EPA-LISS GRANT PROJECT – FINAL REPORT

Phytoplankton Dynamics in Long Island Sound: Influence of environmental factors on naturally occurring assemblages (EPA Grant # X 98 1613-01-1)

Project Period: January, 2002 to January, 2004 (with extension to 2005)
Reporting Period: January, 2002 to January, 2005

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1. PROJECT OBJECTIVES

We have been developing flow-cytometric/ immunochemical methods to examine phytoplankton assemblages of the western, central, and eastern Long Island Sound (LIS), and to determine how nutrient concentrations affect the physiological condition (metabolic activity, viability) and intracellular characteristics (e.g., Ca^{2+}, pH, lipid, protein) of phytoplankton in LIS. Specifically, our study has focused on three primary objectives: 1) to determine how phytoplankton dynamics (e.g. community structures, population abundance, species composition) in LIS differ among sites and change seasonally over a two year period; 2) determine which environmental factors (e.g., nutrients, hypoxia, temperature) are the primary determinants of phytoplankton assemblages and physiological condition of different species; and 3) examine the relationships between phytoplankton assemblages and planktonic grazers.

2. PROJECT SUMMARY

Understanding the patterns and processes of phytoplankton biodiversity in relation to primary production is fundamentally important for sustainable management of coastal ecosystems such as Long Island Sound (LIS). Limited data exist that accurately describe phytoplankton physiological processes associated with natural, internally driven mortality. In fact, most extrapolations are inferred by comparing nutrient levels and the abundance of various population structures, however, such analyses do not examine the actual affect upon the physiology of phytoplankton, which would be a much better indicator of "cause and effect."

In this study we have been developing flow-cytometric/immunochemical methods to examine phytoplankton assemblages of the western, central and eastern LIS, studying how nutrient concentrations affect the physiological condition of these cells. Results suggest that seasonal temperatures, turbidity, dissolved oxygen and salinity affect the composition and abundance of phytoplankton in the Sound less than do nutrient loads. Concentrations of nutrients (e.g., phosphate, nitrate, ammonia) in the summers of 2002 and 2003 generally increased from eastern to western LIS. The presence or absence of phytoplankton genera

appears to be influenced by concentrations of nutrients in the Sound. For example, decreased nutrient concentrations observed in the central and western sound are associated with an increase in *Asterionella*, *Rhizosolenia*, *Thalassiothrix* species. Subsequently, increased concentrations of *Pseudocalanus* occurred in all regions (summer 2003) as well as increases in Chaetognatha, barnacle and fish larvae in the western sound.

Flow cytometry (FC) coupled with biochemical labeling has shown that whereas many biochemical probes are useful for homogenous laboratory controlled experimentation, some probes (e.g. detection of nitrate and phosphate reductase, increased lipids) are better than others when examining heterogeneous, field-collected samples. Because the cell diversity associated with heterogeneous field samples result in the overlap of natural and biochemical signatures, detection of the physiological process of individual cells is proving difficult. The methods we have developed to compare and contrast a heterogeneous population of cells require physical sorting (using FC), followed by the addition of selected biochemical probes and finally sample analyses. This procedure is time consuming, and therefore not yet suitable for general screening of many environmental samples. However, the application of enzyme probes coupled with sorting and analytical flow cytometry permit assessment of some aspects of the physiological status of phytoplankton populations in mixed, natural assemblages. We continue to refine our newly developed techniques which will help reveal the presence of physiological capacities that allow phytoplankton to thrive under a particular set of environmental conditions.

3. PROJECT ACCOMPLISHMENTS

Field work – All collected field samples and their analyses are complete. Re-examination of a number of TOC/DOC (total and dissolved organic carbon) and TPM (total particulate matter) samples and replicates are, however, being repeated for accuracy (Table 1). Due to poor weather conditions and/or instrument malfunction, data are missing from some sites. In January (2003) weather conditions limited sampling to the East (Groton) location only. Sea-Bird instrument analyses were hampered by water leakage which affected some data collected for Greenwich (April 2003), turbidity and chlorophyll for all locations in July (2003), and turbidity, chlorophyll and dissolved oxygen for Bridgeport and Greenwich (October 2003). No data were collected for Groton (October 2003).

Laboratory work – Microscopic phytoplankton and zooplankton analyses for all samples are complete. Composition and abundances indicate similar concentrations each year within each location, but are dissimilar between locations. Summer conditions (2003 & 2004), for example, are associated with similar abundances of *Nitzchia* in the East but are dissimilar to abundances in central and west locations of LIS (Fig. 1. $A_{1\text{-}2}$, $B_{1\text{-}2}$). Similarly, phytoplankton species observed in central and western LIS were not observed in the eastern Sound (e.g. *Euglena*; Fig 1. A_1, B_1). Observations of zooplankton revealed similar compositions of *Acartia*, *Pseudocalanus* and *Temora* among these sites and during each season (Fig 1. A_2, B_2).

Initial results suggest that temperature, salinity and oxygen play a small role in affecting species composition and abundance of phyto- and zooplankton. Summer conditions during 2002 and 2003 (Figs. 2, 3), for example, depict similar temperature, salinity and oxygen profiles compared to nutrient loads (Fig. 4). In the summer of 2002, concentrations of phosphate and nitrate were significantly higher in the western Sound compared to the eastern LIS. Ammonia concentration was higher in the western Sound, but was not significantly different than that of the eastern Sound. Nitrite was undetectable in the eastern Sound, with higher levels in western LIS (Fig. 4). In the summer of 2003, phosphate and nitrate concentrations were higher in the western Sound, but not significantly different than

those of the eastern Sound. Again, nitrite was barely detectable at all locations (Fig. 4). The observed differences in nutrient concentrations between seasons and locality suggest that phytoplankton abundances and compositions are primarily influenced by nutrient fluctuations in the Sound. In turn, zooplankton dominance seems to be affected as their diets shift with changing phytoplankton community structures. The way in which nutrients influence phytoplankton dominance is likely a reflection of how well a particular species metabolizes each of the various compounds (i.e. phosphate, nitrate, etc.). Consequently, we speculated that biochemical stains coupled with flow cytometry could be used to explain the presence of physiological capacities that allow plankton to thrive under a particular set of environmental conditions.

Table 1. Sampling collection and analysis schedule for sites in Long Island Sound. Check-marks indicate completion of sample collection and/or analysis, x-marks reflect incomplete data or data not collected due to poor weather conditions and/or instrument failure.

Parameter	Jun/Jul-2002		Sept/Oct-2002		Dec/Jan-2002/03		Mar/Apr-2003		Jun/Jul-2003		Sept/Oct-2003		Dec/Jan-2003/04	
	C	A	C	A	C	A	C	A	C	A	C	A	C	A
Water Analysis														
Nitrate	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Nitrite	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Phosphate	√	√	√	√	√	√	√	√	√	√	√	√	√	√
TPM	√	√	√	√	√	√	√	√	x	x	x	x	x	x
TOC/DOC	√	√	x	x	x	x	x	x	x	x	x	x	x	x
Phytoplankton: (composition)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
(abundance)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Zooplankton: (composition)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
(abundance)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Sea-Bird Instrument Analysis														
Temperature	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Salinity	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Oxygen	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Depth	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Fluorescence	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Flow	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Flow Cytometry (FC)*														
Phosphate content	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Nitrate content	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Carbon content	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Lipid content	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Viability	√	√	√	√	√	√	√	√	√	√	√	√	√	√

* – Flow Cytometry: Lab experimentation ~%100 complete. Data analysis is still occurring to determine the feasibility of these dyes for use with preserved field samples.

Flow cytometry (FC) – Laboratory "ground-truthing" analyses": We have spent a great deal of time developing FC techniques specific for this project, establishing "proof-of-principle," and gathering base-line data using molecular probes and manipulated phytoplankton cultures (e.g. nutrient replete vs limited). This laboratory work is necessary as we need to know which types of plankton stain with each probe, the degree to which the probe reveals differences in physiological status, and how well each probe works with preserved samples. Despite the laboratory analysis progressing well, it has taken more time than we originally anticipated.

Briefly, we have been conducting experiments to examine the effects of nutrient availability on cellular functions of phytoplankton using five probes. Specifically, we have been examining nitrogen, phosphate, light energy and non-viable cells using the probes CNOB (used to detect nitrate reductase), ELF97 (used to detect alkaline phosphatase), BODIPY (detection of lipids produced from light energy), Carboxy-SNARF 1 (is an

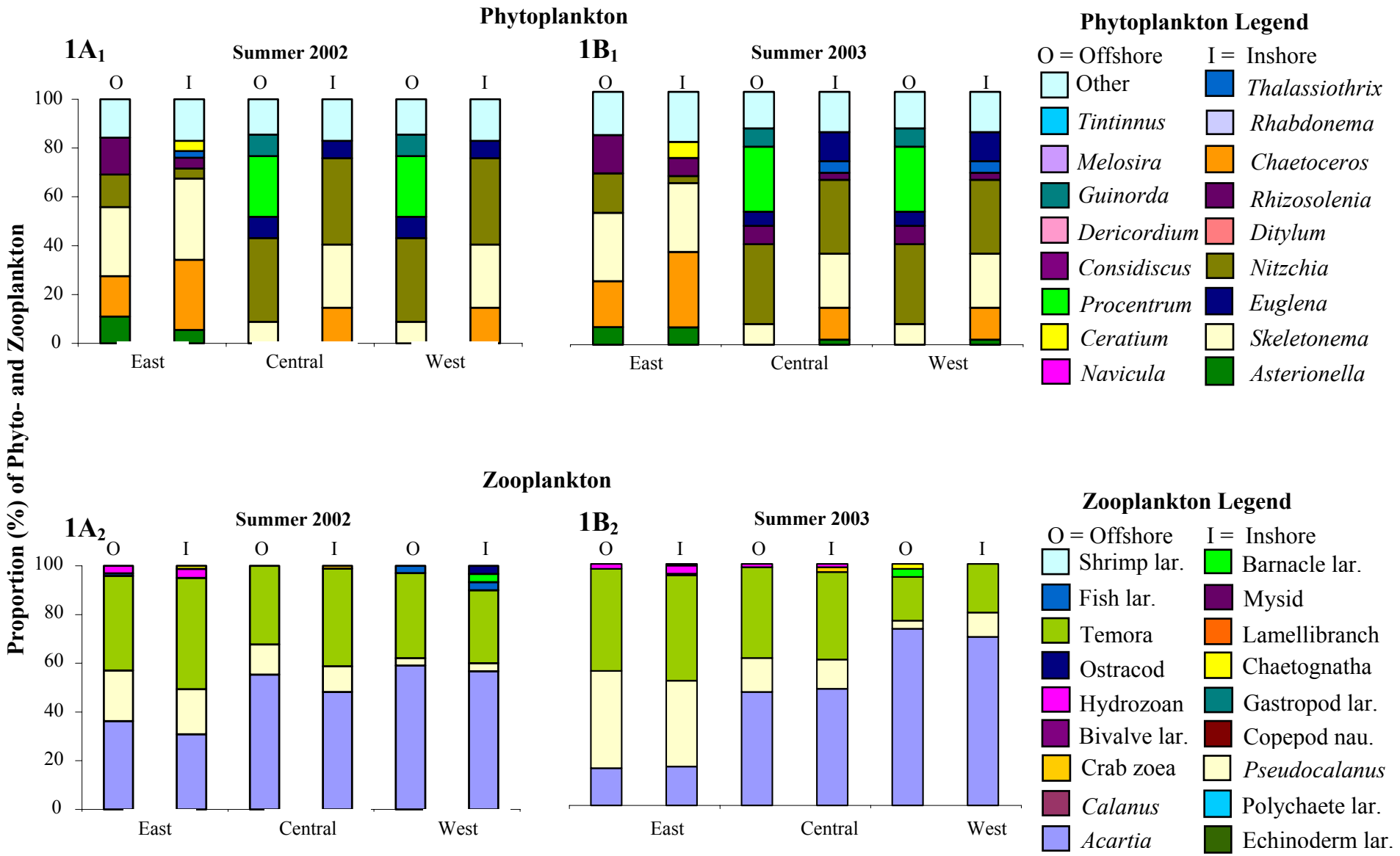

Figure 1. Comparative analyses of Summer 2002 vs. Summer 2003 data collected from East (Groton), Central (Bridgeport) and West (Greenwich) locations in Long Island Sound. Analyses include: (1) mean phytoplankton (A_1 & B_1) and zooplankton (A_2 & B_2) assemblages; In-shore and off-shore samples have been pooled for this presentation.

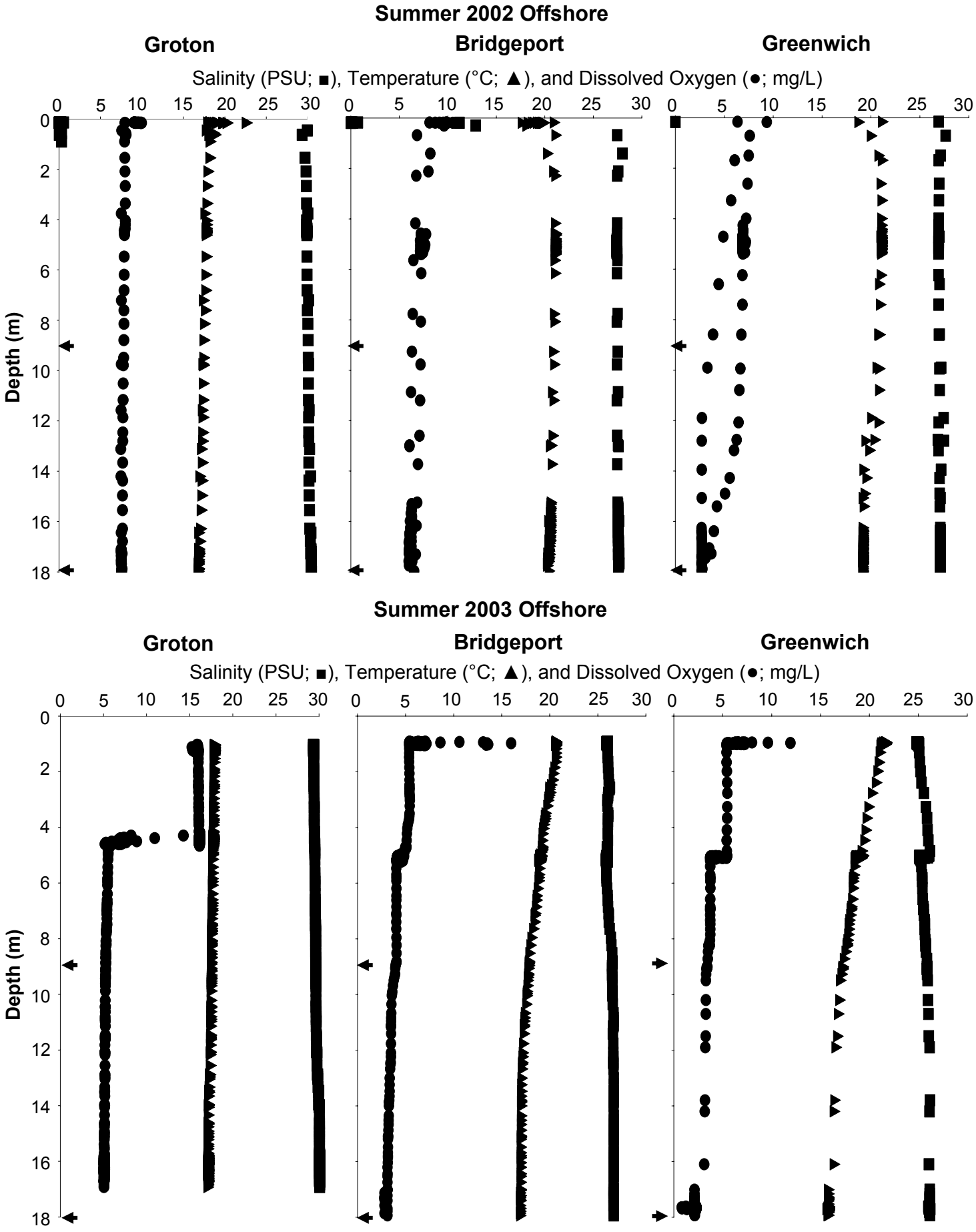

Figure 2. Comparative analyses of Summer 2002 vs. Summer 2003 data collected from East (Groton), Central (Bridgeport) and West (Greenwich) locations in Long Island Sound. Analyses include: Temperature, depth, salinity and dissolved oxygen data collected from Offshore sites. Arrows indicate sampling depth.

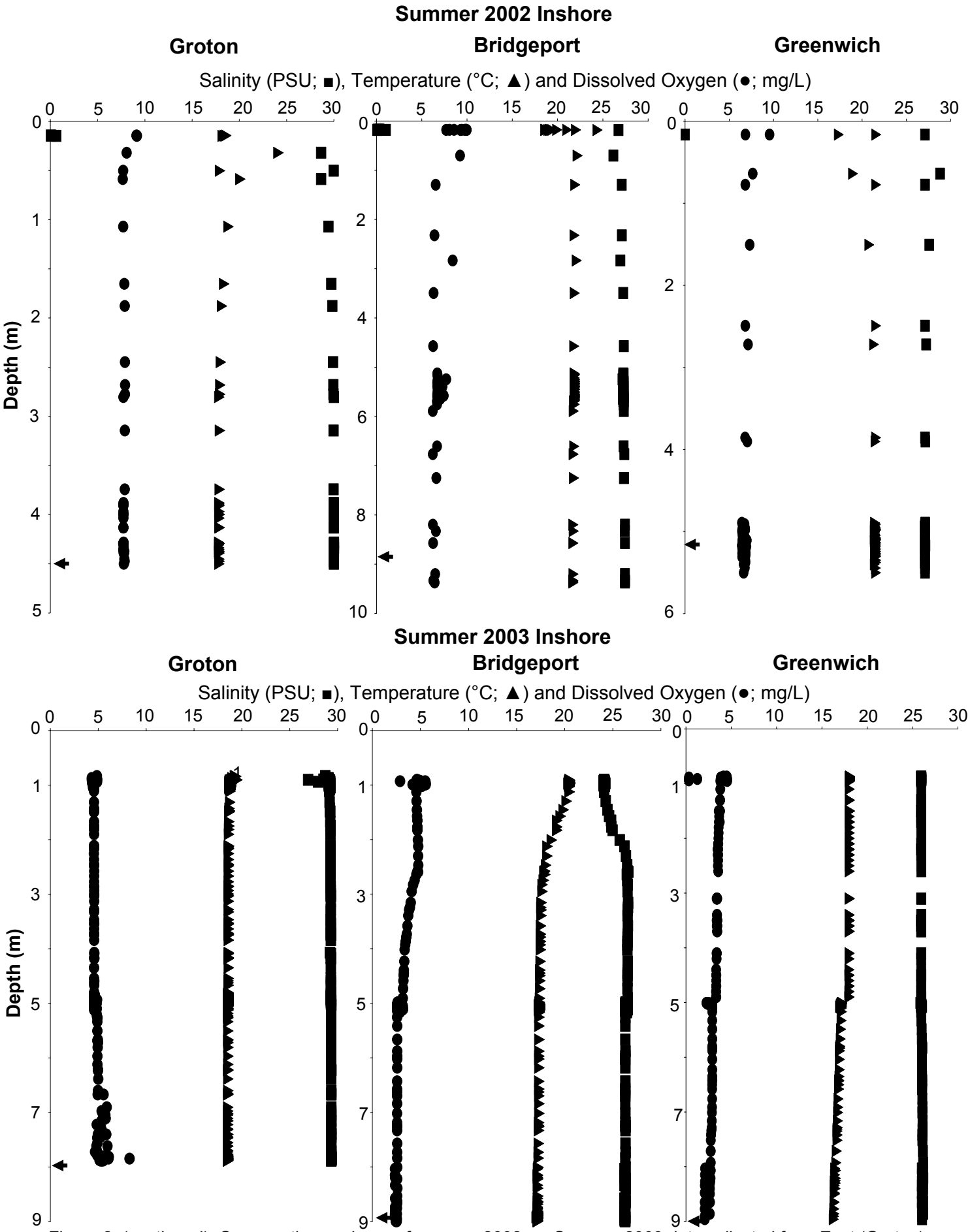

Figure 2. (continued) Comparative analyses of summer 2002 vs. Summer 2003 data collected from East (Groton), Central (Bridgeport) and West (Greenwich) locations in Long Island Sound. Analyses include: Temperature, depth, salinity and dissolved oxygen data collected from inshore sites. Arrows indicate sampling depth.

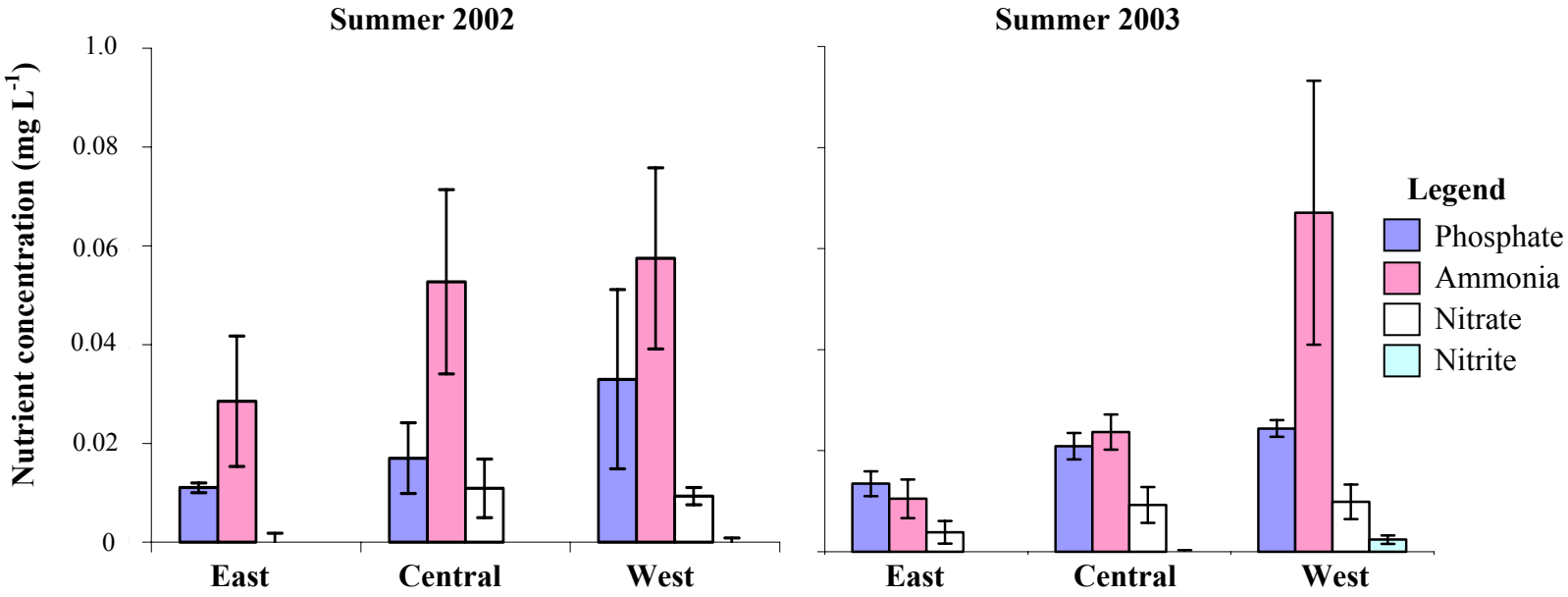

Figure 4. Comparative analyses of Summer 2002 vs. Summer 2003 data collected from East (Groton), Central (Bridgeport) and West (Greenwich) locations in Long Island Sound. Analyses include: phosphate, ammonia, nitrate and nitrite nutrient conditions; some error bars (95% CI) are smaller than data point symbols.

intracellular pH probe also associated with light energy) and Annexin V-*fluor* (detection of apoptotic cells), respectively. These biochemical reagents selectively bind to an enzyme or cell component of interest and fluoresce in a measurable wavelength using a flow cytometer.

Phytoplankton cells that were switched from nitrate to ammonia gradually demonstrated a reduced expression of nitrate reductase (Fig. 5A vs 5B), indicating that such cells are detectable using the CNOB probe.

Observations of *Chlorella*, *Nitzschia* and *Thallasiosira* indicated that no difference in alkaline phosphatase content was discernable when cultures, phosphate replete and phosphate depleted, were stained with ELF-97. We did, however, observe differences for the dinoflagellate, *Prorocentrum minimum* (Fig. 6A & 6B). *Thalassiosira* experiments showed no difference in P-replete vs. low-P conditions; whereas, there was a difference in *P. minimum*.

Experiments conducted to examine the effects of lipid production in the presence of an N-deficient, but light sufficient environment similarly depict increased production of lipids. Increased lipid production was detected using the BODIPY probe (Fig. 7A & 7B).

The Carboxy SNARF 1 probe, used to detect intracellular pH worked equally well (Fig. 8), indicating that it could be useful in comparing samples collected during day or night or from different depths.

Apoptosis, which is a regulated pathway leading to normal as well as non-functional cell development, can be identified using a probe called Annexin V-*fluor*. In this study, a greater proportion of live cells were observed as viable (66%; Fig. 9A) in a test tube not sealed from the atmosphere however, a greater proportion of apoptotic cells (66.9%; Fig. 9B) was observed in test tubes sealed from the atmosphere and, therefore, deprived of carbon dioxide to support photosynthesis, a reduced (33.1% viable cells; Fig. 9B) number of viable cells was found.

Flow cytometry (FC) – Field analyses: The flow-cytometric techniques that we are developing allow rapid and accurate analyses of various physiological conditions of laboratory phytoplankton species exposed to various experimental regimes. The development of these techniques, however, was to not only provide data on the types of phytoplankton that inhabit different regions of LIS, but help reveal the presence of microhabitats allowing particular plankton to thrive under a particular set of environmental conditions. As a result, these types of data and the insights they could afford would provide information inaccessible using traditional bulk field measurements.

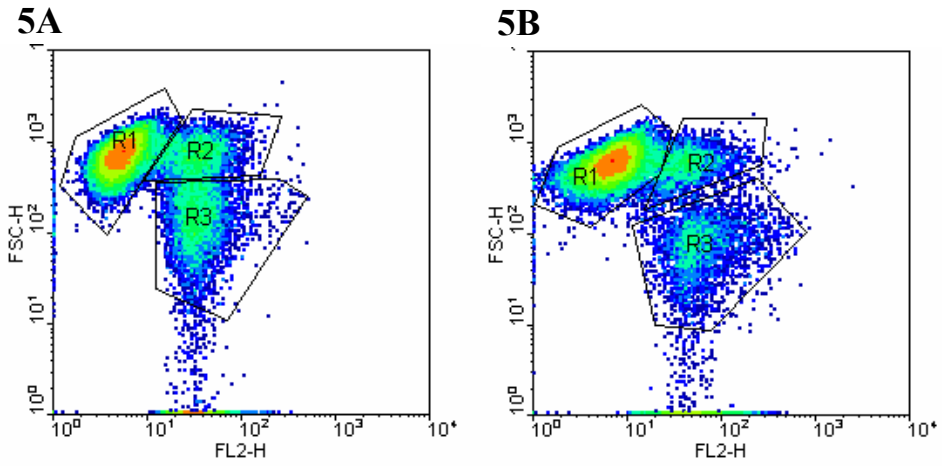

Figure 5. Flow-cytometric analysis of a phytoplankton sample (*Thallasiosira pseudonana*, clone 3H) after exposure to CNOB fluorescent stain. Notice that the cell population depicts a reduced expression of nitrate reductase (regions R2 and R3) in nitrate (2A) vs. ammonia (2B) exposed cells, representing a shift in fluorescent and side-scatter signatures. Red - highest cell concentration; Blue - lowest cell concentration.

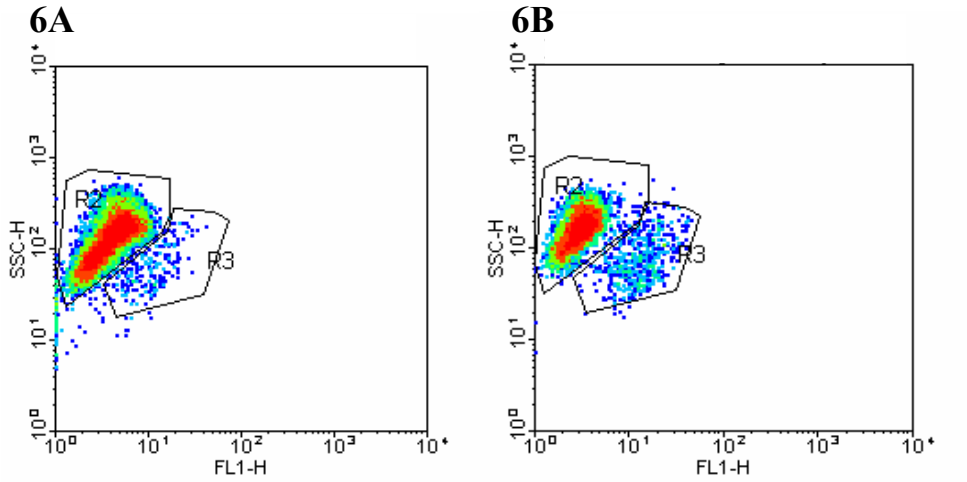

Figure 6. Flow-cytometric analysis of dinoflagellate cells *Prorocentrum* sp. after exposure to the ELF-97 fluorescent stain. Notice that the cell population depicts an increased expression of phosphate reductase (R3 regions) in replete (3A) vs. reduced (3B) exposed cells, representing a shift in fluorescent and side-scatter signatures. Red - highest cell concentration; Blue - lowest cell concentration.

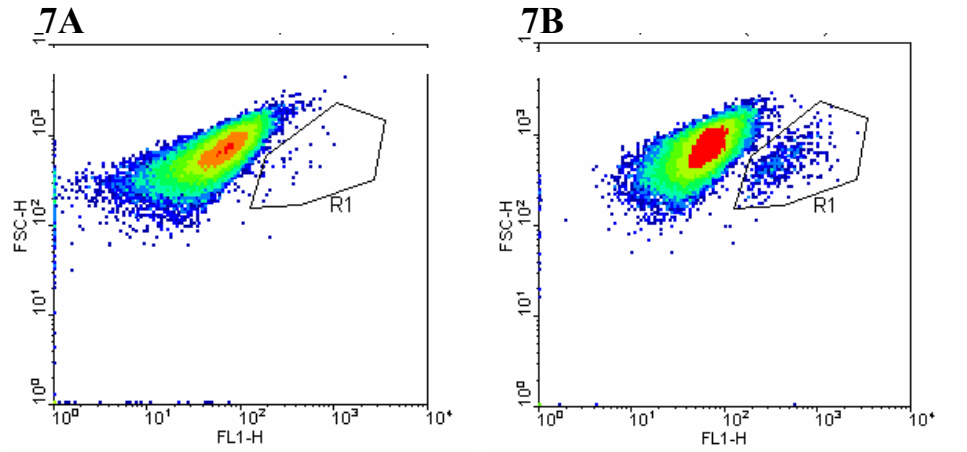

Figure 7. Flow-cytometric analysis of dinoflagellate cells *Prorocentrum* sp. after exposure to the BODIPY fluorescent probe. Notice that the cell population depicts an increased expression of lipid concentration (R1 regions), representing a shift in fluorescent and side-scatter signatures. Red - highest cell concentration; Blue - lowest cell concentration.

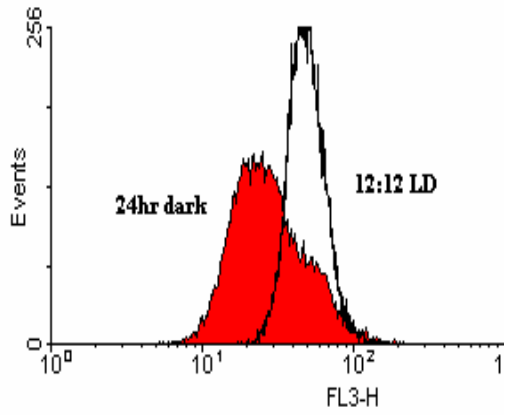

Figure 8. Flow-cytometric analysis of dinoflagellate cells *Prorocentrum* sp. after exposure to the Carboxy SNARF 1 fluorescent probe. Notice that the cell population depicts an increased expression of intracellular pH after a 24 hr dark (red region) vs. 12:12 (light:dark) incubation period.

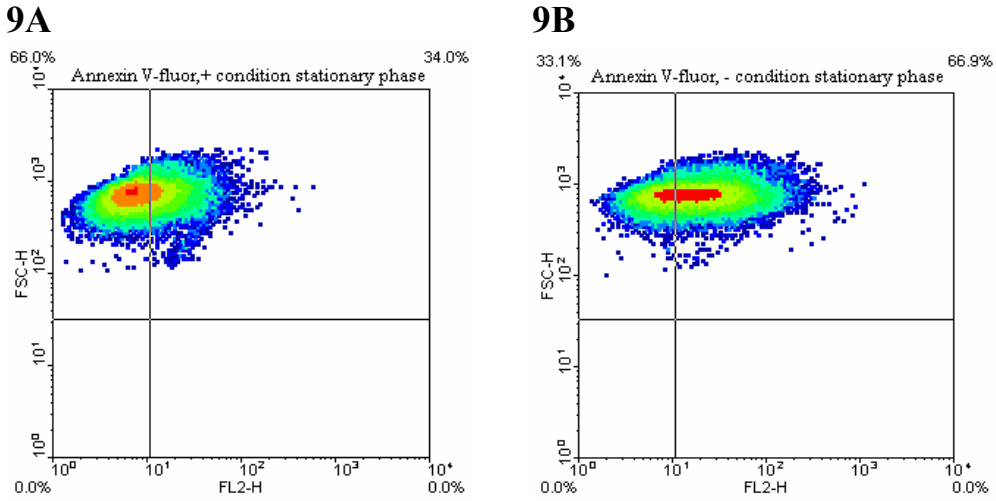

Figure 9. Flow-cytometric analysis of dinoflagellate cells *Prorocentrum* sp. stained with the *Annexin V-fluor* fluorescent probe. Notice that the cell population depicts an increased expression of apoptotic cells from 34% in a positive condition (A) vs. a negative condition (B; 66.9%).

Although our findings are very promising and suggest that flow cytometry coupled with biochemical labeling promotes a better understanding of how nutrient dynamics affects primary production in phytoplankton cells, some of the probes are better indicators with homogenous populations (controlled laboratory experiments) vs. heterogeneous field samples. The diversity associated with heterogeneous field samples makes individual populations difficult to differentiate as there is too much overlap of natural and biochemical labeling of the probes (Fig. 10A & 10B).

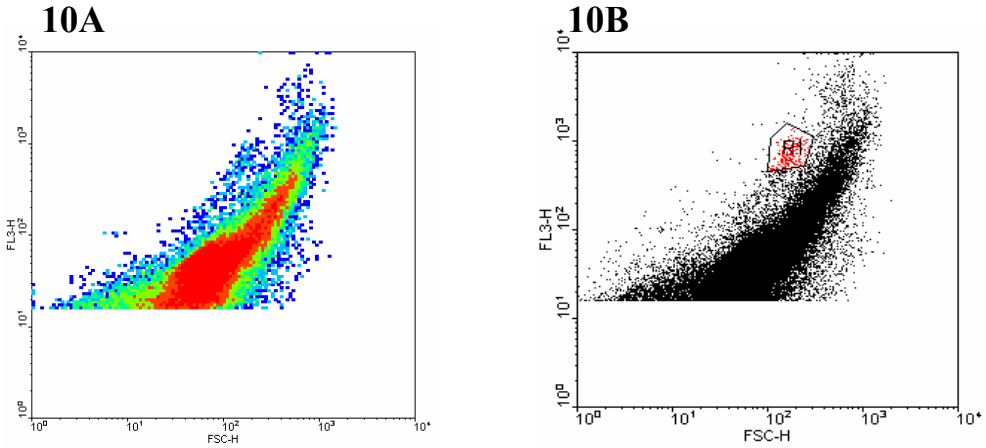

Figure 10. Flow-cytometric analysis of a heterogeneous population of phytoplankton cells collected from field studies show that samples are difficult to differentiate as a result of too much overlap of natural and biochemical labeling of the probes.

We have found that the best method to compare and contrast a heterogeneous population of cells collected from the field is to physically sort the sample using flow cytometry, followed by the addition of selected biochemical probes and finally analyse the samples (Fig. 11). This technique can be very time consuming, however, better protocols are currently under development.

The flow-cytometric techniques that we proposed to examine phytoplankton assemblages

of Long Island Sound are yielding new insights into which plankton are better adapted in nutrient repleat vs. depleted conditions. Thus far, we have shown that some of the biochemical probes are useful in a laboratory setting (Table 2). One of the five probes (Carboxy SNARF-1) does not appear to show distinct differences when examining a heterogenous population of cells. Currently, testing is underway to determine if ELF-97 can be used to label and clearly distinguish phytoplankton cells with more or less alkaline phosphatase from field collected samples (Table 2).

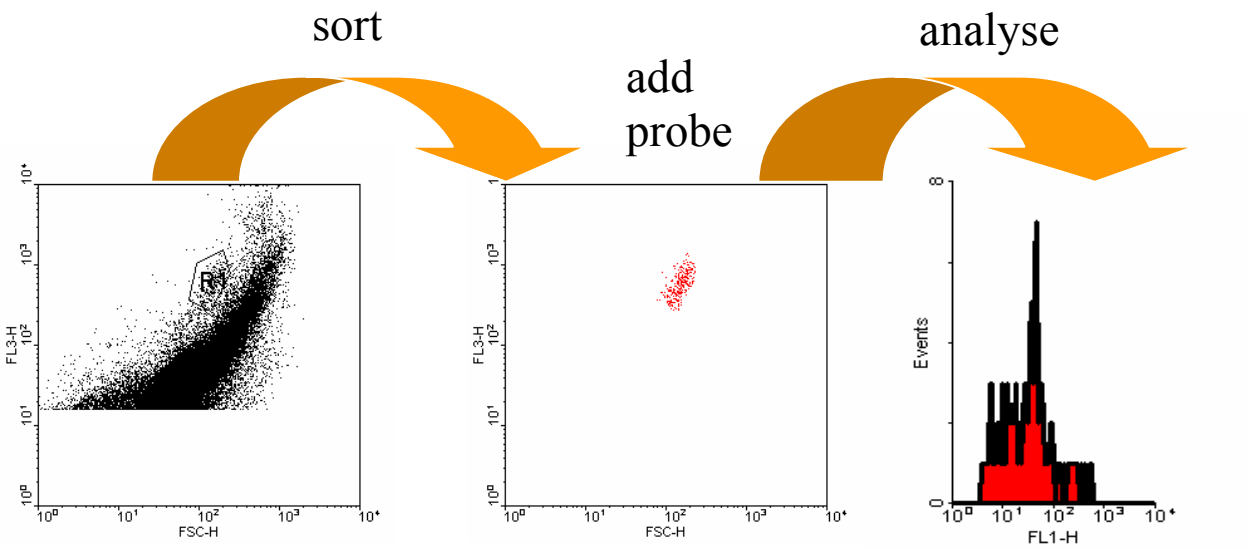

Figure 11. Flow-cytometric analysis of a heterogeneous population of phytoplankton cells are only identifiable by cell "gating", which is a process of selecting a particular region to be sorted and isolated from the remaining population of cells.

Table 2. Biochemical probes used to examine nutrient metabolism in laboratory cultured and field collected plankton cells.

Probe name	Detection ability	Lab usefulness	Field usefulness
CNOB	nitrate reductase	Yes	Yes
ELF97	alkaline phosphatase	Yes	Maybe…
BODIPY	Increased lipids	Yes	Yes
Carboxy SNARF-1	intracellular pH	Yes	No
Annexin V-*fluor*	Apoptosis/viability	Yes	Live samples only

4. PARTICIPANTS

Senior Personnel:

a. J. Evan Ward, Principal Investigator, University of Connecticut. Ward was responsible for helping with the collection of samples, overseeing nutrient analyses, and for general grant administration (e.g., sampling schedule, budget, progress reports, etc.).

b. K. Strychar, Co-Principal Investigator, University of Connecticut. Strychar served as a post-doctoral fellow on the project. He was responsible for all aspects of the proposal, including sample collection, sorting of zooplankton, flow-cytometric analyses of phytoplankton, final data analyses, and writing of manuscripts. Strychar also trained auxiliary personnel who helped with sample analysis (e.g., D. Kach).

c. G. Wikfors, Co-Investigator, National Marine Fisheries Service. Wikfors collaborated on various aspects of the project, including: sample collection, flow-cytometric analyses of phytoplankton, microscopic identification of phytoplankton and data interpretation and work-up.

Other Personnel:

a. B. Holohan, Research Assistant, University of Connecticut. Holohan assisted with sample collection and was responsible for nutrient and dissolved organic carbon analyses.

b. D. Kach, Undergraduate Research Student, University of Connecticut. Kach helped with sample collection, zooplankton sorting, and microscopic identification of phytoplankton (trained by G. Wikfors).

5. PUBLICATIONS & PRESENTATIONS

Presentations:

Strychar, K.B., J.E. Ward and G.H. Wikfors. Phytoplankton dynamics in Long Island Sound: Influence of environmental factors on naturally occurring assemblages. Long Island Sound / New England Estuarine Research Society Conference, Oct. 2002

Strychar, K.B., J.E. Ward and G.H. Wikfors. Flow cytometry: An innovative environmental tool for studies of phytoplankton dynamics and eutrophication in Long Island Sound. NOAA-National Marine Fisheries Service, Nov. 2002

Wikfors, G.H., K.B. Strychar, J.E. Ward, J.H. Alix and B.A. Holohan. Developing new techniques to investigate how nutrients control phytoplankton assemblages in Long Island Sound. Long Island Sound Research Conference, Oct. 2004.

Independent Studies:

Kach, D. Plankton Assemblages in Long Island Sound. Independent study and report. University of Connecticut, Groton, CT, Feb. 2003.

Published Reports and Briefs:

New England Estuaries Research Society, Oct. pp. 24-26. (2002). Phytoplankton dynamics in Long Island Sound: Influence of environmental factors on naturally occurring assemblages.

Connecticut Sea Grant College Program. pg. 14. (2003). Phytoplankton dynamics in Long Island Sound: Influence of environmental factors on naturally occurring assemblages.

Papers in Preparation:

Strychar, K.B., G. Wikfors, D. Kach, and J.E. Ward. (*in prep*.). Plankton dynamics in the Long Island Sound estuary: Influence of environmental factors on dominance in naturally occurring assemblages. To be submitted to "*Estuaries*".

Strychar, K.B., J.E. Ward, and G. Wikfors. (*in prep*.). The development of new techniques to measure metabolic activity of phytoplankton assemblages by flow cytometry using multiple fluorogenic substrates: Understanding nutrient-influenced dominance in marine environments. To be submitted to "*Marine Ecology*".