected field samples from San Francisco Estuary.

The average of replicate *Microcystis* abundance estimates obtained using the FlowCAM was also linearly correlated ($r = 0.88$, $p < 0.01$) with those obtained using the traditional inverted microscope technique, but was slightly lower for this study (Fig. 5). The linear equation describing this association was as follows:

$$\textit{Microcystis}\ \text{cells ml}^{-1} = 0.74\ (\text{FlowCAM cells ml}^{-1}) + 8664$$

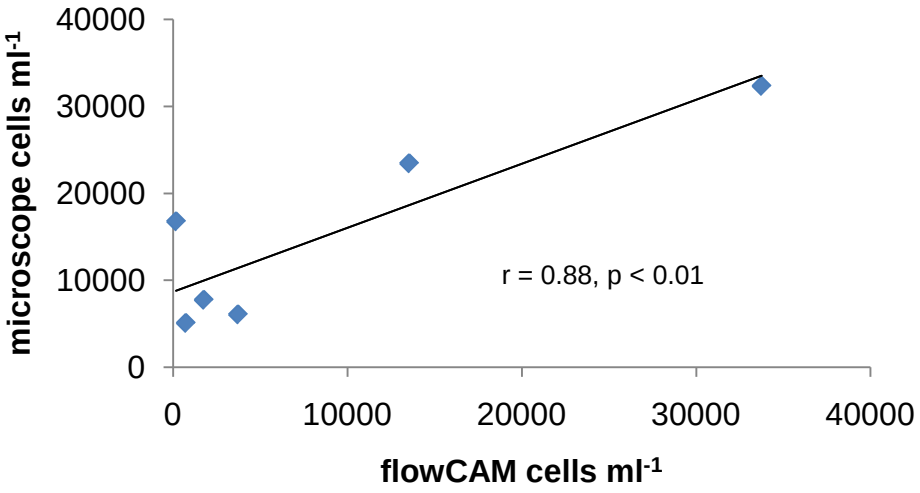

Fig. 5. Correlation between *Microcystis* abundance estimated by FlowCAM and the inverted microscope technique in preserved samples.

Sample processing time

Accurate estimates of cell abundance can be obtained at short sampling processing times with the FlowCAM. Cell abundance estimates were made for each minute over a twenty minute period at each magnification and flow cell combination. There was no significant difference in the abundance estimates computed for each minute at all magnifications and the cumulative sum of the estimates over the 20 minute period was linear (Fig. 6).

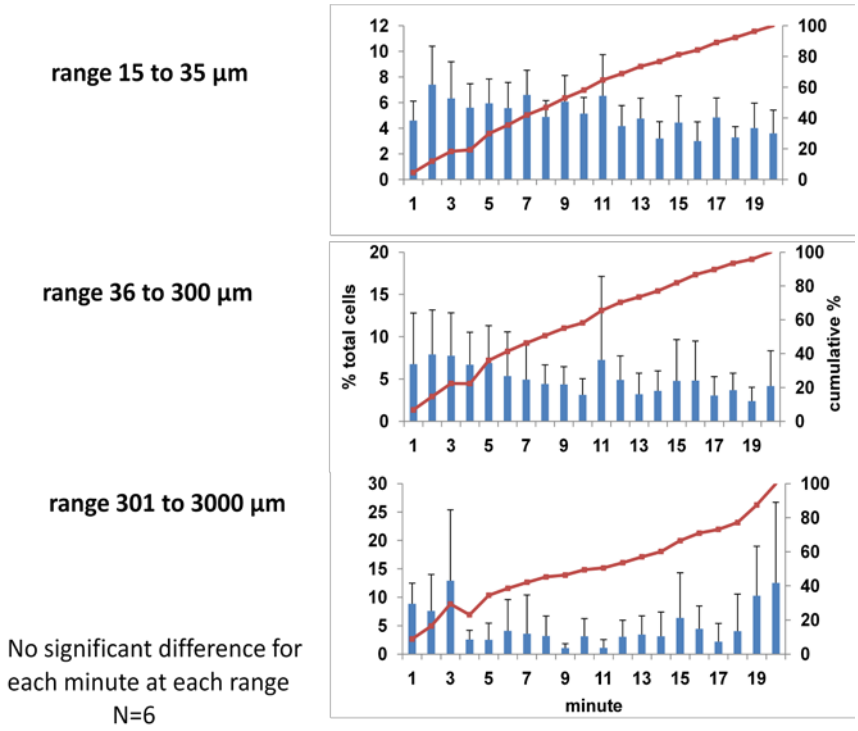

Fig. 6. *Microcystis* cell abundance estimates computed for each minute of sampling at each size fraction.

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2b. Zooplankton

Analysis of zooplankton biomass with FlowCAM

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Goal: The goal of this study was to determine if the flowCAM could be used to quantify zooplankton species composition and biomass.

Summary

The FlowCAM was a rapid and easy way to quantify the biomass of preserved zooplankton from net tow samples. Initial evaluations suggested zooplankton were equally well quantified with the FlowCAM and dissecting microscope techniques. The zooplankton images generated with the FlowCAM allowed identification to taxa (e.g., copepod). Further identification to species depends on the characteristics of the animal. Obvious and large characteristics that can be seen from all angles in the images will easily allow identification to species. Current test were restricted to preserved samples because live sampling is hampered by the movement of zooplankton in the fluid stream. Future work with imobilizing the zooplankton before machine processing is needed to solve this problem.

Sample Protocol

General Comment

Trigger mode was used for processing preserved zooplankton samples. By using trigger mode, the zooplankton trigger the laser to take a picture as they pass by the microscope objective, making it more likely to generate a full image of the animal. In auto image mode there is no way to control the orientation of the particle which leads to a greater number of partial images. Partial images are hard to classify.

Prepare the Sample

1. Measure the zooplankton sample volume from the net tow with a graduated cylinder and then place into a large 2000 ml beaker.
2. Using a Hensen-Stemple volumetric pipette, withdraw 2 ml from a thoroughly mixed sample and dilute up to 100 ml. The best mixing technique is to use a figure eight motion.

Prepare the machine

1. Turn on the Machine by flipping both the Power and Laser switches.
2. Attach the 2x objective.
3. Lay the 600 µm flow cell into the 600 µm flow cell holder; gently screw the top onto the flow cell holder until barely tight.
4. Attach The funnel through the funnel stand to the end of the flow cell
5. Insert the flow cell holder onto the sliding base so that the flow cell is facing the objective.
6. Attach the diffuser onto the light source behind the flow cell.
7. Attach the other end of the flow cell to the 2x pump tubing and wrap the pump tubing into place.
8. Attach tubing to the other end of the pump tubing ; put the tubing through the hole on the side of the machine to a waste beaker.
9. Add distilled water and turn the pump to Forward / Prime until there is no air in the flow cell. Squeeze the tubing below the flow cell and twist the tubing above the flow cell to liberate air bubbles from adductive bondage.
10. Open the Visual Spreadsheet 2x software from the desktop
11. From the main view window under: File select: Load Context…
12. Load: Desktop>Context Files>2x zoo.ctx
13. Under: Setup select: Setup and Focus… this opens the view window.
14. Using the rough focus, move the sliding base until one plane of the flow cell is in focus.
15. Secure the sliding base and record the micrometer reading; using the fine focus adjust until the opposite plane of the cell is in focus. Record the micrometer.
16. Adjust the focus so that it is at the median of the 2 readings or slightly closer to the objective.

Run the sample

1. From the main view window under: Setup select: context…
2. Complete Notes for each run
   Date:
   Sample Name:
   Objective: 2x
   Cell: 600 um
   Dilution: 2:100
   Range : 100-20000
   Special Notes:
3. Calibration:
   Select: Manual Volume Calculation (trigger mode)

Enter Fluid Volume (ml); Flow Cell Width (um); Volume Adjustment Factor
Record any other changes you may have made in the notes section and close the window.

4. From the Main window layout under: Imaging select: Trigger Mode…
   Check your notes and the stop conditions then select: Okay.
   Select the desired save location and turn on the pump to Forward – Slow – 8.
   1. The speed can be varied and should be adjusted to maximize the efficiency of the count and minimize repeat images
5. When the level of water in the funnel reaches the base of the funnel, click save to start the run;
6. Fill the funnel with sample and monitor the process. Continually mix the sample inside the funnel by using an air pump or pipette.
7. Intermittently gently flick/twist the tubing between the funnel and the flow cell to prevent particles clogging and sticking inside the cell.
8. Continue to add sample to the funnel until run time is completed
9. Stop the run: When the sample reaches the base of the funnel, add 2 ml of distilled water and stop the run when the water again reaches the base of the funnel.
10. Rinse the system with distilled water before starting another run.

Table 1. Machine setting for processing zooplankton samples at the 2x objective.

			Calibration	
Capture			Calibration factor (um/pixel)	3.2623
Distance to Nearest Neighbor (um)		75	Calibration Default	3.2771
Closed hole iterations		0	Manual Volume Calculation (trigger mode)	
Convolution Filter		None	Fluid Volume (ml)	100
Collage Image Border Padding(pixels)		2	Flow Cell Width (um)	600
Save collage Images		Check	Volume Adustment Factor	0.02
Save Binary Collage images		Check	Camera	
Save raw Camera Images		No check	AutoImage Rate	7
Segmentation Threshold	Dark	25	Flash Duration	6
	Light	15	Flash Delay	0.1
Both Light and Dark Pixels		Check	Gain	780
Acceptable Region	Left	0	Shutter	45
	Right	1022	Advanced Set-up	

	Top	0
	Bottom	766
Filter		
Use ESD	Min	100
	Max	20000
Stop		
User Terminated		Check
Recalibration Interval (min)		30
Fluorescense/Scatter		
Channel 1 Threshold		400
Channel 2 threshold		400
Channel 3 Threshold		400
Enable Laser		Check

Enable Logging	check
Number of Cameras	1
Buffer Size	3072
Image Acquisition Delay	
Camera Acquisition Delay (ms)	20
File Procession Acquisition Delay (ms)	100
AutoImage Camera Timeout (sec)	3
DSP Camera Delay Timeout	250
Max particles per Display Collage	4096
Max Particles to Graph During Capture	100000
Max Particles per List	100000

Sample analysis

After a run is completed, use the Visual Spreadsheet program to manually or automatically examine the raw image files for zooplankton. Save the cleaned image file and export the final information into an excel spreadsheet.

The FlowCAM cannot image the full sample because the camera has a narrow field of view compared with the entire flow cell. The raw number obtained from the counts must be corrected for accuracy and comparability with the microscope method. There are two ways to do this. Option 1: adjust the internal fluid volume imaged using the equations below before the sample run.

For Trigger mode:

Fluid Volume Imaged =

((1024 pixels*Calibration Factor)/Flow Cell Width)*Fluid Volume

For Autoimage mode:

Fluid Volume Imaged =

(1024 pixels * Calibration Factor) * (768 pixels * Calibration Factor) * Flow Cell Depth* Raw Image total volume

- A conversion factor for cubic microns to milliliters is then applied.

Option 2: adjust the raw animal volume or count values by determining the percentage of the flow cell viewed by the camera and adjust the values; up to one hundred percent.

Adjusted animal count =

Raw animal count* (1+ (1-(Field of view * Calibration factor)/(width of flow cell)(Calibration factor))

Performance evaluation

Dilution effects

The effect of sample dilution on abundance estimates was examined for 5:100, 1:50, 2:100, 1:100 and 1:200 dilutions. The results were similar, with slightly lower values for the 5:100, 1:100 and 1:200 dilutions. The dissecting microscope results were nearly identical for the FlowCAM results at the 1:50 and 2:100 dilutions (Figure 1).

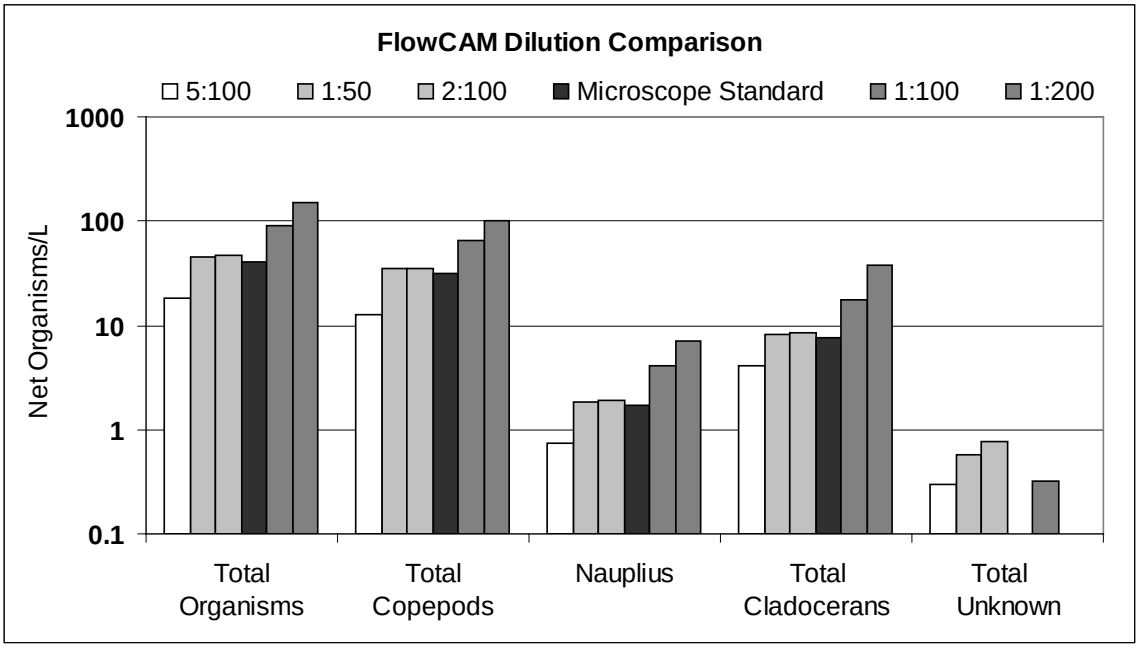
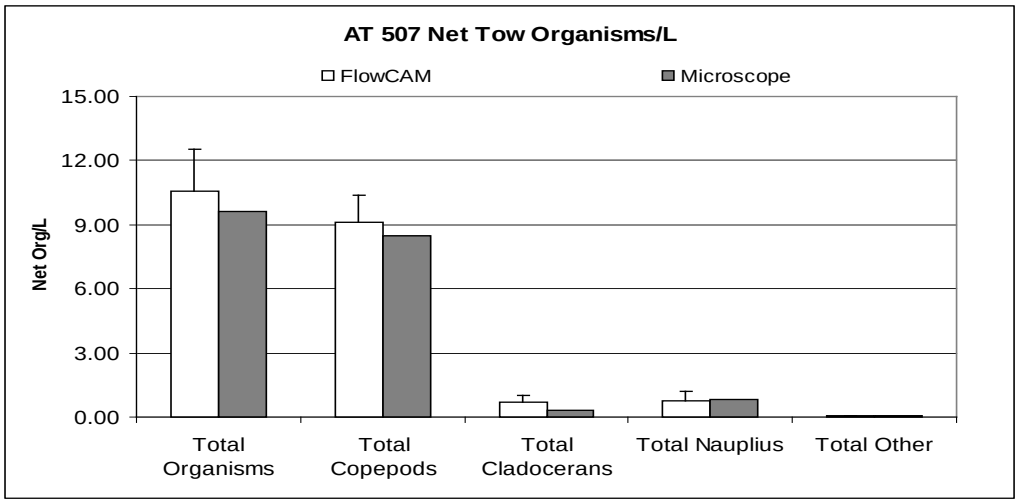


Figure 1. Comparison of FlowCAM and dissecting microscope abundance estimates for different dilutions of zooplankton net tows.

FlowCAM and dissecting microscope comparison

Samples from three different stations were used for comparisons of the FlowCAM and dissecting microscope values. Each sample was replicated at least once. The average and the percent difference between the microscope and FlowCAM animal l^{-1} abundance estimates suggested values were within 30% except for cladocerans at Antioch (Figure 2 and Table 1), which were not abundant.



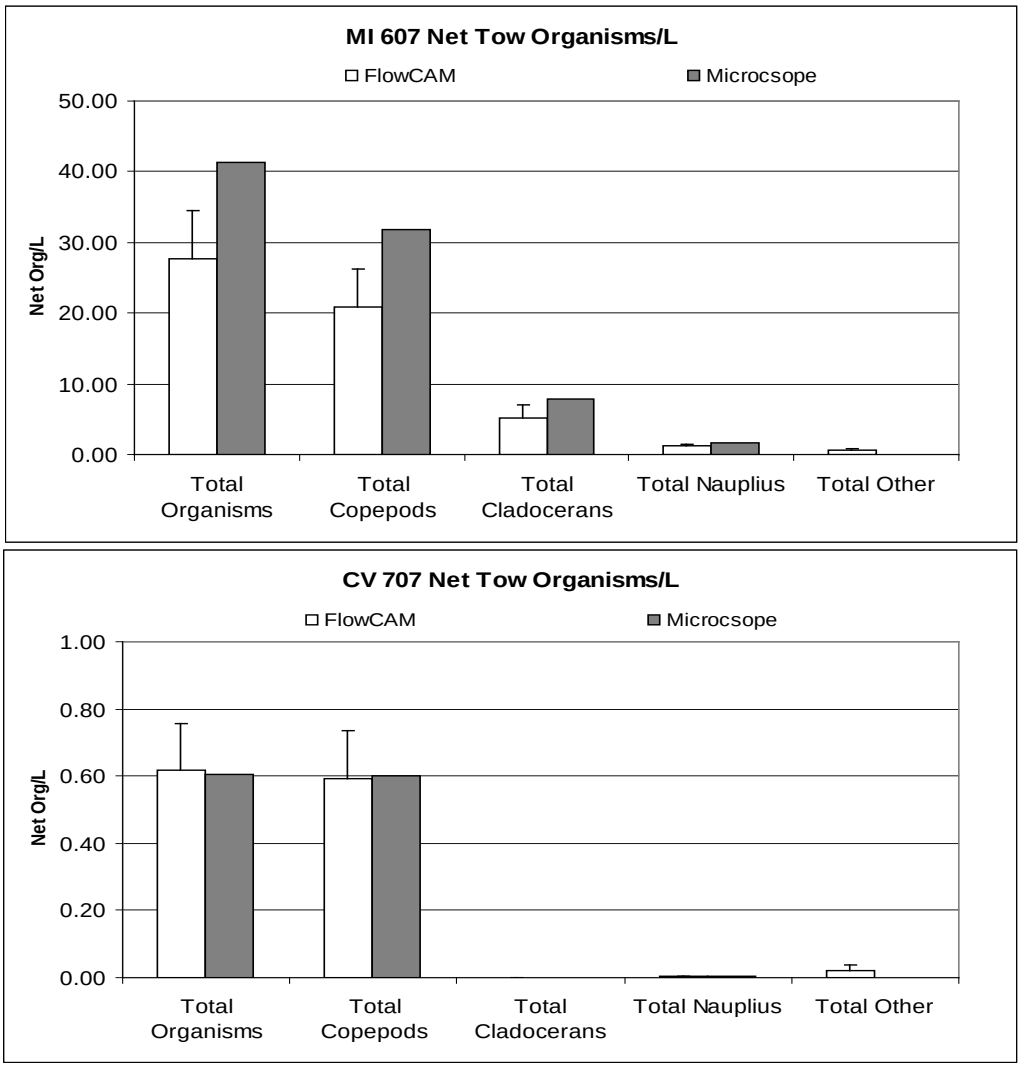


Figure 2. Comparison of zooplankton abundance in net tow samples determined by the FlowCAM and the dissecting microscope at three stations, Antioch (AT), Mildred Island (MI) and Collinsville (CV).

Table 1. The percent difference between the dissecting microscope and FlowCAM estimates of zooplankton abundance as animals l^{-1} at three stations.

Abundance l^{-1}	**MI**	**CV**	**AT**

	June 07	July 07	July 07
Total Animals	33.22	2.10	9.88
Total Copepods	34.65	1.03	7.54
Total Cladocerans	33.28	0.00	133.75
Total Nauplii	33.29	0.78	7.96

Precision

The precision of FlowCAM abundance estimates was evaluated by comparison of three replicates of a sample collected at Antioch in May 2007 (Table 2). Abundance was similar for Copepods, the most abundant group and increased with animals that were rare.

Table 2. Percent difference from the average of abundance estimates for three replicates of a zooplankton sample collected at Antioch (AT) in May 2007.

	AT 1	AT 2	AT 3
Total Organisms	12	9	21
Total Copepods	10	5	15
Total Cladocerans	25	25	50
Total Nauplius	28	43	71

Processing time

Processing samples with the FlowCAM and the dissecting microscope took about the same time. Zooplankton counts were done with a dissecting microscope using a sub-sample of the zooplankton net tow. About 1-2 ml of the zooplankton net tow sample was diluted to a known volume and placed in a zooplankton wheel for counting under a dissecting microscope. One sample, usually took 45 min to 2 hours to process depending on the abundance of organisms and detritus.

Processing zooplankton samples for the FlowCAM also involved taking a sub-sample of the zooplankton net tow and then diluting it to a known volume. Running the sample through the FlowCAM took about 20 to 30 min. The images were classified manually and took anywhere from a few minutes to 30 min depending on the density in the sample. This process can be shortened with the use of library images.

Benefits of zooplankton sample analysis with the FlowCAM

Use of the FlowCAM to process the zooplankton samples was much easier than the dissecting microscope because it produces less stress to the eyes. It also does not require an expert in taxonomy to process the sample and make the initial identifications and biomass estimates. The data are easy to process because they are automatically exported into an excel file. A key benefit is that the image file is permanent and can be reinterpreted later if necessary.

Limitations zooplankton sample analysis with the FlowCAM

The imaging system can lead to error in the estimates. The camera view does not encompass the entire width of the flow cell, so it is necessary to adjust the count to reflect the total volume of the cell, even though this may not perfectly reflect the animals in the sample. The focal plane is in the center of the cell. Any animals that pass more towards the front or the back will be out of focus and possibly unidentifiable. The imaging system can split an animal image or only capture part of the organism, which makes it difficult to identify. In addition, it is not clear at this time how accurate species identifications will be. Biomass estimates are not a problem.

Appendix 3: Using the FlowCAM to characterize spatial and temporal variability

Patchiness in phytoplankton community composition and water quality in San Francisco Estuary

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Summary

Quantifying food resources at the base of the food web is both important and difficult in estuaries where food is often limited and the geographical area is large and structurally complex.The purpose of this research was to determine if food resources associated with phytoplankton community biomass is patchy and if food resource patches are associated with environmental conditions in San Francisco Estuary. Phytoplankton patchiness was determined from high frequency in situ measurements of phytoplankton species composition using FlowCAM digital imaging flow cytometry between April and October 2010. Discrete water samples for live phytoplankton identification and enumeration were collected and immediately analyzed every six minutes along longitudinal transects in the Sacramento and San Joaquin River, the two major rivers in the San Francisco Estuary. Water temperature, pH, specific conductance, dissolved oxygen concentration, chlorophyll *a* fluorescence and turbidity were measured every 10 seconds using YSI water quality sondes. The phytoplankton community was primarily composed of diatoms, cryptophytes and cyanobacteria which comprised 92% to 96% of the biomass. Percent phytoplankton biomass among groups was patchy along river transects where 14% of the biomass peaks were greater or equal to 1 standard deviation unit from the mean and only 5% of the peaks were greater than 2 standard deviation units from the mean. Water quality conditions were also variable along the transects with 18% to 33% of the measurements among variables were equal or greater than 1 standard deviation unit from the mean and only 3% to 4% of the values were

greater than 2 standard deviation units from the mean. Percent phytoplankton biomass and water quality conditions were correlated ($p < 0.01$) with most of the phytoplankton community associated with specific conductance and water temperature in the Sacramento River and turbidity in the San Joaquin River. Among phytoplankton groups, diatoms were associated with cool water temperature in both rivers while cyanobacteria in the Sacramento River and cryptophytes and dinoflagellates in the San Joaquin River were associated with warm water temperature. For both rivers, specific conductance and turbidity were negatively correlated with cyanobacteria, dinoflagellates or cryptophytes. The high frequency patterns of phytoplankton community composition along river transects suggest that high frequency spatial and temporal measurements are needed to gain an accurate estimate of phytoplankton food resources at the base of the food web.

Introduction

Phytoplankton patchiness is an important component of the production of aquatic ecosystems. It has long been thought that phytoplankton are not homogeneously distributed spatially in the water column and that each cell responds to high frequency spatial variation in its surrounding environment conditions (Martin 2003; Hutchinson 1975). Research confirmed the heterogeneous distribution of phytoplankton at small to large spatial scales from 10 to 100 m in marine environments of the Mid Atlantic Bight, Monterey Bay and San Luis Obispo Bay with sub-kilometer scale variability characterizing near shore regions (Blackwell et al. 2008). This heterogeneity can be influenced by variation in phytoplankton growth, species composition, cell diameter, zooplankton grazing and turbulence intensity that occur at high frequency spatial scales (Seruont et a. 1999; Zarauz et al. 2009).

The relative magnitude and frequency of phytoplankton patches is important to energy transfer in the aquatic food web. The magnitude and frequency of phytoplankton patches affect the total carbon in the food web as well as the survival and aggregation of zooplankton and the energy they transfer up the food web to support fish production .

Phytoplankton patches influence energy transfer by influencing zooplankton and fish behavior which affects their ability to find or utilize prey (Seruont 1991; 2001). This process is complex with zooplankton grazing having a greater effect on phytoplankton patchiness than some environmental factors such as small scale turbulence (Calbet 2001; Seruont al. 2001). Importantly, energy transfer through the food web is not necessarily related to the productivity of the aquatic ecosystem (San Martin et al. 2006). Oligotrophic regions with low biomass and high turnover rates can have a high transfer of organic carbon to upper trophic levels.

Large scale pPhytoplankton patchiness has frequently been studied in the ocean. High frequency fixed station sampling at the Mid Atlantic Bight, Monterey Bay and San Luis Obispo Bay indicate variability of cell abundance, turbidity and fluorescence occur at 10 to 100 or more meters (Blackwell et al. 2008). Variability of phytoplankton patch size also varys with distance from shore with patches of 50-200 m at less than 5 km from shore and patches of 5 km at over 20 km from shore (Bissett 2004). Phytoplankton patches of 1-500 km seen in satellite imagery in the ocean are often thought to be produced by lateral stirring and mixing (Martin 2003). Although biological processes such as growth, grazing and behavior as well as non-linear processes are thought to contribute. The high frequency patchiness of phytoplankton is poorly studied in estuaries and no information is available for San Francisco Estuary (SFE).

San Francisco Estuary is a structurally complex and geographically large estuary where only low frequency spatial and temporal sampling has been conducted over the last four decades. It is unknown if this sampling regime is sufficient to accurately quantify the food resources available at the base of the food web. Low frequency, fixed station sampling suggested phytoplankton communities were complex and have high spatial and temporal variability (Lehman 1995; 2000). Species composition can also shift from diatoms to flagellates within 5 km along the San Joaquin River (Lehman et al. 2007). Many of the changes in phytoplankton biomass and community compositions were correlated with environmental factors such as streamflow, water temperature and

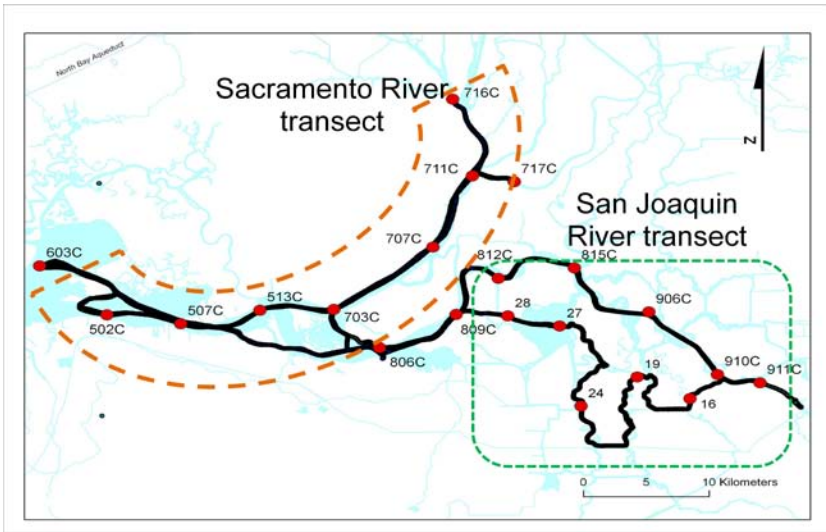
turbidity (Lehman 2004; 2007; Jassby 2005). No information is available on the high frequency spatial variation of phytoplankton biomass and community composition and how this may be influenced by high frequency changes in environmental conditions in the estuary.

The purpose of this study is to characterize the high frequency spatial variation of phytoplankton biomass and community composition along riverine transects in SFE and to determine if this variation is associated with coincident high frequency changes in physical and chemical water quality conditions. Such information is needed to develop management strategies for enhancement of food web production in SFE where the production of historically abundant and native species has declined since 2000 (Sommer et al. 2007).

Site description

Longitudinal transects of water quality and phytoplankton community composition were conducted monthly along a 128 km reach of the Sacramento River and 96 km reach of the San Joaquin River between April and October 2010 (Fig. 1). These reaches occur within the Delta and Suisun and San Pablo Bay portions of the San Francisco Estuary, one of the largest estuaries along the west coast of the United States. The Delta region is formed by the convergence of the Sacramento River on the north and the San Joaquin River on the south and leads seaward into three brackish and marine bays: Suisun Bay, San Pablo Bay and San Francisco Bay. Sacramento River streamflow is the larger of the two rivers that forms the Delta and had an average streamflow of 341 $\pm$ 96 m3 s^{-1} during this study. Average San Joaquin River streamflow was almost four times lower at 87 $\pm$ 50 m3 s^{-1}. The sources of these rivers also differ, with the Sacramento River draining a granitic and the San Joaquin River draining a sedimentary watershed.

Fig. 1.



Methods

Water quality measurements – Water quality conditions were measured at 10 second intervals along longitudinal transects using a YSI (Yellow Spring Harbor) 6600 water quality sonde. Water from 1 m depth was continually pumped by a through-hull pump system into the sonde located on board a boat that traveled at a speed of 15 knots. The sonde was fitted with probes for water temperature, turbidity (NTU), pH, specific conductance, chlorophyll *a* fluorescence and dissolved oxygen concentration. These probes were calibrated to sample standards before each sample run. Measurements were made at 10 second intervals and over the sampling season totaled 13,765 samples and 14,244 samples for the Sacramento and San Joaquin Rivers, respectively. Periodic discrete water samples were collected for analysis of chlorophyll *a* and total suspended solids concentration to calibrate the chlorophyll *a* fluorescence and NTU probe values. In addition, discrete water samples were collected for nutrient analysis from the through-hull pump system at 10 to 11 fixed stations along each river transect between June and October (Fig. 1). Water samples for nitrate, ammonium and soluble phosphate were filtered through HATF nucleopore filters and the filtrate was frozen until analysis (United States Environmental Protection Agency 1983; United States

Geological Survey 1985). Water samples for chlorophyll *a* and phaeophytin pigment concentration were filtered through glass fiber GF/F filters, fixed with 1 ml of magnesium carbonate and the filters were frozen until spectrophotometric analysis (American Public Health Association, American Water Works Association & Water Environment Association, 1998). Unfiltered water samples for total suspended solids analysis were kept at 4°C until analysis using standard methods (American Public Health Association, American Water Works Association & Water Environment Association, 1998).

Phytoplankton community composition – Phytoplankton community composition was quantified at approximately 10 minute intervals along transects from discrete water samples collected at 1 m depth from the through-hull pump system. Water samples were immediately run through a FlowCAM digital imaging flow cytometer (Sieracki et al. 1998) using a fluorescence trigger for 6 min at 10X magnification with a 100 µm flow cell and a speed of Slow 2. This setting allowed imaging of live phytoplankton cells with cell diameter greater than 15 µm, the best detection range of the flow cytometer. Travel and processing times enabled acquisition of approximately 30 samples during each transect for a total of 104 and 133 samples for the Sacramento and San Joaquin Rivers, respectively or an average of one sample every 3 km. Live processing of the samples using a fluorescent trigger was a great advantage of this flow cytometry technique for this estuary where high levels of inorganic and organic suspended sediment interfere with traditional microscope counting techniques.

Analysis

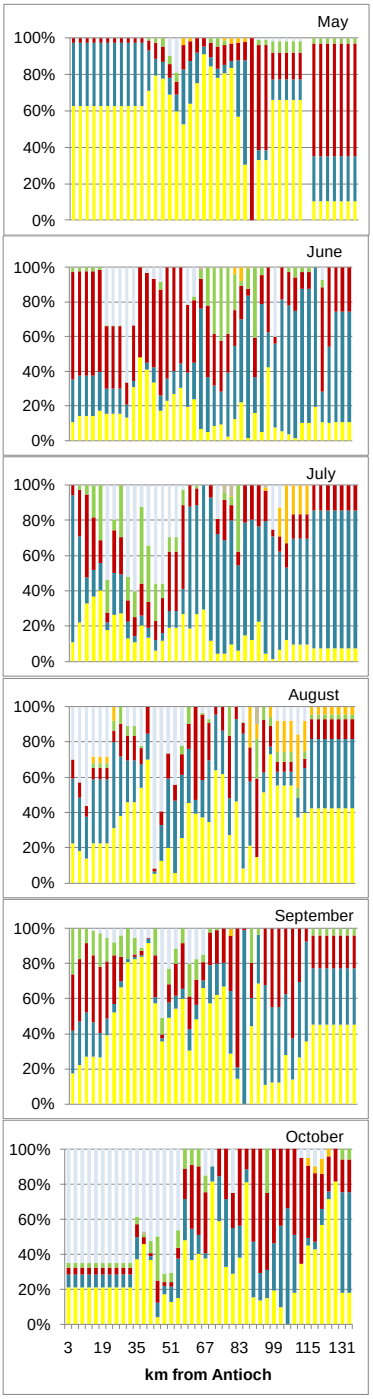
Statistical analyses were conducted using nonparametric statistical programs associated with PRIMERe version 6 (Clarke and Gorley 2006) and Statistical Analysis Software (SAS Institute, Inc., 2004). Phytoplankton cell carbon was computed from ABD volumes generated by the FlowCAM and using equations to compute cell carbon from geometric shapes developed by (Menden-Deuer and Lessard. 2000). The ABD volume was automatically computed from the area of each cell and an assumption of a spherical volume (Sieracki et al. 1998).

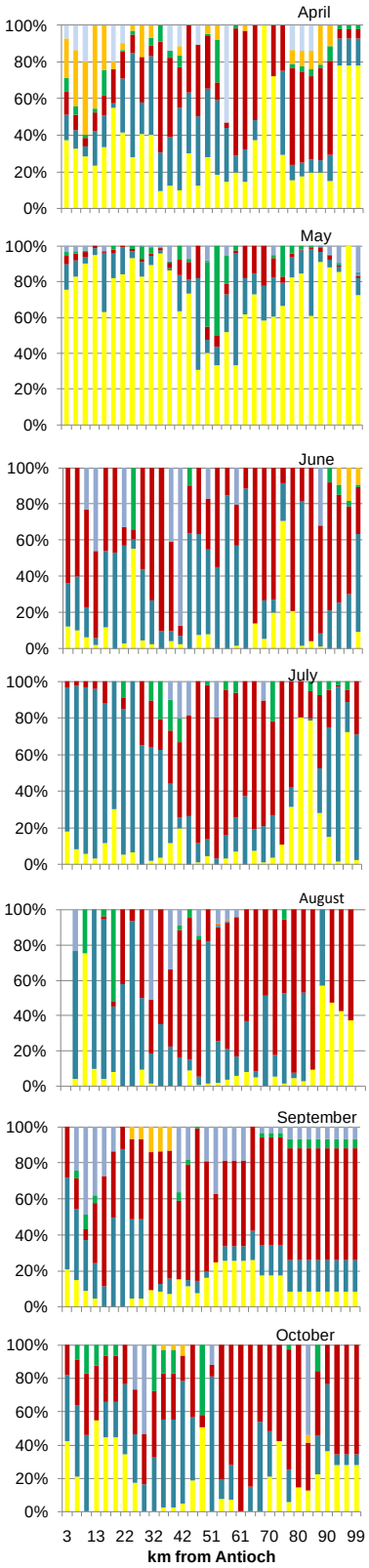
Results

Phytoplankton composition

The phytoplankton community group composition varied at 3 km intervals along the rivers. Most of the phytoplankton biomass was composed of diatoms, cryptophytes and cyanobacteria which comprised 92% to 96% of the biomass in the Sacramento and San Joaquin Rivers, respectively (Fig. 2). The distribution of biomass among groups differed ($p < 0.01$, ANOSIM) between the rivers with diatoms contributing slightly more of the total biomass in the Sacramento River (37% for diatoms, 31% for cyanobacteria and 25% for cryptophytes) and cryptophytes contributing more of the biomass in the San Joaquin River (44% for diatoms, 29% for cyanobacteria and 23% for diatoms).

Fig. 2. Average percent phytoplankton community composition measured at 3 km intervals in reaches of the Sacramento (a) and San Joaquin (b) rivers between April and October 2010.

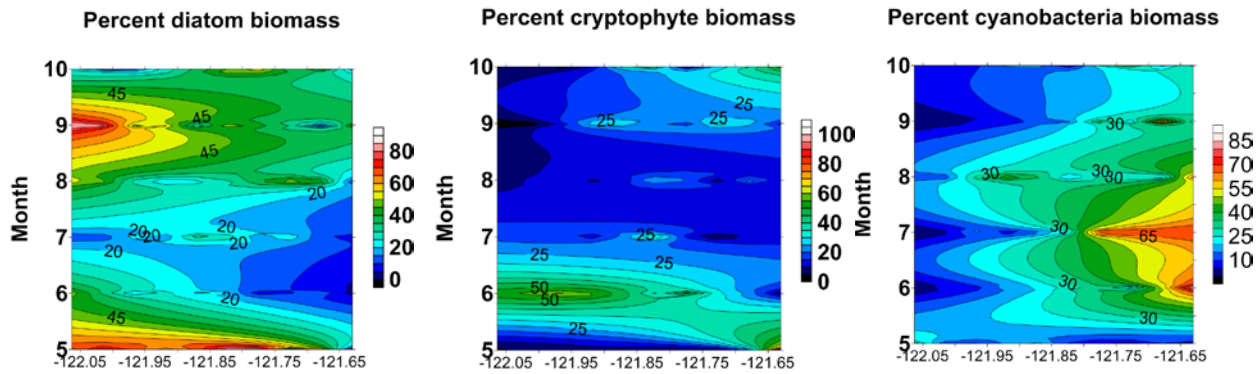

May
June
July
August
September
October
100%
80%
60%
40%
20%
0%
3 19 35 51 67 83 99 115 131
km from Antioch


April
May
June
July
August
September
October
100%
80%
60%
40%
20%
0%
3 13 22 32 42 51 61 70 80 90 99
km from Antioch

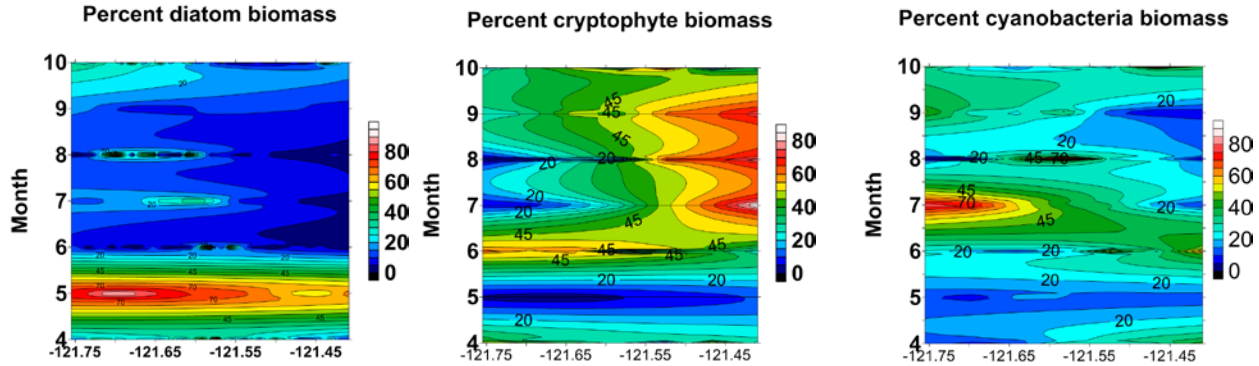
Phytoplankton community composition also varied by month. April and May differed ($p < 0.01$, ANOSIM) from the other months in the San Joaquin River (Fig. 3). May, June and July differed ($p < 0.05$, ANOSIM) from other months in the Sacramento River. Differences among months were largely due to the presence of a large diatom bloom in May and June.

Fig. 3. High frequency measurements of percent phytoplankton community composition along transects of the Sacramento (a) and San Joaquin (b) rivers between April and October 2010 demonstrate a patchy pattern in space and time.

a)



b)



Phytoplankton patchiness

Phytoplankton patches along the river transects ocurred within a background variation of less than 1 standard deviation from the mean. On average, 86% of the biomass peaks in samples among phytoplankton groups were less than 1 standard deviation from the mean for both rivers (Table 1). Biomass peaks for all phytoplankton groups reached 1, 2 and greater than 2 standard deviation units from the mean an average of only 10%, 3% and 1%, respectively of the time. Among phytoplankton groups, biomass peaks were greatest for less represented taxa such as chrysophytes, green algae and dinoflagellates.

Table 1.

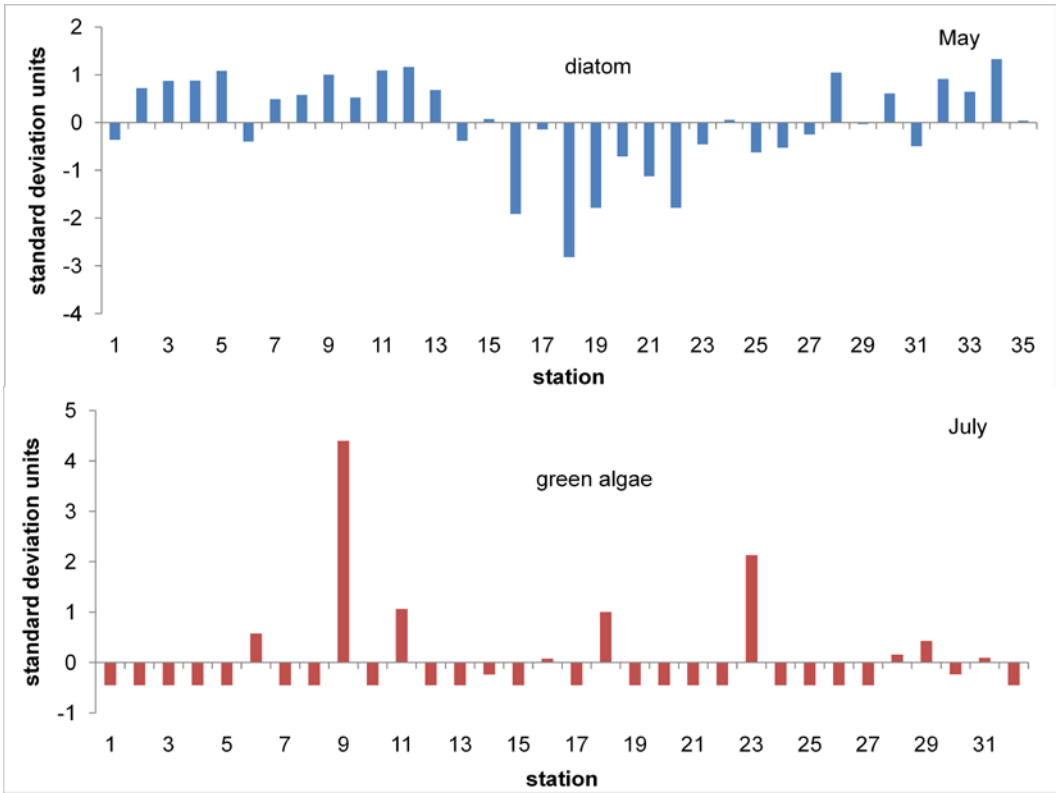
Average percent standard deviation units from the mean of phytoplankton group biomass along transects in the Sacramento and San Joaquin Rivers between April and October 2010.

			Sacramento River					
std dev units	diatom	cryptophyte	cyanobacteria	green algae	chrysophyte	dinoflagellate	ciliate	mean
<1	81.9	87.2	85.8	86.3	93.1	96.9	86.5	88.2
1	15.7	8.0	9.9	5.4	2.1	0.6	8.1	7.1
2	2.4	3.8	2.6	5.9	1.7	0.0	2.6	2.7
3	0.0	1.7	1.7	2.4	0.6	0.0	2.3	1.2
4	0.0	0.0	0.0	0.0	1.4	1.9	0.5	0.5
5	0.0	0.0	0.0	0.0	1.1	0.5	0.0	0.2
			San Joaquin River					
std dev units	diatom	cryptophyte	cyanobacteria	green algae	chrysophyte	dinoflagellate	ciliate	mean
<1	84.5	81.2	82.0	87.1	91.9	99.2	83.5	87.1
1	10.5	17.6	12.5	6.9	0.0	0.4	12.3	8.6
2	3.7	1.2	5.1	3.2	3.2	0.0	1.9	2.6
3	1.3	0.0	0.4	1.1	0.6	0.0	1.7	0.7
4	0.0	0.0	0.0	0.9	0.4	0.0	0.9	0.3
5	0.0	0.0	0.0	0.9	0.9	0.4	0.0	0.3

Peak biomass values reached 5 standard deviation units from the mean in both rivers. For the Sacramento River, a standard deviation unit of 5 occurred in July of 3.45% for chrysophyte samples in the Sacramento River. For the San Joaquin River, peak biomass also reached 5 standard deviations form the mean in June of 3.23% for green and chrysophyte species. Although somewhat lower, green algae and chrysophytes also reached an amplitude of 5 standard deviation units from the mean in August of 2.86%.

Phytoplankton patches were characterized by alternating regions of high and low biomass such as those for diatoms in May or periodic spikes in biomass such as those for green algae in July along the San Joaquin River (Fig. 4). There was no consistent pattern in these peaks by river, phytoplankton group or month.

Fig. 4. Standard deviation units of phytoplankton biomass for diatoms in May and green algae for July 2010 in the San Joaquin River.



Environmental conditions

High frequency measurements of water temperature, specific conductance, pH, dissolved oxygen and total suspended solids demonstrated high spatial and temporal variability along river transects. Most variables differed by at least a factor of two over the sampling season at any given location and could be equally variable within a single day transect (Fig. 5).

Physical and chemical conditions differed ($p < 0.01$, ANOSIM) between rivers and was primary due to differences in water temperature, specific conductance and pH ($r = 0.845$, BEST). Between rivers, most of the variation in the physical and chemical environmental conditions between April and October was associated with specific conductance, pH and dissolved oxygen concentration ($r = 0.86$, BEST) in the Sacramento River and specific conductance, total suspended solids and dissolved oxygen concentration ($r = 0.86$, BEST) in the San Joaquin River ($r = 0.86$, BEST).

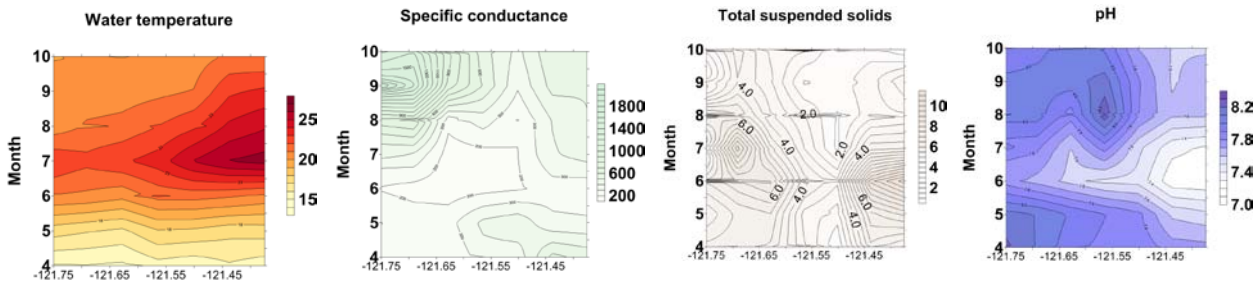
The San Joaquin River results were confounded by the intercorrelation ($r = - 0.70$, $p < 0.01$) between water temperature and dissolved oxygen, which indicated both variables could account for an equal percentage of the variation. Determining the important variables controlling environmental variability was more difficult when nutrients for June through October were included in the analysis because many of these variables were intercorrelated ($r > 0.70$) and could not be considered separately in the analysis. For this smaller data set, dissolved oxygen concentration was intercorrelated with pH ($r > 0.71$, $p < 0.01$) in both rivers. In addition, specific conductance was positively intercorrelated ($r > 0.72$, $p < 0.01$) with soluble phosphorus concentration, nitrate concentration was positively intercorrelated ($r > 0.80$, $p < 0.01$) with soluble phosphorus and total nitrogen concentration, ammonium concentration was intercorrelated with the nitrate to phosphate molar ratio (NP, $r > 0.90$, $p < 0.01$) and soluble phosphorus was

intercorrelated with total nitrogen concentration ($r = 0.71$, $p < 0.01$) for the Sacramento River.

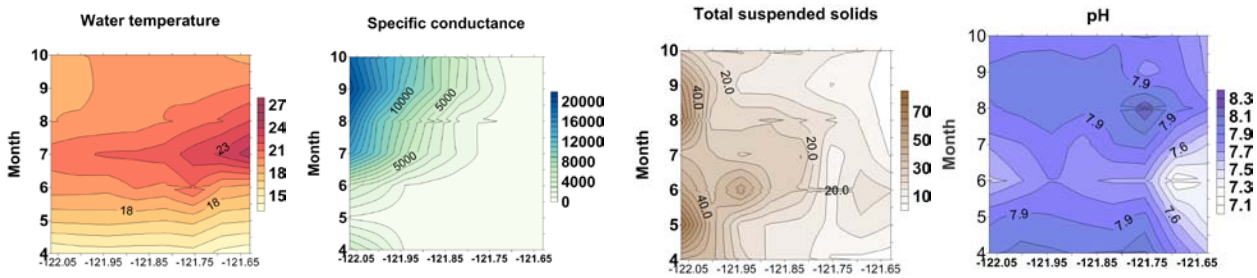
Many intercorrelations also characterized environmental and nutrient variables for the the San Joaquin River data set. Nitrate was intercorrelated ($r > 0.72$) with soluble phosphorus, total dissolved inorganic nitrogen (TN) concentration and the NP ratio and soluble phosphorus was intercorrelated ($r > 0.70$) with TN and NP. When intercorrelated variables (dissolved oxygen, soluble P, NP and TN) were removed from the analysis, most of the variability in the environmental and nutrient conditions in the Sacramento River was associated with specific conductance and pH ($r = 0.80$, BEST). Similarly when the intercorrelated variables (dissolved oxygen, soluble phosphorus, TN and NP) were removed from the analysis, most of the variability in the environmental and nutrient conditions in the San Joaquin River was associated with specific conductance, pH and nitrate ($r = 0.764$, BEST). Physical and chemical conditions also differed among months and were significantly different as a group ($p < 0.01$, ANOSIM) for all months in the Sacramento River and for April, May, June, July and August through October in the San Joaquin River.

Fig. 5. Water quality conditions measured at 10 second intervals along transects of the Sacramento and San Joaquin River between April and October 2010.

a) Sacramento



b) San Joaquin River



Nevertheless, 67% to 82% of the measurements among variables were less than one standard deviation unit from the mean along both the Sacramento and San Joaquin Rivers over the season (Table 2). Only 3% to 4% of the measurements averaged 2 standard deviation units or greater. Among variables, dissolved oxygen and specific conductance in the San Joaquin River and specific conductance and total suspended solids in the Sacramento River averaged somewhat larger deviations. Maximum values reached a negative 7 and a positive 6 standard deviation units for both pH and dissolved oxygen in the Sacramento River and San Joaquin River, respectively.

Table 2. Mean percent standard deviation units from the mean of environmental variables measured along transects in the Sacramento and San Joaquin Rivers between April and October 2010.

Sacramento River

deviation unit	chlorophyll a	dissolved oxygen	specific conductance	pH	water temperature	total suspended solids	mean
<1	67	76	82	68	69	72	72
1	29	21	12	29	27	23	23
2	3	2	5	2	3	4	3
3	0	1	2	0	1	1	1
4	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0

San Joaquin River

deviation unit	chlorophyll *a*	dissolved oxygen	specific conductance	pH	water temperature	total suspended solids	mean
<1	71	77	76	75	59	67	71
1	25	18	19	21	40	30	25
2	4	5	4	2	2	3	3
3	0	0	1	1	0	0	0
4	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0

Correlation environmental phytoplankton and environmental factors

Environmental variables were strongly correlated ($p < 0.01$, ANOSIM) with the percent composition of phytoplankton groups within each river (compare Figs. 3 and 5). For the Sacramento River, water temperature and specific conductance accounted for most of

the variation in the percent composition of phytoplankton groups (r = 0.35, BEST) with most of the correlation associated with specific conductance (r = 0.28, BEST). Specific conductance was also intercorrelated (r > 0.70, p < 0.01) with nitrate, ammonium and soluble phosphorus concentration. For the San Joaquin River, phytoplankton group composition was most closely associated with turbidity (r = 0.18, BEST). Among variables, water temperature was significantly correlated with the percent composition of many phytoplankton groups (Table 3). It was positively correlated with the percent cyanobacteria biomass in the Sacramento River and cryptophyte and dinoflagellate biomass in the San Joaquin River. In contrast, water temperature was negatively correlated with the percent diatom biomass in the Sacramento River and both diatoms and chrysophyte biomass in the San Joaquin River. Among the other variables, specific conductance and turbidity were negatively correlated with cyanobacteria, dinoflagellates or cryptophytes.

Table 3. Spearman rank correlation between phytoplankton group biomass and physical and chemical conditions in the Sacramento and San Joaquin Rivers between April and October 2010. Symbols indicate correlations at the 0.05 (*), 0.01 (**) and non significant (-) levels. Negative correlations are in red and positive correlations are in green type.

	Sacramento			**San Joaquin**		
	WT	**EC**	**NTU**	**WT**	**EC**	**NTU**
diatoms	**	-	-	**	-	-
cryptophyte	-	-	-	**	-	**
cyanobacteria	*	**	-	-	-	-
green algae	-	-	-	-	-	-
chrysophyte	-	-	-	**	-	-
dinoflagellates	-	*	-	*	-	-
ciliates	-	-	-	-	-	-

Comments

Phytoplankton and cyanobacteria biomass and species composition in the San Francisco Delta are characterized by high frequency spatial and temporal variation. Such high frequency spatial and temporal variability requires high frequency sampling in order to quantify the quantity and quality of biomass available at the base of the food web. This study suggests the sampling frequency should be at a minimum of 3 km to gain a understanding of the plankton and water quality variability. However, smaller, sub-kilometer length scales were also determined to be important in coastal waters and could be important here (Blackwell et al. 2008). The high spatial variability in the plankton community suggests that sampling at a higher temporal frequency might reveal even greater variability as well.

The high frequency variation of phytoplankton and cyanobacteria in the delta were correlated with environmental conditions. This suggests the ecosystem variables are tightly coupled. At large spatial scales in the ocean, phytoplankton vary with turbulence and chemical or biological factors are considered to be only contributing factors (Martin 2003). Yet, in the comparatively small delta, water temperature, salinity and turbidity were important correlative variables. In small scale upwelling environments, water quality conditions such as nutrient concentrations can affect phytoplankton patchiness (Martin 2003).

Microzooplankton probably contributed to the high spatial and temporal variability. Phytoplankton and microzooplankton are tightly coupled in the aquatic environment (Martin 2003). Microzooplankton influence phytoplankton patches in freshwater environments through their impact on phytoplankton growth rate (Calbet 2001). However, the presence of phytoplankton patches also affects microzooplankton behavior so that their time in the phytoplankton patch is maximized (Seruont al. 2001).

Quantifying the high frequency spatial and temporal variation in plankton patchiness is critical to an understanding of the structure and function of the food web. Research demonstrated the size of patches affects food web production by influencing sedimentation, growth rate, species composition, and zooplankton behavior (Seruont 1991; Zarauz 2009). These factors can strongly influence trophic transfer and are not a function of the amplitude of production (Martin 2006).

Acknowledgement - Thank you to L. Young, N. Sakata and R. Breuer who assisted with field sampling and administration. This research was funded by a CALFED Science grant number 1074 to Dr. Lehman with supplementary funds from the California Department of Water Resources and US Bureau of Reclamation.

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Appendix 4 : **Comparison of FlowCAM and Fluoroprobe to characterize phytoplankton communities in the Delta**

Comparison of the FlowCAM and Fluoroprobe for identification of in situ phytoplankton communities.

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Introduction

The FlowCAM is a digital imaging flow cytometer that collects digital images of live phytoplankton in the field that can be subsequently identified and enumerated at the genus to species level (Sieracki et al. 1998). The FlowCAM is limited in its ability to measure the picoplankton community accurately because the most effective size range is 12 µm or greater for the version of the machine tested. The Fluoroprobe collects fluorescence spectra of the water within the range of phytoplankton pigments that are used to identify phytoplankton community composition by taxa (Gregor and Marálek 2004). The Fluoroprobe is limited in its ability to separate out taxa due to the overlap of pigments and interference by humic substances but is not limited in size range. The Fluoroprobe can only identify four phytoplankton groups: brown (diatoms, chrysophytes and dinoflagellates), cryptophyte (cryptophye), green (chlorophytes, Euglenophtes) and bluegreen (cyanobacteria). It cannot separate diatoms and dinoflagellates which have similar pigments (See et al. 2005).

The purpose of this study was to determine if the two instruments give similar results.

Methods

The Fluoroprobe and FlowCAM were deployed simultaneously in July 2010 along transects of the Sacramento and San Joaquin rivers (Fig. 1). Discrete samples for FlowCAM analysis were collected approximately every 6 to 10 minutes and immediately

run through the machine. The A total of 29 samples were taken in the Sacramento River and 32 in the San Joaquin River. The Fluoroprobe is submersible and was continuously deployed at 1 m depth. Three discrete phytoplankton samples were collected for microscopic analysis of species composition; these samples have not been analyzed.

Results of the two instruments were compared with non-parametric statistical techniques using Primer-e version 6 software (Clarke and Gorley 2006). The spatial pattern in the data were first identified using multidimensional scaling analysis (MDS ordination). The MDS ordination used a Bray Curtis dissimilarity index and was computed using percent (%) contribution of total phytoplankton biomass for each of the four phytoplankton groups. The same groups were used for each instrument. Differences between the MDS distributions were determined using ANOSIM. Significant differences produced by the ANOSIM test were determined by the value of the *R*-statistic. Lastly, the SIMPER procedure was used to identify which groupings were responsible for the differences among samples within each MDS ordination. The SIMPER analysis also identifies which groups contributed most to the differences between MDS ordinations generated by each method. All statistical tests were deemed significant at the 0.05 level or less.

Results and Discussion

The MDS ordination showed strong differences between the two methods for both river systems (Fig. 2). The ANOSIM test identified a significant difference ($p < 0.01$) between results for the FlowCAM and Fluoroprobe for both rivers as well as a significant difference for each river separately (Sacramento River $R=0.611$, $p<0.01$ and San Joaquin River $R=0.659$, $p<0.01$). Looking further, SIMPER results determined that in the Sacramento River, greens and bluegreens were responsible for about 72.4% of the differences between assemblages (Table 1) whereas in the San Joaquin River, greens

and chryptophytes contributed about 64.5% to the differences between assemblages (Table 1).

Comparison of the phytoplankton groups quantified by each method revealed that the FlowCAM samples had an average similarity of 47.8% for the San Joaquin River and 56.9% for the Sacramento River. Fluoroprobe samples had an average similarity of 74.4% for the Sacramento River and 84% for the San Joaquin River (Table 2). Overall, these values suggest that the FlowCAM samples are able to demonstrate more of the variability found in the system. One should assume that as species composition shifts, the fluorescence signal shifts as well. The greater range of values demonstrated in the MDS ordinations for the FlowCAM suggested it was more successful at describing the range of plankton in the phytoplankton community that was present in July (Figure 3).

Studies comparing Fluoroprobe and FlowCAM are few. See et al. (2005) compared both instruments in addition to HPLC chemical taxonomy analysis, light microscopy and epifluorescence microscopy for phytoplankton along the Texas Gulf coast. Their conclusion was that the Fluoroprobe and FlowCAM are potentially useful for determining the general characteristics of phytoplankton community structure in situ when used in tandem. However, the Fluoroprobe over estimated the chlorophytes and cryptophytes and could not separate diatoms and dinoflagellates due to similar pigment composition. See et al. (2005) also found that FlowCAM is best suited for identification of larger phytoplankton species whereas the Fluoroprobe is designed to detect fluorescence signals of small cells.

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Table 1. Differences between FlowCAM and Fluoroprobe measurements according to SIMPER and percent contribution of each spectral category to differences between methods.

	Sacramento River		**San Joaquin River**		
Average Difference	**58.1%**		**68.7%**		
Species	***%***	***Cum.%***		***%***	***Cum.%***
Green	**38.9**	**38.9**	**Green**	**40.9**	**40.9**
BlueGreen	**33.5**	**72.4**	**Cryptophyte**	**23.5**	**64.5**
Brown	**14.9**	**87.3**	**Bluegreen**	**23.5**	**88.0**
Cryptophyte	**12.7**	**100.0**	**Brown**	**12.0**	**100.0**

Table 2. Similarity Index, expressed as percentage (%) within methods for each river systems.

River	**FlowCAM**	**Fluroprobe**
Sacramento River	56.9	74.38
San Joaquin River	47.8	83.99

Fig. 1. Map of Sacramento-San Joaquin Delta indicating sampling transects for the Sacramento River (green marks) and the San Joaquin River (red marks).

Fig. 2. Multidimensional scaling ordinations for samples collected along July transects for the Sacramento and San Joaquin Rivers by the flowCAM (green triangle) and the Fluoroprobe (blue triangle).

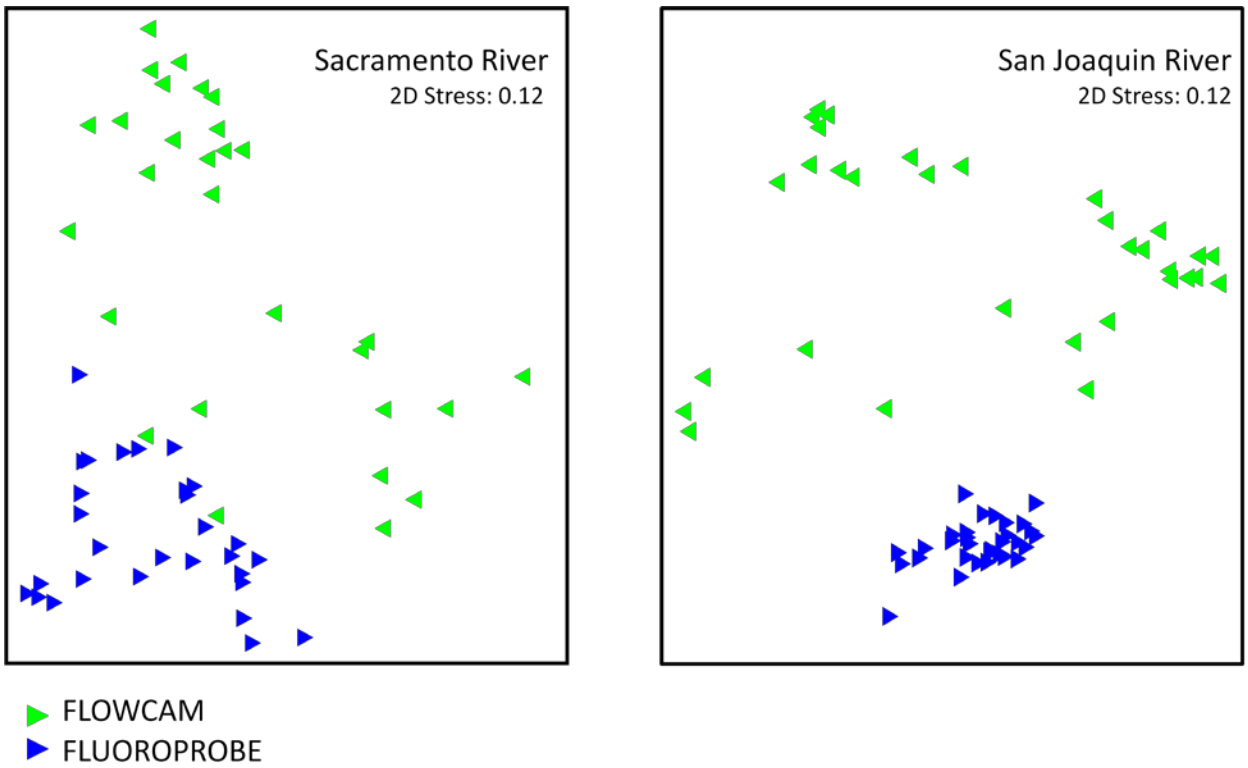
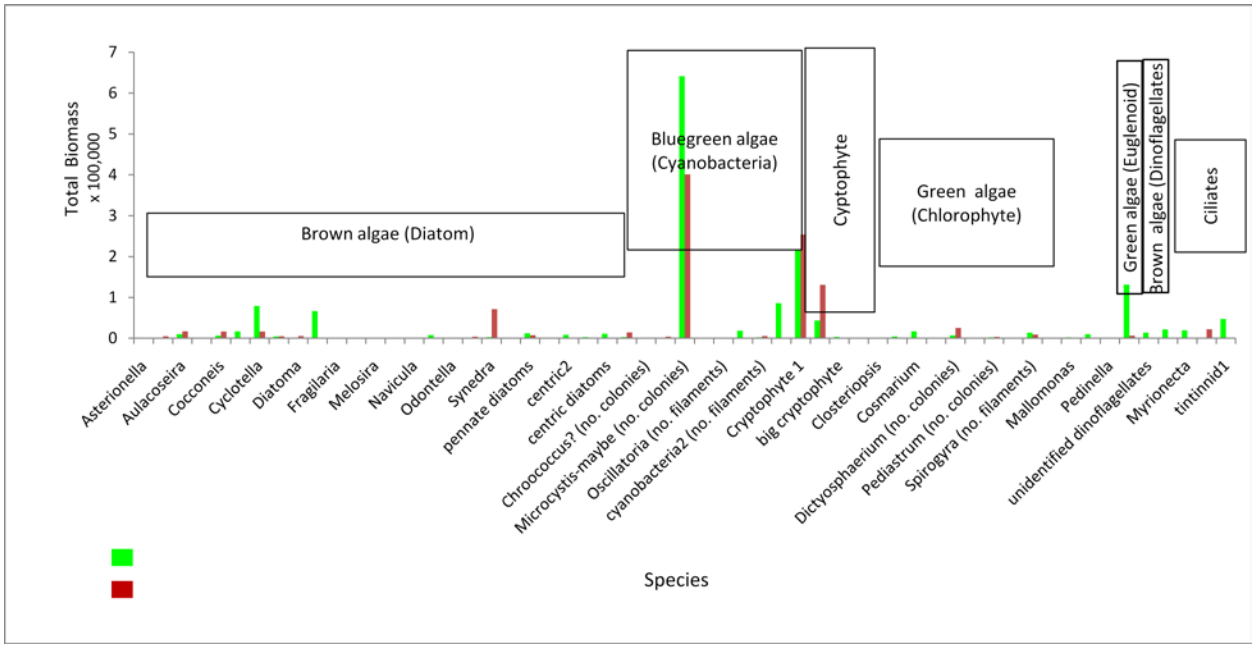
Fig. 3. Biomass of phytoplankton genera among taxa identified by the FlowCAM within water quality samples collected in July along the Sacramento (green bar) and San Joaquin rivers (red bar).