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Flow Cytometry of Alexandrium catenella from Elkhorn Slough, California.

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FLOW CYTOMETRY OF *ALEXANDRIUM CATENELLA* FROM ELKHORN SLOUGH, CALIFORNIA.

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ABSTRACT
FLOW CYTOMETRY OF *ALEXANDRIUM CATENELLA* FROM ELKHORN SLOUGH, CALIFORNIA.

by Phillip R. Hawkins

This study describes the use of flow cytometry for the enumeration of the toxic marine dinoflagellate *Alexandrium catenella* in both estuarine samples from Elkhorn Slough, California and from sea water samples from inner Monterey Bay. Samples were subjected to a density-barrier sample enrichment technique employing percoll to separate debris from phytoplankton prior to sample fixation, labeling and analysis. Clarified, enriched preparations of phytoplankton were subjected to whole cell Fluorescent *In Situ* Hybridization (FISH) using a ribosomal DNA (rDNA) probe specific for the North American ribotype (NA1) of the dinoflagellate genus *Alexandrium* and analysis by flow cytometry. Flow cytometry was validated using epifluorescence microscopy on paired samples. Density-barrier sample enrichment and flow cytometry employing multi-parametric logical gating enabled detection *Alexandrium catenella* down to concentrations of 10 cells L^{-1}.

Samples were taken semimonthly from 10 stations along the entire length of Elkhorn Slough and 1 station a mile offshore of the Moss Landing harbor entrance over a two year period from July 2006 to July 2008. In samples taken from the entrance to Elkhorn Slough, *Alexandrium catenella* was detected in low concentrations by flow cytometry and epifluorescence microscopy. In samples taken from the inland portions of Elkhorn Slough, rare occurrences of a few *A. catenella* cells were detected by flow cytometry while no *A. catenella* was detected by epifluorescent microscopy.

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PREFACE

This thesis consists of three chapters, appendices and references cited. Chapter I is a detailed introduction to the general effects of Harmful Algal Blooms, description of the toxic marine dinoflagellate *Alexandrium catenella*, characterization of Monterey Bay and Elkhorn Slough, current methods of detection for *Alexandrium*, flow cytometry, and environmental sample labeling. Chapter II is presented in manuscript format consistent with the journal "Harmful Algae." Chapter II reports the application of flow cytometry to detect *A. catenella* in both open water and estuarine environments. A density–barrier enrichment technique was used as a sample preparative step prior to Fluorescent *In Situ* Hybridization (FISH) of the samples followed by flow cytometry and epifluorescence microscopy. Chapter III consists of general conclusions about the use of flow cytometry in the field of Biological Oceanography, the use of flow cytometry as an environmental monitoring tool, a survey of other instrumentation designed specifically to monitor for species causing Harmful Algal Blooms (HABs), and possible future applications to flow cytometry in monitoring for HABs. The appendices provide additional supportive data, techniques, methods of data analysis, locations and bathometry of sample sites for both Elkhorn Slough and Monterey Bay as well as a description novel equipment manufactured for the acquisition of vertical samples in Elkhorn Slough presented in this research.

CHAPTER I
INTRODUCTION

1. Introduction

1.1. Harmful Algal Blooms

Phytoplankton are microscopic single celled, free-floating aquatic plants that are the primary producers in the marine environment. Phytoplankton, through photosynthesis, release oxygen from carbon dioxide and water using light energy produced via photosynthetic pigments. After viruses and bacteria, phytoplankton are the most numerous organisms in the ocean and form the base of trophic interactions in the marine environment. Some phytoplankton produce toxins. Under favorable conditions, toxic phytoplankton (algae) can form dense surface populations in coastal waters often referred to as Harmful Algal Blooms (HABs) that are associated with finfish death and contamination of shellfish that are consumed by humans. HABs are caused by harmful marine phytoplankton of the taxonomic division Dinophyta, often referred to as dinoflagellates (Greek *dinos,* whirling flagella).

Dinoflagellates are phytoplankton characterized by having two flagella, one transverse and the other longitudinal, which give the cell its characteristic whirling motion. Dinoflagellates are an ancient group of organisms, first appearing in the fossil record 240 million years ago. Taxonomically dinoflagellates were once considered an intermediate kingdom ("mesokaryota") due to having a nucleus lacking histones and having condensed chromosomes throughout the cell division cycle (Hackett et al., 2004). Dinoflagellates have chloroplasts containing the pigments chlorophyll *a* and *c*, β-carotene and peridinin. Peridinin is a taxonomic marker accessory pigment found only in dinoflagellates (Loblich, 1984). Dinoflagellates can be autotrophic, mixotrophic or in

some cases heterotrophic (Stoecker, 1999). Under bloom conditions, dinoflagellates can discolor sea surface water to form red-tides that are associated with shellfish contamination (Shumway, 1990).

1.2. Alexandrium catenella

One genus of dinoflagellates, *Alexandrium*, produces potent neurotoxins that can cause paralytic shellfish poisoning (PSP) in humans who have eaten contaminated shellfish (Carreto et al., 2001). The symptoms of PSP in humans include numbness in the mouth and extremities, ataxia, dizziness, headache, respiratory distress, paralysis and death (Whittle and Gallacher, 2000). One of the earliest recorded PSP-related events on the west coast occurred in 1793 in British Columbia during the Vancouver expedition. Several crew-members suffered deaths caused by paralysis and asphyxiation after eating shellfish taken from what is now known as "Poison Cove". In 1927 PSP-related deaths in San Francisco were attributed to human consumption of mussels contaminated with *Alexandrium* (Horner et al., 1997).

The genus *Alexandrium* consists of more that 30 different species worldwide (Steidinger et al., 1997). In the genus *Alexandrium*, 3 species (*A. catenella, A. tamarense and A. fundyense*) are found off the North American coasts with *A. catenella* being found off the Pacific coast and in Monterey Bay (Scholin et al., 1995). *Alexandrium catenella* (Whedon & Kofoid) Balech 1985, is a moderately sized phytoplankter ranging from 35 to 45 μm in diameter and 30 -35 μm in length, and is a chain-forming, toxin producing species. Blooms of *Alexandrium* may be associated with relaxation of seasonal marine upwellings in conjunction with shifts in wind patterns in late summer. These shifts in

wind patterns force warmer offshore water, with associated populations of *Alexandrium*, onshore (Horner et al., 1997). HABs that occur near the surface of the water can be influenced by wind waves and tangential water flow forming "stripes" of concentrated phytoplankton, called Langmuir cells (Evans and Taylor, 1980). Because of Langmuir circulation, concentrations of HAB- forming species, may be sporadic on surface water affecting the direct monitoring of HABs.

1.3. Monterey Bay and Elkhorn Slough

Monterey Bay, off the coast of Central California, is best known for its unique the deep submarine canyon that is in close proximity to the shore. From the Monterey Submarine Canyon deep, cold nutrient-rich water upwells to the surface under the influence of the California Current System (Collins et al., 2003). Elkhorn Slough is an estuary which is connected to Monterey Bay and the Salinas River, and has a large tidal prism (water exchange) with Monterey Bay. The mouth of Elkhorn Slough (termed the lower slough) interacts with Monterey Bay in a way more typical of California coastal marine conditions of cold, deeper waters. In contrast to the lower slough, the inland region of Elkhorn Slough (termed the upper slough) is shallower and more turbid with warmer water temperatures. These contrasting conditions in Elkhorn Slough exhibit a range of phytoplankton diversity in which the upper slough is biologically less diverse than the lower slough (Welschmeyer, 2007). Because *A. catenella* is typically found in colder, nearshore waters (and not in warmer environments) the upper slough is not an ideal environment to support a population of *A. catenella*. This dichotomy of environmental conditions within Elkhorn Slough with respect to water temperature,

turbidity, biological diversity and depth, present challenges to detecting *A. catenella* in seawater samples using flow cytometry. *Alexandrium catenella* is known to exist in Monterey Bay and the colder, more nutrient replete waters off-shore are ideal for *A. catenella*.

1.4. Alexandrium catenella detection

Current methods for the detection *Alexandrium* include monitoring seawater samples for *Alexandrium catenella* that has been fluorescently stained for enumeration by epifluorescent microscopy. Other methods of detection for *A. catenella* include monitoring seawater samples or animal tissue for the presence of proxy indicator toxins called saxitoxins. *Alexandrium* saxitoxins in shellfish extracts are detected by high performance liquid chromatography (HPLC) (Yu et al., 1998). Some other biochemical tests for detecting *Alexandrium* include, fast fluorometric assay (FFA) for saxitoxin determination (Gerdts et al. 2002), and a mouse bioassay (MBA) (AOAC 1999). HPLC can be used to detect of dinoflagellate-specific pigments such as peridinin (Caretto et al., 2001). A ship-board method, the Maritime *in vitro* Shellfish Test (MIST) Alert™, can be used to determine the presence of saxitoxin (Jellet et al., 2002). Molecular based methods for detection of *Alexandrium* include an enzyme-linked immunosorbant assay (ELISA) and the polymerase chain reaction (PCR) (Penna and Magnani, 1999). A novel method to detect *Alexandrium* in environmental samples uses DNA microarrays. This method detects cDNA derived from total RNA from samples that hybridizes with *Alexandrium* specific oligonucleotides spotted onto a solid substrate (Gescher et al., 2008).

1.5. Flow cytometry

Modern methods of detecting HAB forming species in marine samples employ short processing times and automation. Flow cytometry is an automated cell enumeration method used extensively in the fields of medical technology, cell biology and immunology. The instrument interrogates each cell as it flows through a laser beam. The interaction between the laser and each cell is measured with photodiodes and photo-detectors, and characterizes each cell within a complex mixture. Flow cytometers were developed primarily for differential analysis of human white blood cells (WBC) using small sample volumes, typically 50 μL - 500 μL. Since normal human blood contains on average 4,500 to 10,000 WBC's μL^{-1}, very little blood is needed for flow cytometric analysis. Using flow cytometry for environmental marine samples with low cell densities requires concentrating cells within the sample. Some species of phytoplankton have cell densities as low as a 5-10 cells per liter. Detecting a few cells of a species of interest in an environmental marine sample containing high concentrations of other cells and suspended debris is challenging. Since the flow cytometer is highly sensitive and has a small orifice (typically 100 μm), suspended debris and sediment must be removed prior to applying the sample to the instrument. In this study we employed a two-step centrifugation strategy that enabled concentrating phytoplankton cells and separating them from debris and sediment in the sample prior to analyzing the sample on the flow cytometer.

A flow cytometer measures fluorescence emission as well as physical cellular properties such as size and granularity (complexity) (Collier, 2000). Size is measured by

diffraction of light scattered by the cell which is termed forward scatter (FSC). Granularity of a cell (measured by the refraction and reflection of light) is termed side scatter (SSC). In a blood sample, the properties of forward scatter and side scatter can differentiate between granulocytes and lymphocyte subpopulations, but cannot differentiate lymphocytes such as T lymphocytes from B lymphocytes. To identify and enumerate cell populations of similar size and morphology by flow cytometry, other cellular parameters such as intrinsic or induced fluorescence emission must be used. Fluorescence emission can be accomplished using specific antibodies, labeled with a fluorophore. T lymphocytes and B lymphocytes are identical in size and granularity. Antibodies specific to each cell type can be labeled with different fluorescent chemicals (fluorophores) such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). Using this technique T lymphocytes and B lymphocytes in the same blood sample can be distinctly enumerated with a flow cytometer (Calvelli et al., 1993).

A flow cytometer uses an argon laser to excite fluorescently labeled cells. The argon laser incorporated within many flow cytometers has a peak excitation wavelength of 488 nm. This laser is used to excite a number of fluorophores that are conjugated to DNA molecules, antibodies, or lectins that can adhere to cellular surfaces (Anderson et al., 1999; Costas et al., 1994). A typical flow cytometer, such as a Beckton and Dickenson ^{TM} FACScalibur flow cytometer, has three or four different fluorescent photo-detectors, depending on the model, along with forward and side scatter detectors. Most of the photo-detectors are photomultiplier tubes (PMTs) with the exception of the forward scatter detector which is a photo-diode. PMTs convert incident photonic energy

into electrical charge (photoelectric effect). A photocathode, amplifies that electric charge, and converts the charge into a current pulse with the use of an anode (Hamamatsu K.K, 2006). The analog signal produced by the PMT pulse current is converted into a digital signal with an A to D converter. The digital signal is then processed and recorded by computer (BD Biosciences, 2000). A PMT detects incident photons within a broad specified range of wavelengths, typically from 200 nm to 900 nm. For detection of discrete wavelengths via a PMT, filters are placed in front of each detector that limits the fluorescence emission permitted to interact with the detector.

The Becton-Dickenson family of flow cytometers, the FACscan™, FACSsort™ , and the FACScalibur™ all use at least three PMT's for signal detection and are capable of analyzing forward scatter (FSC), side scatter (SSC) and fluorescence. Each fluorescence detector within the flow cytometer is designed to respond to a specific wavelength band. Fluorescence detector-1 (FL-1) is sensitive to 530±30 nm, fluorescence detector #2 (FL-2) is sensitive to 585±42 nm, and fluorescence detector-3 (FL-3) is sensitive to $\geq$670 nm. Light emitted from each cell is measured with respect to the parameters FSC, SSC, FL-1, FL-2 and FL-3 simultaneously, and is termed an event. Noise in a flow cytometer can be caused by inert particles reflecting and refracting light. This sample "noise" can be removed by setting a threshold value for one or more of the parameters allowing only light with values above a cut-off value to be recorded. It is desirable to achieve a high signal to noise ratio to accurately identify and enumerate cells of interest.

For a flow cytometer to identify and enumerate single cells within a complex sample, cells must be interrogated one at a time. This is accomplished through

hydrodynamic focusing in which a stream of unordered cells is constricted into a narrow stream of ordered, single cells for interrogation by the laser (Yang et al., 2007). The samples are interrogated in a flow cell at a defined rate of 60 μL min^{-1}. This defined flow rate allows for quantitative analysis and calculation of a cell concentration in cells mL^{-1}.

Studies pertaining to species complexity of phytoplankton communities from environmental samples have been performed using flow cytometry. Flow cytometry has been used to enumerate phytoplankton by measuring autofluorescence of naturally occurring pigments within the algal cells. These algal pigments include alloxanthin and chlorophyll *a*, which are detected on a flow cytometers FL-2 and FL-3 PMT's respectively (Welschmeyer, 2007). Using auto-fluorescent properties, along with FSC and SSC parameters, enables the use of multiparametric logical gating to enumerate phytoplankton in moderate concentrations within the samples (Sinigalliano et al., 2009).

Signal amplification of a target species can be achieved with the use of a DNA probe that is labeled with FITC and can be detected by the flow cytometers FL-1 detector. With the use of species-specific FITC labeled probe, it is possible to expand a multiparametric logical gate to include enhanced FL-1 signaling thereby increasing the signal to noise ratio. Using a multiparametric logical gating scheme, an event must fall within each of the pre-defined parameters for it to be recorded as a positively detected event. A logical gate used in sample analysis would then include auto-fluorescent signaling from chlorophyll *a* (FL-3), FITC labeled probe (FL-1) as well as forward scatter and side scatter. Using these four parameters in a multiparametric logical gate

analysis, it may be possible to enumerate a rare phytoplankton species within a densely populated environmental sample with a flow cytometer (Sinigalliano et al., 2009).

1.6. Sample Labeling

To accurately identify *Alexandrium* cells in environmental samples, a species-specific DNA probe was used on fixed cells (Sako et al., 2004). DNA probes were hybridized to fixed samples at specific temperatures and conditions for optimal specific binding of the probe to the correct cell and minimize non-specific reactions. Fixing the target cells prior to hybridization maintains their morphology. The process of fixing the cells followed by probe hybridization is called fluorescent *in situ* hybridization (FISH). Cells subjected to DNA-directed FISH can be identified by their acquired fluorescence, size and morphology using both epifluorescent microscopy and flow cytometry (Adachi et al., 1996). Epifluorescent microscopy is accurate and extremely time consuming, whereas flow cytometry is fast and amenable to automation.

Because of health and economic concerns of HABs and their recent increase in frequency in nearshore waters, there is an important need for a quick, simple and automated method of detecting of HABs (Hallegraeff et al., 1995). On the east coast of the United States, the Massachusetts Water Resources Authority has developed a contingency plan that describes a caution level for *Alexandrium tamarense* as 100 cells L^{-1} for nearshore fisheries (Hornbook, 2002). In recent years Monterey Bay has had several large HABs consisting of different dinoflagellate species, such as *Akashiwo sanguinea* and *Cochlodinium* spp. (Curtiss et al., 2008; Kudela et al., 2008). Large concentrations of *A. catenella* cells in seawater samples, as well as saxitoxins in finfish

and shellfish tissue, have been detected in Monterey Bay (Jester et al., 2009). With the presence of *Alexandrium* in Monterey Bay, there is a need for robust, quantitative and simple monitoring methods. Monitoring environmental samples using NA1-directed FISH and flow cytometry may be useful in rapidly evaluating a large number of environmental samples for *A. catenella*.

In this study a DNA probe (NA1) is homologous to a small portion of the large ribosomal sub-unit of *Alexandrium catenella/tamarense* North American ribotype (Scholin et al., 1994) (hereafter referred to as *A. catenella*). The probe was used to detect *A. catenella* in environmental samples taken from Elkhorn Slough and Monterey Bay. The NA1 probe is an oligonucleotide which is dual-labeled at the 5' and the 3' end with FITC. The probe was used for DNA-directed FISH of the samples which were analyzed by epifluorescence microscopy and flow cytometry to detect NA1-FITC labeled *A. catenella*. In our samples, epifluorescent microscopy showed that the upper Elkhorn Slough was devoid of *A. catenella*, whereas flow cytometry detected a few "events". However in samples from the lower slough, and analyzed by flow cytometry and epifluorescent microscopy, *A. catenella* was consistently detected in moderate concentrations. The flow cytometry results obtained were highly correlated to observed epifluorescent microscopy results. Samples were processed in a novel way to concentrate phytoplankton yet remove debris and sediment. This method enabled us to analyze samples without fouling the flow cytometer and reduced false positive results due to non-specific binding of the NA1 probe to debris.

CHAPTER II

FLOW CYTOMETRIC DETECTION OF *ALEXANDRIUM CATENELLA* FROM ELKHORN SLOUGH AND MONTERY BAY, CAIFORNIA. (USA).

FLOW CYTOMETRIC DETECTION OF *ALEXANDRIUM CATENELLA* FROM ELKHORN SLOUGH AND MONTEREY BAY, CALIFORNIA. (USA).

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ABSTRACT

Flow cytometry, originally designed for use in clinical research in human blood analysis and human cell biology, has since been adapted by various groups for fast analysis of specific target populations of bacteria and phytoplankton in environmental samples. We used the ribosomal DNA (rDNA) sequence-based probe specific for *Alexandrium* North American ribotype (NA1), natural chlorophyll fluorescence, size and granularity in quantitative flow cytometry to enumerate *Alexandruim catenella*. To concentrate the samples for analysis by flow cytometry a new preparative technique was developed and applied to the detection of *A. catenella* in seawater samples of moderate turbidity. Environmental samples of 500 mL were collected semi-monthly from July 2006 to July 2008 from eleven stations in Elkhorn Slough, and Monterey Bay, CA. Samples were concentrated to 1 mL using a density-barrier enrichment protocol, with the use of percoll as the density-barrier media. The resulting sample, enriched for phytoplankton, was devoid of debris. Whole cell fluorescence *in situ* hybridization (FISH) of the enriched samples was done using the NA1 probe and the universal control probe uniR, then analyzed by flow cytometry and epifluorescent microscopy for the presence of *A. catenella*. Using multiparametric sequential logical gating in analyzing the flow cytometric results, *A. catenella* within the enriched samples could be detected to a resolution of 10 cells L^{-1}. *A. catenella* was consistently detected in low concentrations (40-50 cells L^{-1}) in samples taken from entrance of Elkhorn Slough. In samples taken from the shallower inland regions of Elkhorn Slough, no *A. catenella* were detected by

epifluorescent microscopy, while rare occurrences of a few events were detected by flow cytometry. Flow cytometry, augmented with density–barrier sample enrichment and multiparametric logical gating, was shown to accurately enumerate *A. catenella* from a wide range of sample environments.

1. Introduction

Dense concentrations of nearshore toxic marine phytoplankton leading to formation of Harmful Algal Blooms (HABs) are reported to be increasing worldwide in coastal areas, bays and estuaries (Hallegraeff et al., 1995; Heisler et al., 2008). HABs due to the dinoflagellate *Alexandrium catenella* can cause Paralytic Shellfish Poisoning (PSP) (Tillman et al., 2002). Increased incidence of HABs is an economic and public health concern which affects humans that consume shellfish contaminated with *Alexandrium* (Shumway, 1990).

Dinoflagellates of the genus *Alexandrium* occur on both the west and east coasts of the United States as well as temperate waters of Western Europe, Australia and the Japanese Islands (Scholin et al., 1995). The genus *Alexandrium* consists of more than 20 species that are classified by morphometric features, such as plate tabulation (Steidinger et al., 1997). The *Alexandrium* North American species complex is a grouping of *Alexandrium* found in North America which consists of the species *A. catenella* and its ribotype relatives (*A. tamarense* and *A. fundyense*). *Alexandrium catenella* is exclusively distributed along the west coast and *A. fundyense* is found on the east coast, while *A. tamarense* is distributed on both the west and east coast of the United States (Scholin et al., 1995). *Alexandrium catenella/tamarense* occurs in open nearshore high

salinity zones and are transported into bays and estuaries by tidal influences (Cloern and Dufford, 2005). Most of the species within the *Alexandrium* genus produce a potent array of voltage-gated sodium ion-channel blocking neurotoxins such as saxitoxin (STX), neosaxitoxins (neoSTX) and gonyotoxins (GTX) (Kao et al., 1982). These toxins are produced by *Alexandrium* and are sequestered in filter feeding bivalves that ingest them. Subsequent human ingestion of these bivalves can lead to PSP. The symptoms of PSP in humans include numbness in the mouth and extremities, ataxia, dizziness, headache, respiratory distress, paralysis and death (Whittle and Gallacher, 2000).

Current methods of detection of toxic *Alexandrium* include high performance liquid chromatography (HPLC) to detect the presence of the STX toxin in seawater (Yu, 1998), HPLC to detect dinoflagellate-specific pigments such as peridinin in seawater samples (Zapata et al., 2000; Caretto et al., 2001), a fast fluorometric assay (FFA) for STX determination (Gerdts et al., 2002), mouse bioassay (MBA) (AOAC, 1999), and the Maritime *In Vitro* Shellfish test (MIST) Alert^{TM} to determine the presence of STX in real-time (Jellet et al., 2002). Molecular methods for detecting of *Alexandrium* include polymerase chain reaction (PCR) and an enzyme-linked immunosorbant assay (ELISA) (Penna and Magnani 1999; Penna and Magnani 2000; Peperzak et al., 2000). A remote *in situ* ELISA system for detecting harmful algal species termed the Environmental Sample Processor (ESP) has been used for automated detection of *Alexandrium* as well as other HAB species (Greenfield et al., 2008).

Flow cytometry is a fast, quantitative method that can give insight into the ecology and presence of an individual algal species during HABs (Collier, 2000). Flow

cytometry was developed for clinical laboratory analysis of human blood in order to enumerate and differentiate white blood cells, and was later adapted for analysis of algal cells under environmental conditions (Cunningham, 1986). Flow cytometry characterizes and enumerates cells based upon size and granularity using laser diffraction and refraction (Collier, 2000). In the fields of oceanography and environmental biology, flow cytometry has recently made a large impact as a versatile quantitative instrument. Early work in flow cytometry on environmental samples was limited by the size of cells and autofluorescence spectra of naturally occurring algal pigments (Phinney and Cuccu, 1989). Cells of an individual species that has been tagged with a specific fluorescent probe can also be enumerated based on relative fluorescence.

Complex populations of phytoplankton have been differentiated by flow cytometry by detecting multiple natural auto-fluorescent pigments contained within the phytoplankter (Sinigalliano, 2009). Automated methods to enumerate phytoplankton include the use of solid-phase cytometry (SPC) using the Chem*Scan*^{TM} system (Chemunex, Irvy, France) (Lemarchand, 2001) and the FlowCAM system (Fluid Imaging Technologies) (Buskey and Hyatt, 2006). Future refinements in flow cytometry applications in the marine environment that include specific probes may enhance our ability to characterize and enumerate individual species within a complex mixture in a rapid fashion to evaluate their changes over time. To increase fluorescence intensity, DNA binding dyes such as SYTOX Green and SYBER Green have been used in flow cytometry (Marie et al., 1997; Veldhuis et al., 1997). Flow cytometric cell sorting has augmented investigations in metabolic studies using ^{13}C tracer uptake by phytoplankton

(Roel et al., 2004). Trophic level interactions in plankton have been investigated with the use of flow cytometry (Pires et al., 2004). Viruses and other parasites that internally infect microplankton have also been detected by flow cytometry (Brussaard et al., 1999).

Specific molecular probes such as monoclonal antibodies specific to *Alexandrium* (Aguilera et al., 1996; Anderson et al., 1999), plant lectins which bind to *Alexandium's* outer theca (Costas and Rodas, 1994; Rhodes et al., 1995), and nucleic acid sequences specific to *Alexandrium* rRNA have been employed using epifluorescent microscopy and flow cytometry. Fluorescent *in situ* hybridization (FISH) (Adachi et al. 1996; Miller and Scholin, 1998, Vreiling et al., 1994) has the advantage of specifically staining fixed cells directly prior to visual detection by fluorescent microscopy or by flow cytometry. Fluorescein isothiocyanate (FITC), with its 495 nm absorption peak and 520 nm emission peak, allows for detection of the hybridized target cells by instruments that incorporate both an argon laser (peak excitation at 488 nm) and a detector sensitive to 530 nm ± 30 nm (Allman et al., 1990).

A major impediment to using flow cytometry for environmental monitoring is the highly variable amounts of suspended particles and sediment that may be present in the sample. Turbidity in bays and estuaries caused by suspended particles, eutrophication from increased nutrient loading and mixing due to tidal prism, make direct detection of HAB forming species using flow cytometry problematic. Flow cytometers, originally designed for use on human blood samples are easily fowled by turbid environmental samples with suspended debris and sediment. Suspended debris includes seasonal

zooplankters such as copepods, invertebrate larvae, and larger multi-cellular colonial phytoplankton.

Another issue in adapting flow cytometry for environmental assessment is that flow cytometers in clinical settings typically analyze small sample volumes (usually less than 200 μL. Concentrating the sample prior to analysis by flow cytometry is important when monitoring minority populations during HABs or species in non-bloom conditions where large sample volumes may be required. A recent study has evaluated the use of filtration and centrifugation of natural seawater samples prior to hybridization to reduce debris and concentrate microorganisms in the sample (Hosoi-Tanabe and Sako, 2005). Inevitably these methods incur cell losses leading to underestimates of ground-truth environmental cell concentrations. To decrease the loss of phytoplankton in samples collected, we developed an adaptation of a density-enrichment method originally designed to retrieve dinoflagellate cysts from sediment (Swinghamer, 1991). We employed this method to enrich surface seawater samples for phytoplankton while removing debris. The goal of this study was to evaluate the distribution and seasonal variation of *A. catenella* in estuarine and inshore marine environments in Elkhorn Slough and Monterey Bay, CA. using rDNA probe directed FISH and flow cytometry.

2. Materials and methods

2.1. Dinoflagellate cell culture isolation

Dinoflagellate cells used in this study, were isolated from Elkhorn Slough, CA (*Alexandrium catenella*, *Prorocentrum micans*), and South San Francisco Bay (*Heterocapsa triquetra* and *Karenia mikimotoi*), and brought to unialgal culture. Individual cells for each

species were identified microscopically in seawater samples and captured with a P-20 micropette tip. A single cell was then transferred to a six-well microtiter plate with each well containing 3 mL of filtered seawater and incubated for one week at 15^{o}C, under 12:12 L/D cool white fluorescent lights with a photon flux density of 70 μmol m^{-2} s^{-1}. After this initial incubation period, 3 mL of f/2 media at pH 8.0 was added to wells containing viable, motile cells. If cells were found to be viable, an additional 3 mL of f/2 media was added and allowed to incubate for an additional week. After two weeks, 2.0 mL viable cell cultures were transferred to 15 mL polypropylene Falcon™ tubes each containing 5 mL of f/2 media at pH 8.0 and incubated further. One week later an additional 5 mL of f/2 media was added to actively replicating cultures and the cultures, were incubated to confluency. Confluent cultures were split 1:40 in f/2 medium, and maintained in continuous log phase growth.

2.2. Sampling sites

Seven samples from Monterey Bay were taken off the R/V Point Sur outside the entrance of Elkhorn Slough along the northern edge of the Monterey canyon on 14 March 2007 (Fig. 1A).

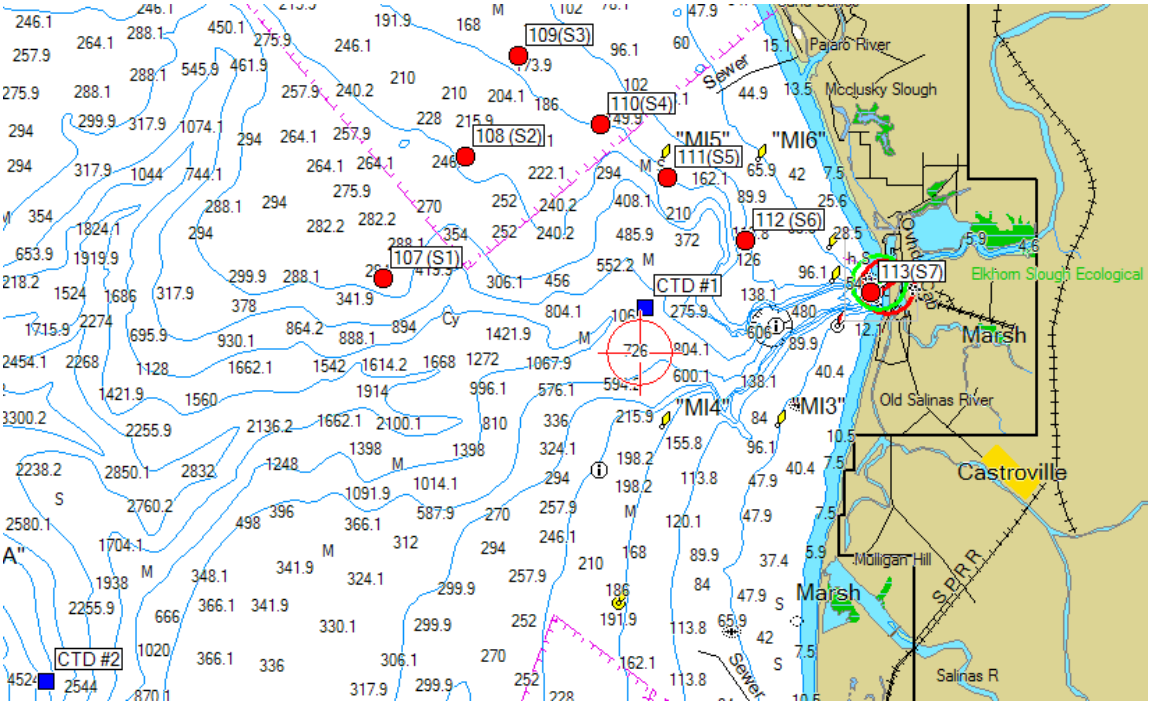

Fig 1A. Location of seven surface marine sample sites in Monterey Bay selected for analysis by NA1-directed FISH flow cytometry and epifluorescent microscopy.

Nearshore marine and estuarine samples were collected from one station in Monterey Bay and ten stations from Elkhorn Slough, in Monterey County, California a tidal salt marsh connected to Monterey Bay and the Salinas River. Surface samples were taken twice a month over a two year period. All samples were prepared as described below and analyzed by flow cytometry and epifluorescent microscopy (Fig. 5).

Fig. 1B. Location of eleven sample sites (one Monterey Bay and ten Elkhorn Slough) selected for analysis by NA1–directed FISH flow cytometry and epifluorescence microscopy.

2.3. Sample preparation

Near surface samples from Elkhorn Slough and Monterey Bay were collected in 1 liter dark bottles. Samples were taken semimonthly from July 2006 to July 2008 at high tide +/- 1 hr, stored at 4^{o}C, and processed within a week. A 500 mL aliquot of each sample was initially filtered through a 70 μm nylon cell strainer (Becton Dickenson, Franklin Lakes, NJ). A pre-filtered 500 mL aliquot of sample was centrifuged at 300 x g at 16^{o}C for 15 min. and resuspended in 5.0 mL of sample in filtered seawater. The concentrated seawater sample (representing a 500 mL sample volume) was then

overlayed dropwise onto 5.0 mL of Percoll™ (density 1.130 g/mL) (GE Healthcare, Piscataway, NJ) (Fig. 2). The tubes were then centrifuged at 280 x g at 16^{o}C for 30 minutes and decelerated with no brake as not to disturb the Percoll™ -sample interface. Located in the bottom of this 6 ml fraction, at the interface between the denser percoll layer and the seawater layer, is a discrete band of phytoplankton dominated by dinoflagellates, cryptophytes and smaller diatoms, referred to as the "phytoplankton band". Following this rate-zonal centrifugation, the top 6 mL including of the interface was collected and transferred to a new 15 mL falcon tube. All other debris along with larger, more dense diatoms are concentrated at the bottom of the tube below the percoll layer (Fig. 2). The phytoplankton band and the upper layer were transferred to a new 15 mL falcon tube.

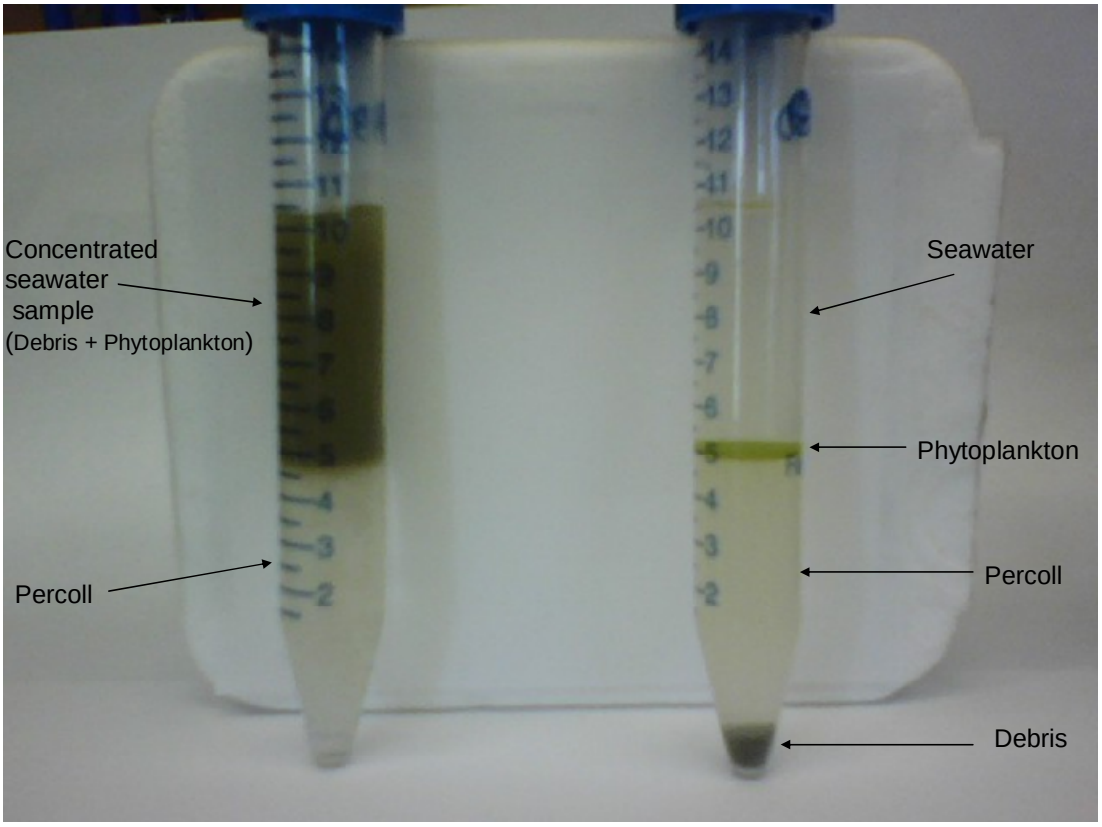

Fig. 2. Preparations of seawater samples of a resuspended pellet from 250 mL for FISH analysis by density-barrier enrichment before (left) and after (right) centrifugation.

Six milliliters of 0.2 μm filtered seawater was then added to the concentrated sample to dilute out any residual Percoll™ and the tube was centrifuged at 280 x g for 15 min. The phytoplankton pellet was then resuspended in 1.0 mL of filtered seawater and transferred to a 1.6 mL microfuge tube for FISH hybridization. The sample was then split equally into two 1.6 mL microfuge tubes (500 μL each). Each one these duplicate samples were then hybridized using the centrifuge based FISH protocol for flow cytometrc analysis and for epifluorescence microscopy (Sako et al., 2004). One tube was hybridized to the NA1

Alexandrium genus specific DNA probe and the other tube hybridized to the uniR universal negative control DNA probe (Baker and Cowan, 2004).

2.4. Sample hybridization

The hybridization temperature was adjusted from 40^{o}C to 37^{0}C, for the centrifuge-based FISH protocol using the NA1 probe and the hybridization wash step temperatures were adjusted from 50^{o}C to 47^{o}C. Completed hybridizations of 750 μL in 5x SSC (83 mM NaCl, 83 mM Sodium Citrate) were split into two aliquots for a side-by-side comparisons of flow cytometry (375 uL) and epifluorescent microscopy (375 uL).

To monitor hybridization efficiency, cultured *Alexandrium,* was hybridized to the uniR probe (universal negative control), uniC probe (universal positive control) and a hybridization containing no probe. To test for probe cross-reactivity during hybridization, three marine armored dinoflagellates, *Heterocapsa triquetra, Prorocentrum micans and Karenia mikimotoi* were used in addition to the cultured *Alexandrium*. DNA probes were synthesized with 6-FAM (FITC) fluorochromes on both the 5' and the 3' ends of the synthesized DNA oligonucleotide and PAGE-purified (Oligos Etc. Wilsonville, OR). Three probes that were used, NA1 probe specific for *Alexandrium* North American ribotype (FITC 5'-AGT GCA ACA CTC CCA CCA-3' FITC) , uniC positive universal control for conserved 18S ribosome (FITC 5'- GWA TTA CCG CGG CKG CTG-3' FITC) and the negative universal control uniR, (FITC 5'-CAG CMG CCG CGG UAA UWC-3' FITC) the reverse complement to the uniC probe (Baker and Cowan, 2004; Suziki and Giovannoni,1996). Using the centrifuge-based FISH protocol described by Sako et al. 2004, 0.5 μM of each probe was used in each

hybridization in this study. Along side of these probe hybridizations a no probe control was processed in order to scale relative fluorescence. For sample controls 2500 cultured *Alexandrium* cells in log phase growth were hybridized to the probes NA1, uniR and uniC. Data from paired samples prepared for flow cytometry and epifluorescent microscopy as described above were compared to evaluate efficacy of flow cytometric detection of *Alexandrium* in turbid bay and estuarine waters.

2.5. Flow cytometry data acquisition and analysis

Flow cytometry on environmental samples was performed on a FACScan™ flow cytometer (Becton-Dickenson, San Jose, CA.) using distilled water as sheath fluid. Flow cytometer data acquisition parameters used for sample enumeration were as follows: Forward scatter (FSC) photodiode voltage set to E-1, Side Scatter (SSC) photomultiplier tube (PMT) voltage set to a value of 220, FL-1 (FITC Channel) photomultiplier voltage set to 451, FL-3 (Chlorophyll a channel) photomultiplier tube voltage set to 308. Threshold was assigned to FL-3 at a value of 130. Flow cell rate was set on high (60 μL min^{-1}) and samples were collected for 5.0 min. Data analysis was done with CellQuest™, with multiparameter logical gating of defined regions using laboratory cultured *A. catenella*. Region #1 (FSC vs. SSC) defines the physical characteristic of *Alexandrium* with respect to size and granularity (Fig. 3A). Region #2 (FL1 vs. SSC) defines the population of *Alexandrium* labeled with NA1 FITC labeled probe (Fig. 3B). Region #3 (FL3 vs. SSC) defines that region of *Alexandrium* that exhibits chlorophyll *a* fluorescence (Fig.3C). Gate #1 was a logical gate of R1 and R2 and R3, and was

represented on a scatter plot of FL1 vs. FL3. Events arising from environmental samples that fell within gate #1 were designated as *Alexandrium* cells (Fig. 3 Box D).

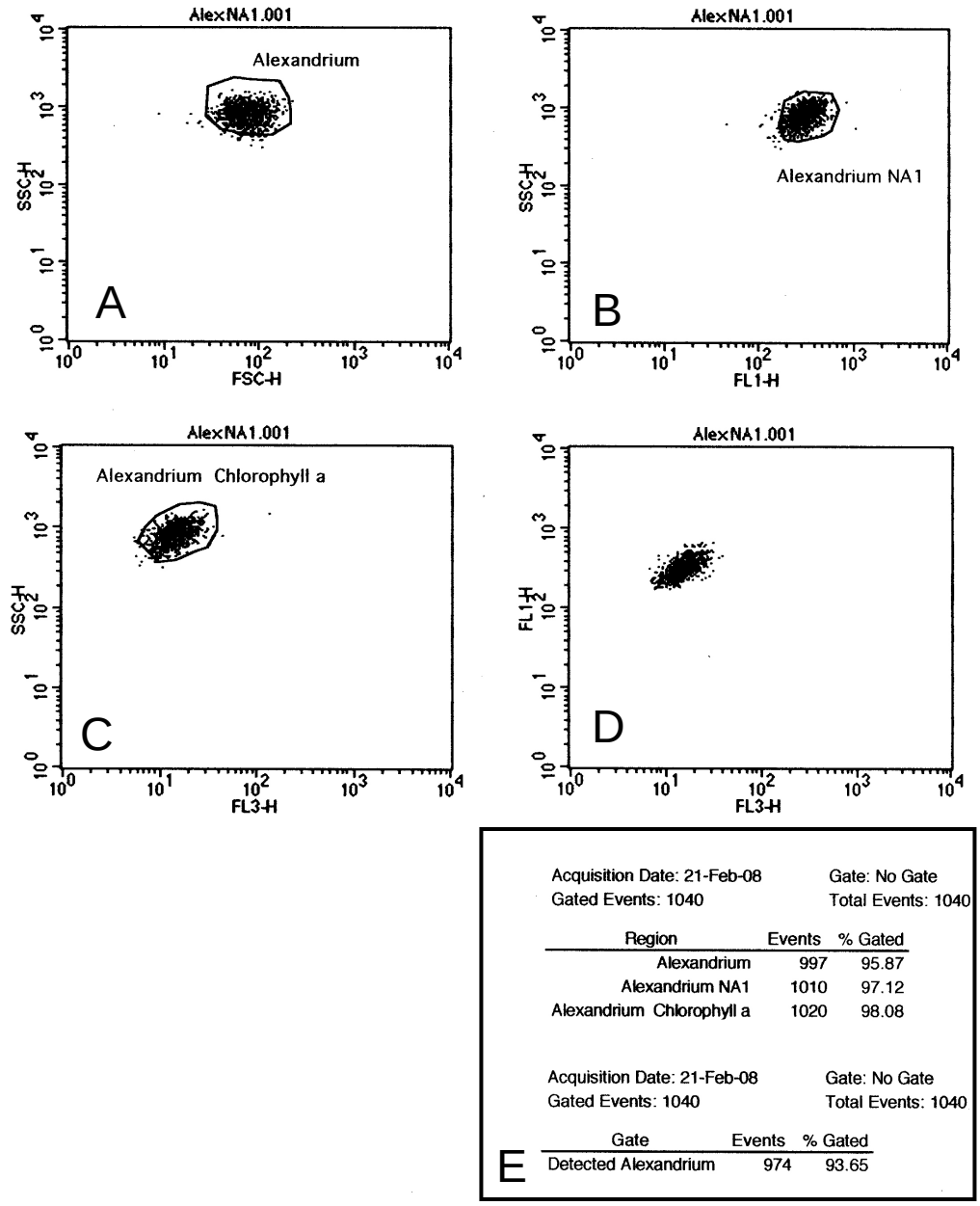

Fig. 3. Flow cytometry dot plots of a sample prepared from a pure laboratory culture of *Alexandrium* subjected to NA1-directed FISH and sequential logical gating.

Box A: Size and granularity, Box B: *Alexandrium* NA1 probe hybridized, Box C: *Alexandrium* intrinsic chlorophyll a fluorescence, Box D: Gated *Alexandrium.*

Alexandrium concentrations in environmental samples are calculated from the equation adapted from (Paul, 2001) where N equals the number of *Alexandrium* cells existing in the environment in cells L^{-1}, C is the number of events acquired, T is the duration of analysis in minutes, and R is sample delivery rate (µl min^{-1}) (Paul, 2001).

Eq. 1 $N = [C \times (Vc/T\ R)]/CF$ where $CF = Va/Ve$

Ve is the environmental cell sample volume, Va is the volume of unconcentrated sample analyzed and Vc is the volume of concentrated sample applied to the cytometer. The CF is the coefficient of the fractional volume of sample used for flow cytometry, where total environmental sample volume is 1000 mL (Ve) (used in cells L^{-1}calculation), and the analyzed environmental sample volume (Va) represents the unconcentrated volume (125 mL) interrogated by the flow cytometer ; therefore CF = 125 mL/ 1000 mL = 0.125.

In this procedure (using the NA1 or the uniR probe) 500 mL of an environmental sample was processed by density-barrier centrifugation and concentrated to 1.0 mL. This concentrated sample was then split equally into two 500 µL samples in which one was NA1–directed FISH treated and the other uniR directed FISH treated. Following hybridization both sampled were resuspended in 750 µL and split into two 375 µL aliquots to be analyzed. To one aliquot, 300 µL was analyzed by flow cytometry and to the other aliquot 300 µL of cells were layered on to a polycarbonate filter and analyzed by epifluorescent microscopy. Using these calculations, one event (C in the equation

above) reported by the flow cytometer is equivalent to 10 cells L^{-1} in environmental concentration.

3. Results

In preparation for flow cytometry and epifluorescent microscopy, starting samples of 500 mL seawater containing zooplankton, phytoplankton, suspended debris were concentrated to 5 mL then over-layed on top of percoll for density barrier enrichment in which phytoplankton were segregated into a distinct band (Fig. 2). The resulting phytoplankton bands included dinoflagellates (naked and armored) as well as ciliates, chlorophytes, cryptophytes and some smaller bacillariophytes (data not shown) and a debris pellet consisting of particulate matter, sediment and most large bacillariophytes. Preliminary tests using pure *Alexandrium catenella* cultures, found that 94% of *A. catenella* was recovered in the phytoplankton band (data not shown).

In NA1-directed FISH samples from Elkhorn Slough and Monterey Bay, the probe showed some cross-reactivity with *Akashiwo sanguinea* during a large HAB formation of *A. sanguinea* in Monterey Bay during November 2007 (Fig.4A).

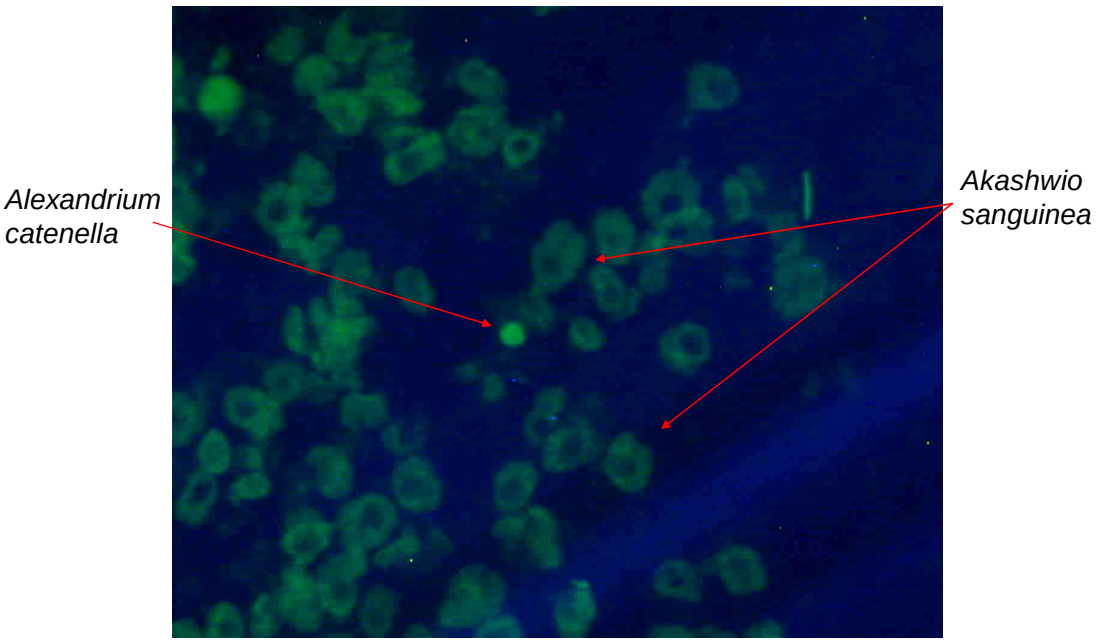

Fig. 4A. Single *Alexandrium* cell showing increased fluorescence during 17 November 2007 *Akashiwo sanguenia* bloom in Elkhorn Slough after NA1-directed FISH.

This cross-reactivity of the NA1 probe with *A. sanguinea* was observed at the hybridization temperature of 37^{o}C. During the density-barrier centrifugation process in enriching the samples and the FISH centrifugation protocol, naked dinoflagellates such as *A. sanguinea* were shown to remain intact. *Alexandrium catenella*, which is smaller and more spherical in shape than *A. sanguinea*, has symmetrical epitheca and hypotheca which remain intact during density-barrier enrichment centrifugation, allowing for identification of *A. catenella* by staining and morphological characteristics by epifluorescent microscopy.

Pure *A. catenella* laboratory cultures, which had been subjected to NA1-directed FISH and analyzed by flow cytometry, were used as reference controls for analyzed environmental samples. Multiparameter, logical gating with respect to granularity and size (SSC vs. FSC), granularity and NA1 fluorescence (SSC vs. FL-1), granularity and chlorophyll *a* auto-fluorescence (SSC vs. FL-3) was used to define an *A. catenella* specific region in complex environmental samples. These control laboratory culture-defined regions detect *A. catenella* in complex mixtures of phytoplankton (Fig. 4B).

Our analysis of seawater samples taken from the mouth Elkhorn Slough during the November 2008 *Akashiwo sanguinea* algal bloom in Monterey Bay, (Jessup et al., 2009) revealed a minority population of *A. catenella*. These samples were subjected to NA1-directed FISH and analyzed by epifluorescence microscopy and flow cytometry. Multiparametric logical gating detected the presence of the minority population of *A. catenella* cells within this algal bloom. Within this *A. sanguineum* algal bloom, in our analysis, the minority population of *A. catenella* was at a concentration of 40 cells L^{-1} (Fig 4B, box E). These data were confirmed with epifluorescent microscopic enumeration of the same sample (Fig. 4A).

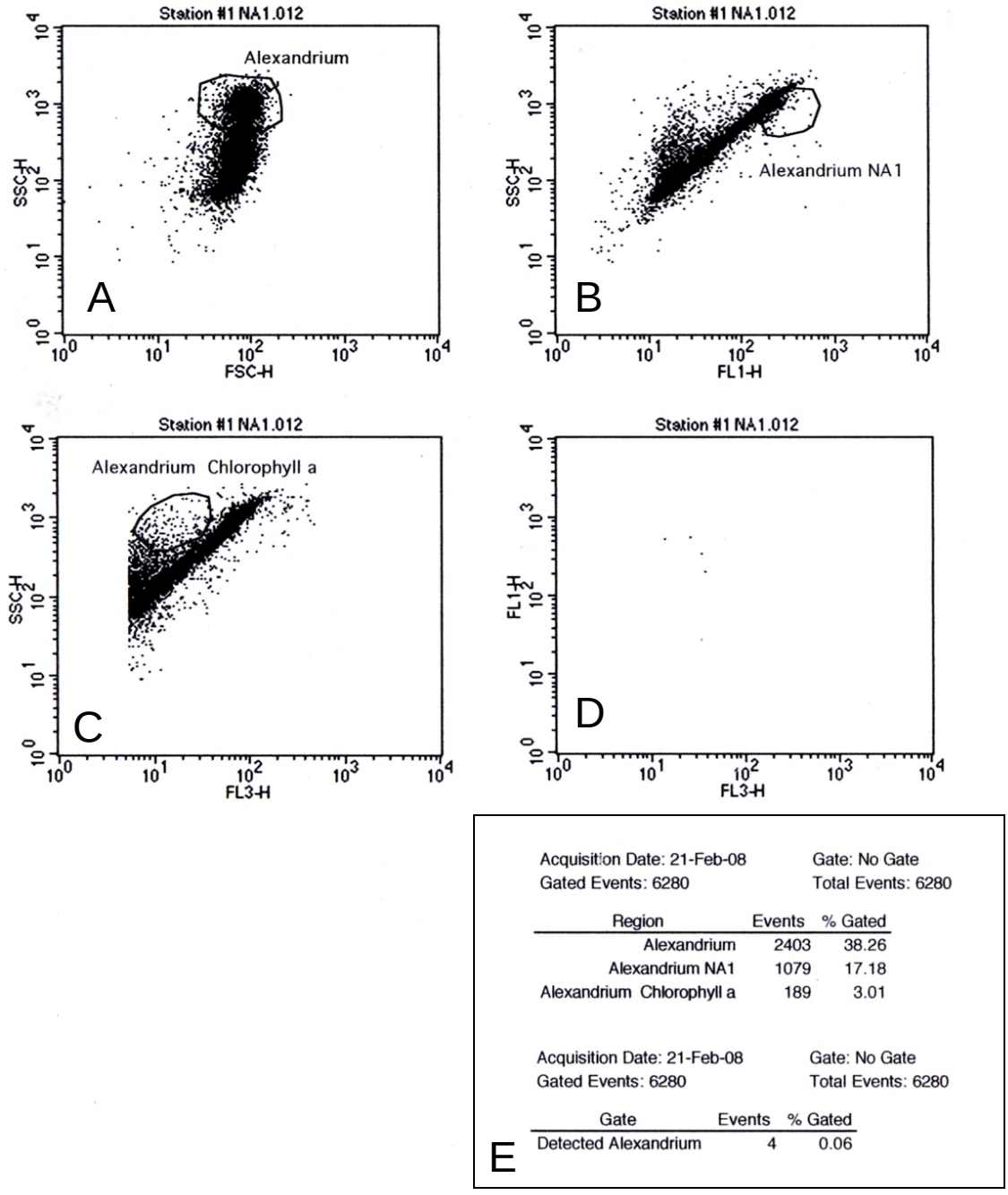

Fig. 4B. Flow cytometry dot plots of a field sample from station #1 subjected to NA1-directed FISH and sequential logical gating. (see Fig. 4A. for epifluorescence microscopy of sample).

Seven near-shore surface samples from Monterey Bay were analyzed using NA1-directed FISH followed by flow cytometry and epifluorescent microscopy. In a

comparison of flow cytometry and the epifluorescent microscopy results, taken from Monterey Bay, all seven samples had good agreement (r^2 correlation of 0.8924). For stations 107-109, the samples from the most offshore sites had lower concentrations (0 - 10 cells L^{-1}) of *A. catenella* than the nearshore samples (30- 50 cells L^{-1}). (Fig.1A and 5).

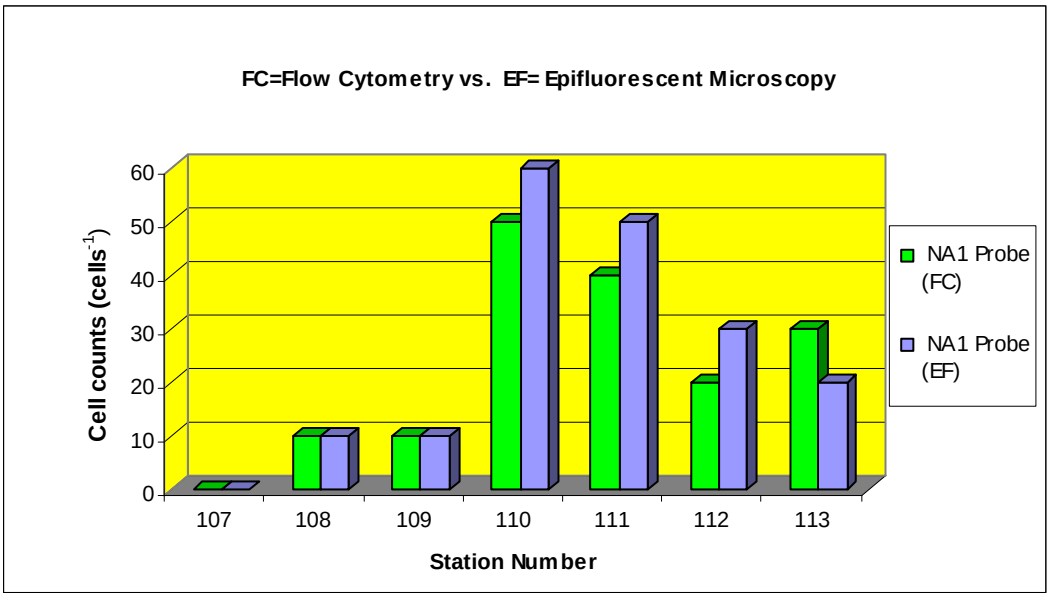

Fig. 5. Comparison of NA1-directed FISH flow cytometry and epifluorescent microscopy results from surface samples collected at seven sites in Monterey Bay.

The highest concentration of *A. catenella* was found in samples from the sites near a sewage out-flow pipe associated with the Watsonville treatment plant and effluent from the Pajaro River (samples 110 and 111). In these Monterey Bay samples the maximum concentration observed was 50 cells L^{-1} as measured by flow cytometry and 60 cells L^{-1} as measured by epifluorescent microscopy. To characterize how deeply an *A .catenella* population could establish in an estuary, eleven sample sites (one Monterey Bay and ten

from Elkhorn Slough) were selected for analysis over a two year period by NA1-directed FISH flow cytometry and epifluorescent microscopy. Comparison of the NA1-directed FISH flow cytometry and epifluorescent microscopy results from samples collected from these 11 sites showed that the highest concentrations of *A. catenella* occurred at station 1 (180 cells L^{-1}, Fig. 6B) which is one nautical mile from the entrance to Elkhorn Slough (Fig.1B).

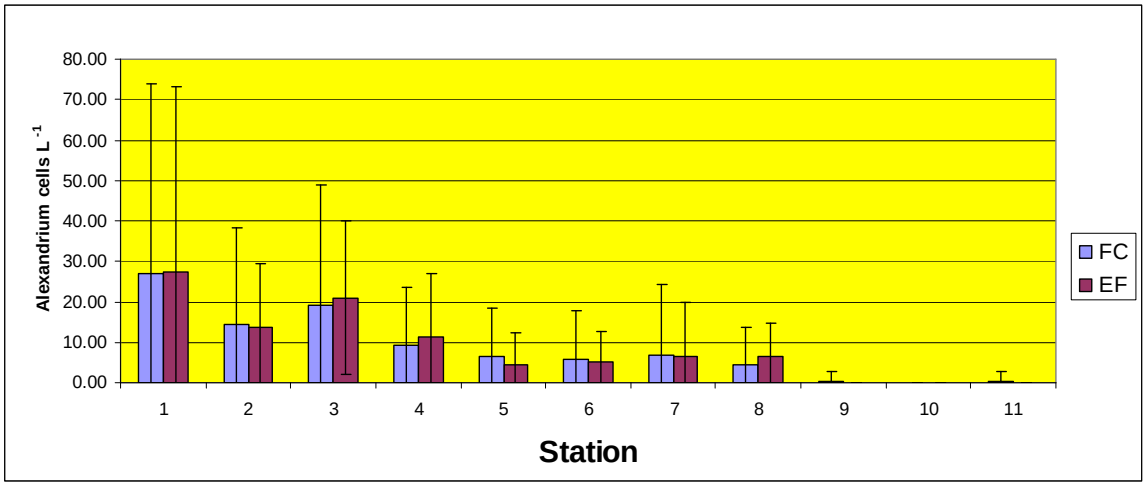

Fig.6A. Comparison of NA1-directed FISH flow cytometry and epifluorescence microscopy results from samples collected at one station in Monterey Bay and ten stations in Elkhorn Slough; FC = Flow cytometry, EF = Epifluorescent Microscopy.

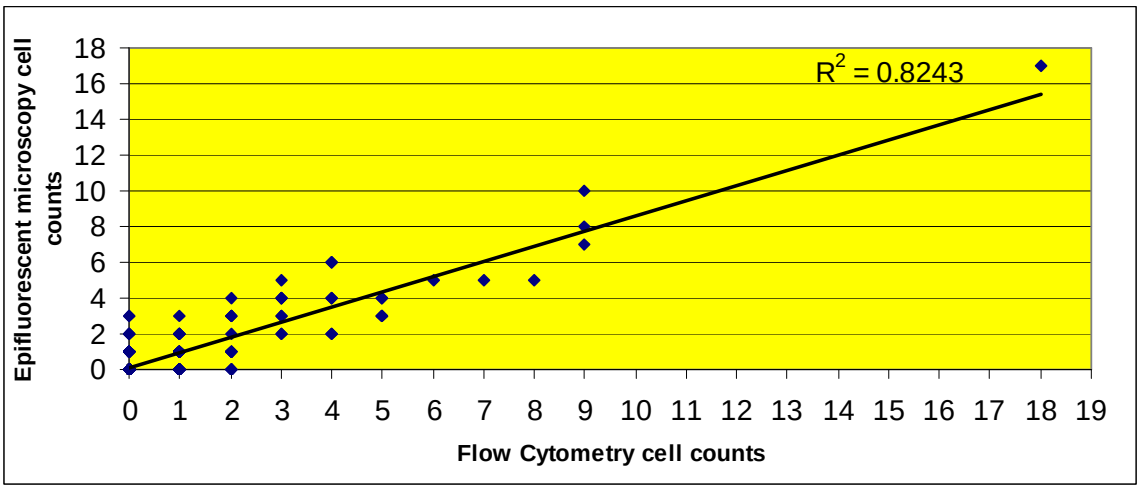

Fig. 6B. Correlation of all 253 NA1-directed FISH flow cytometry and epifluorescence microscopy data from Monterey Bay and Elkhorn Slough samples. (P (≥3 events) = 0.868).

The P value (Fig. 6B) was calculated from those samples containing equal to or more than 3 events of detected *Alexandrium* as observed by flow cytometry and epifluorescent microscopy. The correlation value r^2 for these data is 0.8243 and was obtained by comparing epifluorescent microscopy results vs. flow cytometry results of all samples from Elkhorn slough and Monterey Bay. In this graph, the 31 data points are representative of all 253 samples where many of the data-points are redundant observed values of multiple observations. In the flow cytometry and the epifluorescent microscopy results, because of the degree of concentrating the sample with the density barrier enrichment protocol, each cell count on the flow cytometer is equal to a calculated environmental concentration of 10 cells L^{-1}.

Alexandrium catenella concentrations were lowest in the samples that were taken more inland on Elkhorn Slough as compared to the samples nearest to Monterey Bay. Concentrations of *A. catenella* were highest at the lower Elkhorn Slough stations with

deeper water (stations 1 to 3) closest to Monterey Bay as compared to the more inland, shallower upper Elkhorn Slough stations (stations 9 to 11) having no occurrences of *A. catenella* as observed by epifluorescent microscopy and few occurrences as observed by flow cytometry.

In this study, three Elkhorn Slough stations (stations 3, 7 and 10) that were sampled have automated sensor moorings that record environmental factors (**www.mbari.org/lobo/lobovis.htm**). Relative temperature, salinity, nitrate, and chlorophyll concentrations were extracted from these sensor data for each sample date. The environmental factor data from these sample stations; station 3 (Fig 7A), station 7 (Fig. 7B) and station 10 (Fig. 7C) was collated with *A. catenella* concentration results determined by flow cytometry and epifluorescent microscopy. These data show that temperature varied with season in a similar pattern at all three stations with the highest temperature occurring in the summer and the lowest temperatures occurring in the fall and the winter. Highest temperatures were observed at station 10 in the upper slough compared to the lower slough and Monterey Bay. Similar salinity values were observed at all three stations with some seasonal variation. The most seasonal and inter-sample variability in salinity values occurred at station 10. This station also had the highest average salinity values of all the stations in the summer months.

Fig. 7A.

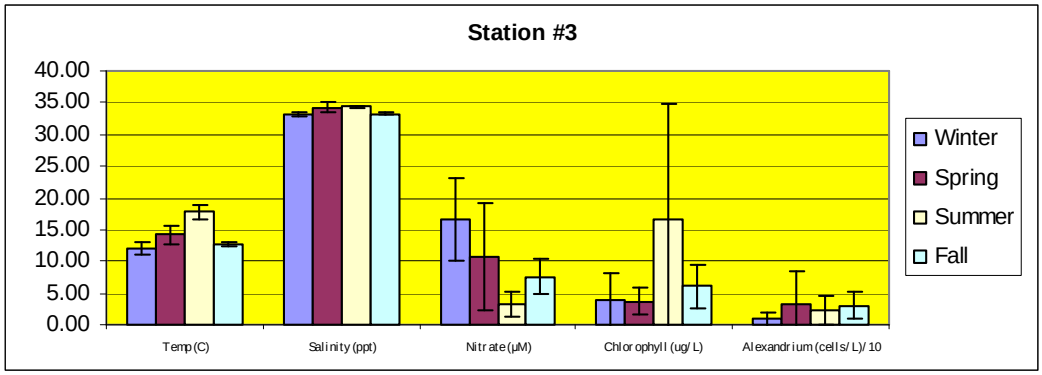

Fig. 7B.

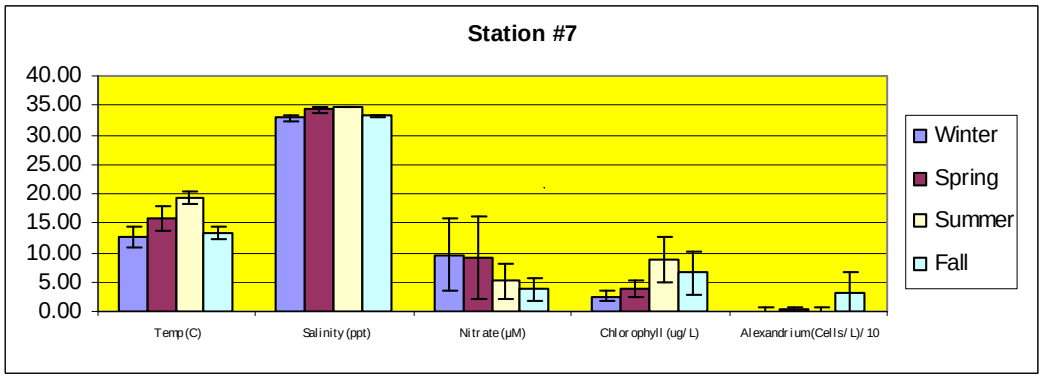

Fig. 7C.

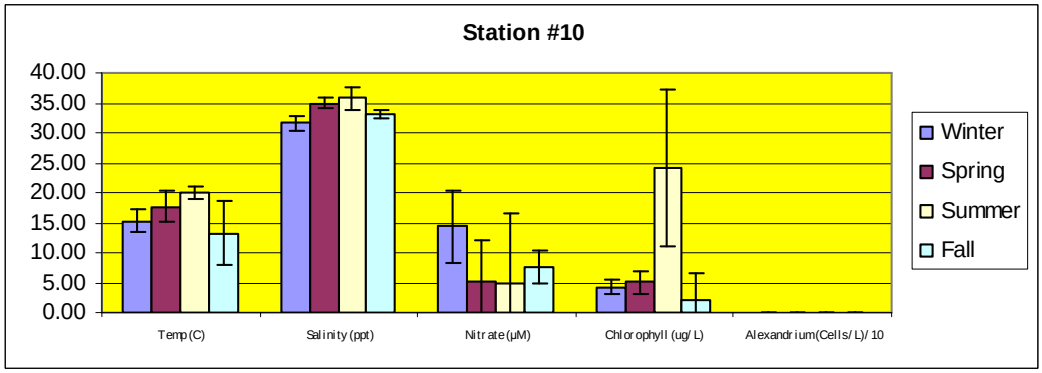

Fig. 7. Relative temperature, salinity, nitrate, chlorophyll a and *Alexandrium* concentrations in Winter, Spring, Summer and Fall at three different sampling sites in Elkhorn Slough. Temperature , Salinity, Nitrate and chlorophyll concentrations were extracted from MBARI automated buoy sensors (data : www.mbari.org/lobo/lobovis.htm).

Nitrate concentrations were highly variable and demonstrated a seasonal pattern, with the levels highest in the winter months. The chlorophyll concentrations were highest in the summer at all three stations. Chlorophyll concentrations were remarkably higher and more variable at stations 3 and 10 during the summer. In general chlorophyll concentrations were higher in the upper slough than in the lower slough. No *A. catenella* was observed using either flow cytometry or epifluorescent microscopy at any time of the year in the upper slough station #10. In the lower portion of Elkhorn Slough (station 3) the highest concentrations of *A. catenella* were observed in the spring and the lowest concentrations were observed in the winter months.

4. Discussion

The goal of this study was to determine concentrations of *Alexandrium catenella* in a variety of marine and estuarine environments during normal, seasonal and HAB conditions using flow cytometry. The use of automated instrumentation provides researchers with the opportunities to study individual species concentrations during HAB and non-HAB conditions. Algal blooms, being generally dominated by one species of alga, may be composed of toxic as well as non-toxic nuisance species (Gescher et al., 2008). Algal demographics during bloom conditions can change due to eutrophication and algal grazing (Smayda, 2008). It is important to monitor populations of toxin-producing species within an algal bloom to learn more about the conditions leading to their ascendance causing the formation of HABs.

Within an algal bloom, toxin producing or other harmful phytoplankton may cause poisoning or detrimental effects in marine mammals, fish populations and shellfish

harvested for human consumption. The complexity of nearshore phytoplankton dynamics due to mixing and complex current systems has made enumeration of a single phytoplankton species over a wide area difficult. Horizontal as well as vertical concentrations of a specific algal species may vary greatly. These variations in phytoplankton concentration may be due to physical conditions such as localized onshore wind conditions, time of day, sunlight availability, temperature and nutrient upwelling. Biological responses, such a phototaxis and chemotaxis, affect vertical and horizontal migration of phytoplankton (MacIntyre et al., 1997). These physical and biological characteristics tend to cause phytoplankton to form localized patches making any sampling regime complex. The distribution of nearshore phytoplankton populations may also change due to anthropogenic influences such as nutrient-laden agricultural run-off nearby watersheds (Los Huertos et al., 2006). In this study we found elevated concentrations of A. *catenella* in nearshore waters proximate the Watsonville sewage outflow and effluent from the Pajaro River, which drains water from a major agricultural community. Both of these nutrient filled water sources, as well as Elkhorn Slough, drain into Monterey Bay and may influence local nearshore phytoplankton concentrations (Fig. 1A and Fig. 5).

To study phytoplankton population dynamics with good resolution, a well designed sampling scheme and high numbers of samples are required. Single species enumeration by microscopy is time consuming and labor intensive. Automated monitoring systems for HAB species are under development with the goal of increasing sample throughput and increasing analytical capacity. For an automated system to be

effective in evaluating phytoplankton in environmental samples it must be sensitive, quantitative, specific for an individual species, robust and fast. Ideally, an automated system should identify and enumerate multiple species simultaneously in a single tube. Systems such as the environmental sample processor (ESP) developed by Monterey Bay Aquarium Research Institute (MBARI), currently deployed in Monterey Bay for the remote monitoring of HAB species exemplifies this approach (Greenfield et al., 2008). Use of DNA microarrays utilizing multiple specific, fluorescently labeled DNA probes to detect multiple species via PCR products also has great potential (Gescher et al., 2008).

Other algal monitoring systems are based on chemical detection of saxitoxins, but do not quantify the number of toxin–producing cells within a sample. Receptor-binding assays on seawater or shellfish samples can quantify saxitoxin content measured in ug STXeq 100 g^{-1} for extracted tissues, and ng STXeq L^{-1} for seawater samples (Jester et al., 2009). Paralytic Shellfish Poisoning (PSP) toxin composition in the genus *Alexandrium* differs between species and depends on growth conditions (Carretto et al., 2001; Kim et al., 1993). Consequently, methods that detect saxitoxin in seawater samples, which are used as a proxy for cell concentration, seem to correlate well with quantitative cell counts of *Alexandrium* sp. from offshore samples. However, in nearshore samples the correlation between toxin levels and cell counts is highly variable (Jester et al., 2009). The detection of PSP toxins, either by HPLC, or receptor-binding assays, infers the presence of a substantial population of *A. catenella* producing high levels of toxins. With such sample evaluation methods, the detection of *A. catenella* below the limits of toxin detection, might go undetected and may form a future HAB.

In our observations, many of the samples are indicative of low concentrations of *Alexandrium* of less than 50 cells L^{-1}. In previously published data from samples taken at the Santa Cruz Warf and Monterey Bay, *Alexandrium* concentrations from 2003 to 2005 rarely reached concentrations above 1000 cells L^{-1} (Jester et al., 2009). Determining the concentration of *Alexandrium* cells within an environmental sample, even in low concentrations (< 100 cells L^{-1}) may be important in evaluating the potential for *A. catenella* induced HABs. Specific probe-directed FISH, in conjunction with methods that characterize individual cells such as epifluorescent microscopy and flow cytometry, may provide for tools for assaying individual phytoplankton species population dynamics over time.

Analysis using epifluorescence microscopy on probe–directed FISH samples for phytoplankton enumeration is accurate and sensitive. Epifluorescence microscopy of seawater samples is used to quantify cells of individual species, but is time consuming labor intensive and subjective. If a large number of samples are to be enumerated, analysis by epifluorescent microscopy becomes impractical. Flow cytometry been used in laboratories as well as aboard research vessels for real-time enumeration of phytoplankton populations (Li, 1989). Flow cytometry quantifies cells and has potential to be automated, fast and accurate. With the use of multiple fluorochromes conjugated to different species specific DNA probes, it may be possible to investigate trophic interactions as well as HAB population dynamics in environmental samples within a single analysis.

Optimizing *Alexandrium*-specific FISH flow cytometry for this study presented two predominant challenges: probe specificity and sample preparation. Removal of debris was critical in preparing samples of moderate turbidity for probe-directed FISH and flow cytometry. This density-barrier sample enrichment step avoided fouling the flow cytometer and also increased the signal to noise ratio between the NA1 hybridized *A. catenella* and other phytoplankton. Cell losses of 6% occurred during the density barrier enrichment technique (data not shown) resulting in an underestimation the ground-truth density of *A. catenella* within the samples analyzed (Hawkins, 2010). Many different dinoflagellate species were observed by light microscopy prior to analysis. Since the NA1 probe demonstrated cross-reactivity with several dinoflagellates including *Akashiwo sanguinea*, and *Prorocentrum micans* (data not shown), non-specific probe binding using the universal control probe uniR was assessed (Hawkins, 2010). Our flow cytometry analysis protocol used a dot-plot format to define a region using cultured *Alexandrium* as a control for each parameter making the enumeration of *Alexandrium* in environmental samples of high biodiversity possible (Fig.3).

No *A. catenella* were observed in samples taken from upper Elkhorn Slough when analyzed by epifluorescence microscopy. The few samples that showed positive events in samples from upper Elkhorn Slough by flow cytometry may represent actual enumerated *A. catenella* or maybe due to NA1 cross reactivity probe with other phytoplankters of similar size, shape and chlorophyll *a* content. Losses in sensitivity may occur as an artifact of splitting samples for epifluorescent microscopic and flow cytometric enumeration where the environmental concentration of *A. catenella* is low

(<20 cells L^{-1}). *Alexandrium catenella* was routinely detected in greater numbers in samples taken from the lower Elkhorn Slough stations by both flow cytometry and epifluorescent microscopy. Multiparametric logical gating during analysis, however, enabled us to accurately detect low populations of *A. catenella* in these samples using flow cytometry.

Flow cytometric specificity may be further increased by using newer species specific probes currently used in DNA microarray analysis (Gescher et al., 2008). With the advancement of techniques used in molecular biology and the growing size of DNA sequence database repositories with respect to ribosomal sequences of phytoplankton, fluorescently labeled DNA probes can be designed for many species for enumeration or trophic-level investigations.

The occurrence of *A. catenella* in Monterey Bay is well documented (Jester et al. 2009) and monitored monthly by the California biotoxin monitoring program (California Department of Health). *Alexandrium catenella* also occurs along the entire west coast of the United States including Alaska (Trainer, 2002), as well as the Japanese Archipelago (Scholin et al., 1994). In this study we found *A. catenella* in Monterey Bay and Lower Elkhorn Slough using flow cytometry and epifluorescent microscopy.

Elkhorn Slough has been studied with respect to phytoplankton population dynamics and trophic interactions (Welschmeyer et al., 2004). In our study variations in *A. catenella* concentrations in Elkhorn Slough changed with season, temperature and nitrogen availability. Samples taken from the surface at one meter and two meters simultaneously at the entrance to Elkhorn Slough demonstrated that *A. catenella* sub-

surface concentrations often exceeded surface concentrations (data not shown) (Hawkins, 2010).

Our flow cytometry results for *A. catenella* compared well with epifluorescent microscopy. Populations of *A. catenella* were absent in true estuarine environments existing in upper Elkhorn Slough, and are cosmopolitan at low concentrations in nearshore environments in Monterey Bay and lower Elkhorn Slough. The lower regions of Elkhorn Slough (stations 1-5) are nutrient saturated and readily mix with Monterey Bay. The upper regions of Elkhorn Slough (stations 8-11) are dominated by cryptophytes and subject to bacterial denitrification that decreases the amount of available nitrate (Caffery et al,. 2003; Francis et al., 2005). The upper regions exhibit a low diversity of phytoplankton speciation (Welschmeyer, 2007). These regions of Elkhorn Slough are also subject to agricultural run-off from the Salinas River and nearby agricultural operations that increases the Nitrogen/Phosphorus ratio well above the Redfield N/P ratio of 16:1 thus increasing the possibility of creating algal bloom conditions (Welschmeyer, 2007).

Samples of moderate turbidity, as defined by a Secchi depth of less than 1 meter, could be analyzed by flow cytometry when coupled with density-gradient centrifugation. Utilizing multiparametric logical gating in a flow cytometry protocol is critical in accurately detecting and enumerating cells of multiple HAB forming species within the same sample. With the increase of red-tide and HAB occurrences on a global scale, the importance of more advanced automated monitoring systems for HAB forming species is increasing. Combining the tools of molecular biology and immunology, with other

chemical and spectroscopic methods will increase the ability to characterize HAB species samples in inshore estuarine environments with high resolution.

In this two-year study the concentrations of *A. catenella* followed a seasonal pattern. In our study *A. catenella* was observed year round in the lower portions of Elkhorn Slough (Stations 1 to 6), with the highest concentrations of *A. catenella* occurring in the spring. In samples taken from the upper slough (Stations 7-11) *A. catenella*, occurred only in the fall months, and in far less concentrations than the lower slough. In the lower slough, tidal forcing causes a daily replenishment of water from Monterey Bay, while the Upper Slough water circulation is for the most part independent (Breaker et al., 2008). Since Elkhorn Slough is an ebb-dominated estuary, this supports the notion that *A. catenella* should not be found in the shallower portions of the upper slough.

This study represents the first long-term characterization of *A. catenella* along the entire length Elkhorn Slough, a seasonal estuary. The tracking of an individual HAB species, with the use of specific probes, is necessary in nearshore environments surrounded by fertile agricultural watersheds which could be subject to eutrophication and possible algal blooms from *Alexandrium* seed populations. Nutrient run-off into Elkhorn Slough is influenced by two highly productive agricultural watersheds, the Pajaro watershed and the Elkhorn Slough watershed, in which the latter passes through Elkhorn Slough (Los Huertos et al., 2006). These watersheds have been compromised by high level of both nitrogen (Nitrate-N) and soluble reactive phosphorus (SRPs) due to urban and agricultural usage (Los Huertos et al., 2006). The stimulation of an algal

bloom may not be related to the measured concentration of a single nutrient, but rather a ratio of the concentrations between two chemicals, such as nitrogen and phosphorus (Anderson et al., 2008). Our survey of *A. catenella* in Elkhorn Slough over a two year period, found the year round presence of *A. catenella* in low concentrations in the lower slough and the absence of *A. catenella* in the upper slough. We also discovered that surface sampling alone may not accurately reflect *A. catenella* abundance and distribution in low environmental concentrations. The detection of a HAB species, during non-bloom conditions is important in areas of high nutrient loading due to agricultural and urban activities. The presence of a HAB forming species in such areas, and in low concentrations, has the potential of forming an algal bloom from seed populations either from resting cysts in the sediment or from vegetative cells. With this potential, along with increasing anthropogenic eutrophism in nearshore estuaries and tidal embayments, the need for extensive environmental monitoring for HAB forming species will become critical.

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CHAPTER III

CONCLUSIONS

Conclusions

Flow cytometers have become important instruments in biological oceanography for the real time assessment of environmental samples both in laboratories and on research vessels (Collier, 2000; Li, 1989). This study showed that a flow cytometer, designed to be used on clinical samples, was applied successfully for the enumeration of *Alexandrium catenella* in marine and estuarine samples. Flow cytometry detected *A. catenella* when concentrations were low during non-bloom conditions. Monitoring systems for HABs which use probe-directed FISH prior to enumeration utilizing epifluorescent microscopy is labor intensive and time consuming. Flow cytometry, being automated and fast, can efficiently analyze a large number of samples. Our flow cytometric analysis results of *A. catenella* concentration from Elkhorn Slough and Monterey Bay correlate well with epifluorescent microscopy. This indicates that flow cytometry using DNA-directed FISH may be the method of choice for analyzing individual species populations during HABs. Additionally, surface sampling, without attention to vertical depth may be inadequate for reliable detection of HABs forming species.

An important aspect of this work is the use of a novel method for enriching and concentrating phytoplankton in marine samples which effectively decreases both background noise and sample volume. This method increases the concentration of *Alexandrium catenella* within the final sample to a volume appropriate for flow cytometry. A second important aspect that enabled us to detect *A. catenella* within a complex phytoplankton population was the use of multiparametric logical gating. These

two contributions (sample enrichment/concentration and logical gating) have enabled us to overcome the major obstacles to using flow cytometry for HAB monitoring. Sample loss during sample preparation remains a valid concern for HAB monitoring using this method. Sample loss may lead to underestimating the true concentration *A. catenella* in marine samples.

Methods other than epifluorescent microscopy such as high performance liquid chromatography (HPLC) could have been used for comparison. Although *A. catenella* produces a unique repertoire of algal pigments, the small differences between dinoflagellate species are not easily discernable using HPLC. Unlike flow cytometry, HPLC does not give a quantitative enumeration of cells, but rather concentration of pigments per unit volume and is not specific for *A. catenella*. Epifluorescent microscopy quantifies number of cells and was useful in this study. Advanced molecular techniques such as quantitative PCR (QPCR) could have been used for comparison. QPCR provides for the real-time monitoring of targeted population of cells with the use of specific oligonucleotides, similar to those used in standard PCR, but unlike flow cytometry, QPCR cannot enumerate cells directly, but can only quantify the number of gene copies a cell has. QPCR also uses a fluorescently labeled reporter probe. With the QPCR system, it would have been possible not only to detect *A. catenella*, but also assess the relative concentration of *A. catenella* within a sample, and compared to flow cytometry results.

Elkhorn Slough and Monterey Bay provided optimal proof-of-concept study sites for detecting of *A. catenella* by flow cytometry due to the diverse array of environmental conditions between the upper slough, the lower slough and the open waters Monterey

Bay. As expected *A. catenella* was detected in low level concentrations in the lower slough and Monterey Bay, but not detected in the upper slough. These data lead to the conclusion that flow cytometry could be used as an effective tool to monitor for *A. catenella* in samples of moderate turbidity (Secchi depth less than two feet). Since *A. catenella* was successfully detected in Elkhorn Slough, it follows that this technique could be used to monitor other marine sites with a wide array of environmental conditions. For example San Francisco Bay, an important economic waterway, could be monitored for invasive forms of dinoflagellates belonging to the genus *Alexandrium* that possibly could be introduced by release of ballast seawater from large cargo vessels. *Alexandrium* spp. found in coastal waters of Asia, Europe and the Americas are of unique "ribotypes" (organisms encoding a particular 16S rDNA sequence; analogous to genotype or phenotype) which are found in Asia and are not found off the Western and Eastern seaboard of the United States. Each species of *Alexandrium* have different toxin production profiles and can present economic and health concerns if they become established in new waterways, bays and coastal waters. Since ballast water from large cargo vessels tends to be turbid, methods described in this work, may be useful in real-time monitoring of ballast water for invasive *A. catenella* ribotypes.

In this study, we focused on the detection of *A. catenella* in Elkhorn Slough and Monterey Bay and are known producers of PSP (Caretto, 2001). Other HAB-forming species of economic and health concerns include the dinoflagellates *Cochlodinium* sp., *Dinophysis* spp. which causes diuretic shellfish poisoning (DSP), and in Southern

California *Lingulodinium polehedra* another toxin producing dinoflagellate. All these HAB forming organisms could be enumerated simultaneously using flow cytometry.

Application of flow cytometry to monitoring HABs is not limited. HAB's can form from diatoms as well, such as the case with *Pseudo-nitzschia* spp. This marine diatom is the cause of Amnesic Shellfish Poisoning (ASP) and is causal to mortality in marine mammals in Monterey Bay. *Pseudo-nitzschia* may present a problem to flow cytometry analysis. This problem is due to its pennate morphology (approximately 6-8 μm in width and 72-144 μm in length). At these dimensions, *Pseudo-nitzschia* may obstruct the flow cell orifice within the flow cytometer.

With increased use of flow cytometers in cell biology, immunology and clinical sciences, a number of instruments are available. Clinical laboratories and Universities often prefer flow cytometers from Becton and Dickenson which produce the FACScalibur™, FACScan™ and the FACSsort™. Routine maintenance, technical help and reagents for these flow cytometers are readily available worldwide. Newer flow cytometers are entering the marketplace, and used instruments can be purchased at a moderate cost. Instruments marketed for oceanographic research include the FLOWcam (Fluid Imaging Technologies, Yarmouth, ME.) are not as common as the more popular Becton and Dickenson flow cytometry instruments and maintenance as well as repair service for the FLOWcam is more limited.

A flow cytometer is a versatile instrument of immerging importance in biological oceanography and environmental biology. It has proven useful in characterizing trophic level interactions of complex aquatic communities and HABs. Like any sensitive

instrument, there will be limitations in its use, some of which may be overcome with innovative sample processing and instrument protocols. In this work, we have shown that flow cytometry can be used successfully quantify a specific phytoplankton species in low concentrations in near-shore bodies of water of moderate turbidity.

APPENDICES

APPENDIX A. MONTEREY BAY SAMPLING STATIONS DEPTHS AND LOCATIONS (FIG. 1A**).**

Station	Depth (ft)	Latitude	Longitude
107	300	N36 48.454	W121 55.176
108	250	N36 49.912	W121 53.900
109	180	N36 51.187	W121 53.030
110	180	N36 50.367	W121 51.706
111	200	N36 49.716	W121 50.698
112	120	N36 48.929	W121 49.407
113	65	N36 48.071	W121 47.523
CTD#1	800	N36 47.862	W121 50.967
CTD#2	3800	N36 43.465	W122 00.422

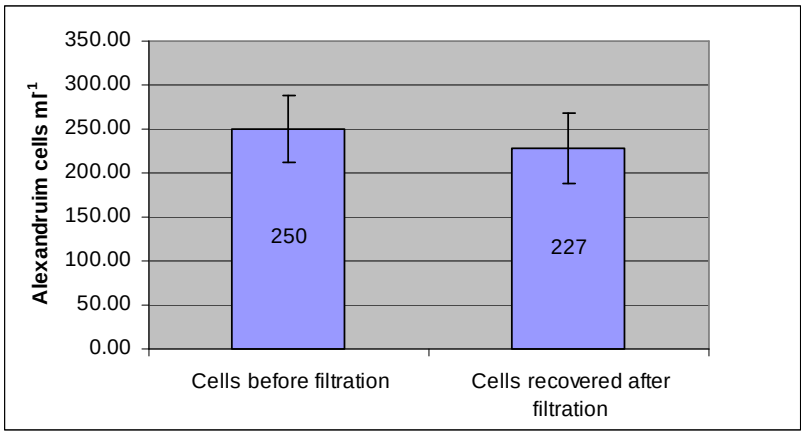
APPENDIX B. ELKHORN SLOUGH SAMPLING STATIONS DEPTHS AND LOCATIONS (FIG.1B).

Station	Depth (ft) high tide	Latitude	Longitude
1	65	N36 47.930	W121 48.083
2	37	N36 48.558	W121 47.155
3	32	N36 48.749	W121 46.462
4	30	N36 48.872	W121 45.951
5	26	N36 48.809	W121 45.449
6	23	N36 48.975	W121 44.892
7	23	N36 49.337	W121 44.744
8	19	N36 49.753	W121 44.857
9	19	N36 50.178	W121 44.505
10	16	N36 50.430	W121 44.894
11	15	N36 50.641	W121 45.234

APPENDIX C. SAMPLING DEVISE USED FOR SIMULTANEOUS ACQUISITION OF SAMPLES FROM SURFACE, 1 METER AND 2 METERS IN DEPTHS.

This devise was used to sample from three different depths simultaneously for near surface populations of *Alexandrium catenella*. Because *Alexandrium* populations are sporadic and somewhat rare when not in bloom conditions, obtaining samples at different depths may be a more robust sampling method. The devise is constructed to trigger all three sample bottles simultaneously with the use of fishing line attached to sealed elastic plugs. Sample release is done with the use of three independent ball valves which release the sample (800 mL) into collection bottles.

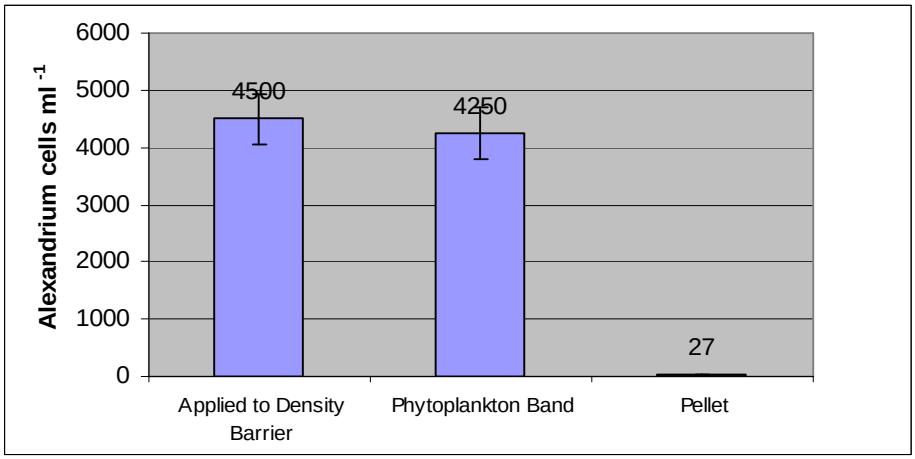
APPENDIX D. COMPARISON OF CULTURED *ALEXANDRIUM* CONCENTRATION BEFORE AND AFTER FILTERING AN EQUAL VOLUME OF *ALEXANDRIUM* CELLS THROUGH A 70 MICOMETER FILTER TO DETERMINE LOSS ESTIMATED DUE TO FILTRATION PRIOR TO NA1-DIRECTED FISH AND FLOW CYTOMETRY (N=3).



In this study, the effect of losses due to sample pre-filtering prior to flow cytometry was assessed. An average loss of 9.2 % [1-(227/250)] is observed when laboratory cultured *Alexandrium* is passed through a 70 μm filter prior to density barrier sample concentration.

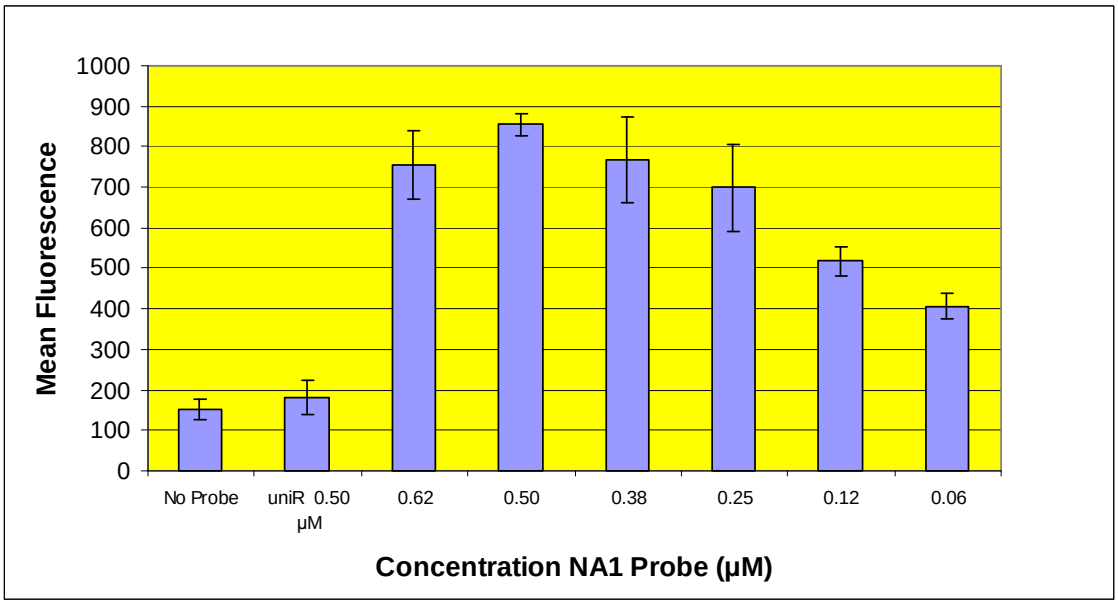
Pre-filtering samples with a 70 μm filter was done to reduce orifice plugging since the flow cytometer has a flow cell orifice diameter of 100 μm. Anything larger that 100 μm will fowl the flow cytometer flow cell orifice. *Alexandrium* size is 35-45 μm. The standard of deviation for all three trials for both the filtered counts is large enough to infer little difference between the filtered and unfiltered samples (P= 0.665). Cell counts of *Alexandrium* were done before and after filtration with a light microscope counting using a hemocytometer. Losses may be attributed to error in cell counting, *Alexandrium* adhering to filter, and loss of *Alexandrium* in concentrating for cell enumeration.

APPENDIX E. COMPARISON OF CELL COUNTS OF LABORATORY CULTURED *ALEXANDRIUM* CELLS APPLIED TO AND RECOVERED AFTER DENSITY-BARRIER ENRICHMENT, FROM THE BARRIER LAYER AND THE CELL PELLET AFTER CENTRIFUGATION (N=3, SEE FIG. 2).



In three separate trials to determine the loss of sample attributed to the density barrier enrichment procedure, an average of 94% recovery [(4250/4500) x100] of *Alexandrium* is observed in the recovered phytoplankton band. Some cells, (average 0.6% [(27/4500) x 100], were found in the pellet. Some cell loss may occur in extracting the phytoplankton band away from the percoll layer as the band was extracted visually, leaving some of the sample behind which was discarded. The upper seawater layer and the phytoplankton interface band were removed simultaneously from the percoll layer in order to limit losses as some of the phytoplankton, being motile, migrate upward and away from the phytoplankton band just after centrifugation. The combined seawater and phytoplankton band after removal from the percoll layer were then differentially centrifuged and supernatant discarded. Removal of this supernatant if not carefully done, could also lead to loss of cell.

APPENDIX F. MEAN FLUORESCENCE AND COEFFICIENT OF VARIATION VALUES FOR LABORATORY SAMPLES PREPARED FROM A PURE CULTURE OF *ALEXANDRIUM*: UNSTAINED CONTROLS (NO PROBE), NEGATIVE CONTROL (UNIR PROBE), AND SAMPLES SUBJECTED TO NA1-DIRECTED FISH USING DIFFERENT CONCENTRATIONS OF NA1 PROBE N=3, 3000 CELLS, 6000 CELLS, AND 12000 CELLS.

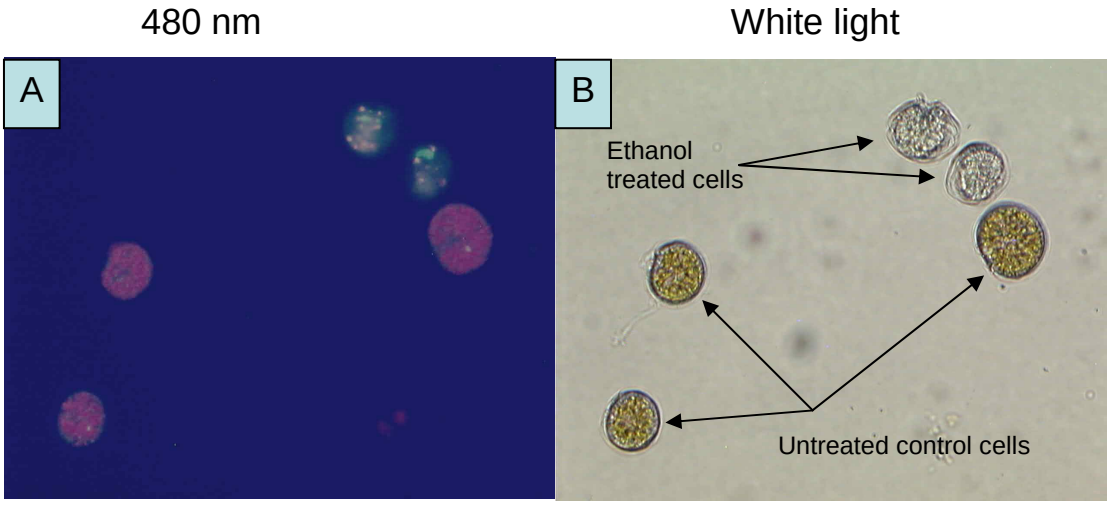


All probes used in this study (NA1 and uniR) are both dual labeled with FITC ant the 5' and the 3' end. This dual-labeling increases the signal from the FISH probed *Alexandrium catenella.* To determine the optimal amount of probe to be used in NA1-directed FISH prior to flow cytometric analysis, three different *Alexandrium* cell concentrations were used in the NA1-directed FISH probe titration analysis, these concentrations were 3000, 6000 and 12,000 cells. Optimal NA1 probe concentrations for *Alexandrium* NA1-directed FISH in this experiment was 0.50 µM. In dilutions that were analyzed, the coefficient of variation was demonstrated to be the lowest at 0.50 µM NA1 probe concentration in all three trials. *Alexandrium* subjected to NA1-directed FISH is had an average mean fluorescence that was 4.7 times brighter than *Alexandrium*

subjected to uniR- directed FISH. *Alexandrium* subjected to NA1-directed FISH had an average mean fluorescence which was 5.6 times brighter than *Alexandrium* subjected to no probe.

In this study, the optimal probe concentration for use in centrifugal FISH protocol adapted for flow cytometry was best achieved at 0.50 μM, the same probe concentration that was previously described (Sako et al., 2004). The optimal probe concentration for FISH directed flow cytometry using the dual FITC labeled probe, 0.50 μM, also resulted in the lowest coefficient of variation among all probe concentrations analyzed.

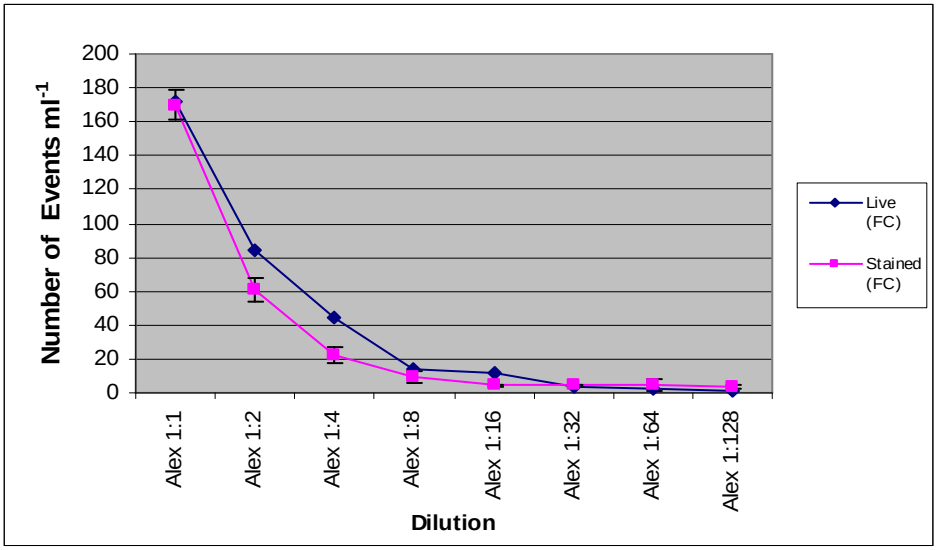
APPENDIX G. A MIXTURE OF TWO LABORATORY PREPARATIONS OF *ALEXANDRIUM* (ETHANOL TREATED AND UNTREATED CONTROL) OBSERVED UNDER EPIFLUORESCENCE (A) AND BRIGHT-FIELD (B) MICROSCOPY.



In the procedure for fluorescent *in situ* hybridization (FISH), prior to fixation with paraformaldeyhe, cells are dehydrated with 80% ethanol. Cultured *Alexandrium* treated with 80 % ethanol for 5 minutes on ice results showed a 15.7 fold decrease in endogenous mean chlorophyll a fluorescence as compared to non-treated *Alexandrium* cells as analyzed by flow cytometry of the two mixed populations (flow cytometry data not shown)

Treatment of *Alexandrium* with 80 % ethanol for 5 minutes quenches most (20%), but not all of the chlorophyll *a* signal (Hosoi-Tanabe and Sako., 2005) which is acquired by the FL-3 flow cytometry detector. Over-quenching for 1 hr with 80% ethanol would effectively extract all the chlorophylls from *Alexandrium*, and would remove one parameter from a multiparameter sequential logical gating scheme.

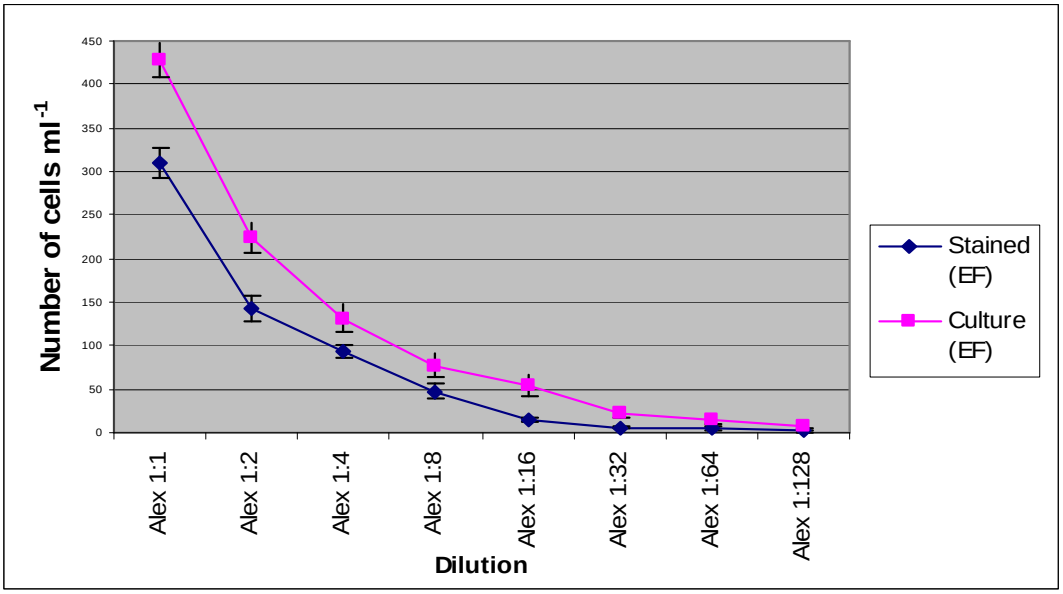
APPENDIX H. COMPARISON OF EQUAL INITIAL CELL CONCENTRATIONS AND SERIALLY DILUTED, LABORATORY CULTURES OF *ALEXANDRIUM* (LIVE) AND NA1-DIRECTED FISH TREATED *ALEXANDRIUM* (STAINED) BOTH ANALYZED BY FLOW CYTOMETRY AND GATED FOR INTACT CELLS (PREVIOUSLY HEALTHY PRIOR TO ANALYSIS).



A dilution series of NA1 FITC stained cell enumeration based on gated hybridized healthy *Alexandrium* cells. Undiluted cells indicate little difference in cell numbers between live (untreated) and NA1-directed FISH treated cells, average (N=3) loss equal to 2%. [Largest average loss observed at a 1:4 dilution, with an average loss of 52%.] As dilution increases, the loss of cells also increases.

At larger cell concentrations losses are less pronounced. This may be due to removal of reagents from a subsequent centrifugation steps. Some of the pellet may get removed with the discarded supernatant. Sample preparation technique affects cell loss from the sample. Care must be taken after centrifugation steps, when removing supernatants away from pelleted cells as this is the major source of error due to cell loss.

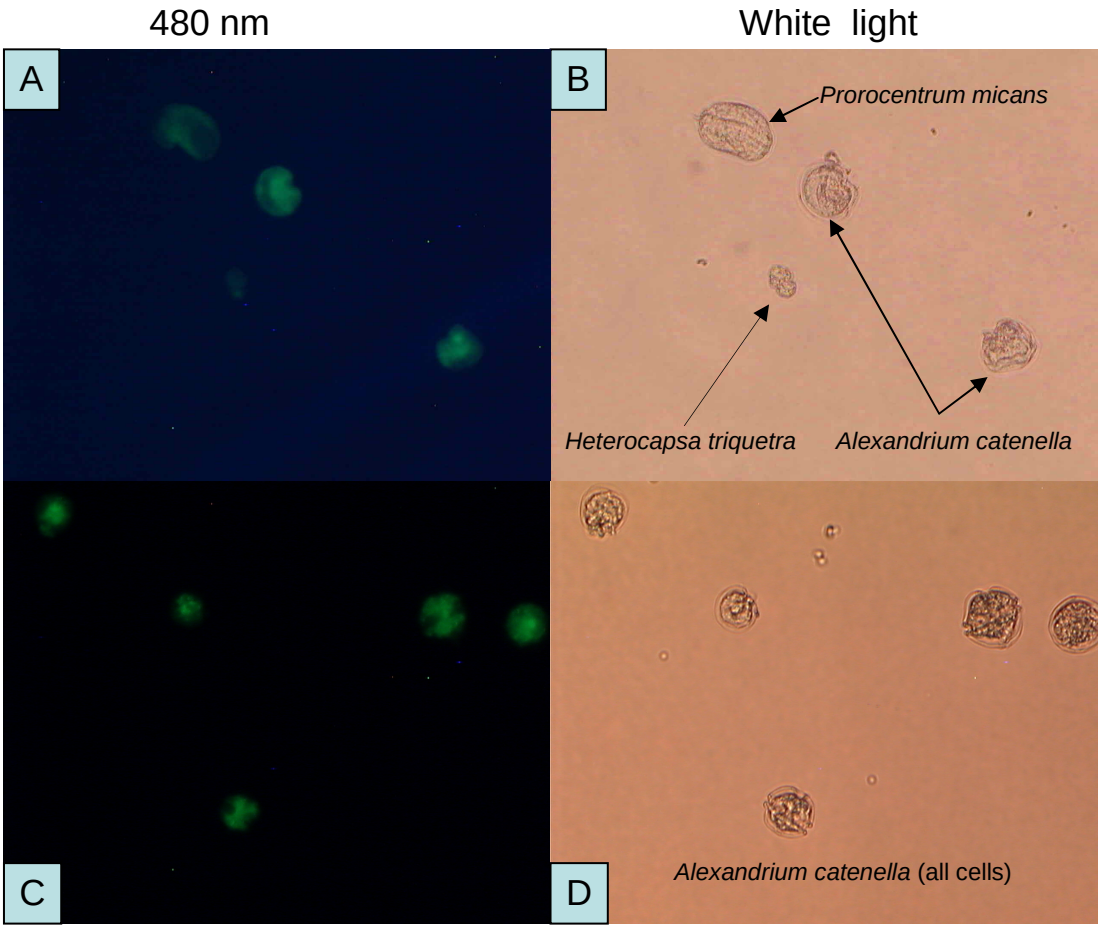
APPENDIX I. COMPARISON OF EQUAL INITIAL CELL CONCENTRATIONS, AND SERIAL DILUTED, LABORATORY CULTURES OF *ALEXANDRIUM* (LIVE) AND NA1-DIRECTED FISH TREATED *ALEXANDRIUM* (STAINED) BOTH ANALYZED BY EPIFLUORESCENCE MICROSCOPY.



In undiluted cell suspensions, NA1-directed FISH of *Alexandrium* using the centrifugation FISH protocol demonstrates and average of 28% loss. Observed losses decrease as dilution increases.

The disparity in cell counts between the live culture enumeration and the NA1-directed FISH stained cells as analyzed by epifluorescence microscopy may be caused by the existence within sample of non-viable unstained cells incapable of uptake of the NA1 probe due to degraded ribosomes to which the NA1 probe hybridizes. As with any multi-step process, losses may also occur due to sample preparation error.

APPENDIX J. PREPARATIONS OF LABORATORY CULTURES CONTAINING A MIXTURE OF PHYTOPLANKTON (A AND B) AND A PURE *ALEXANDRIUM* CULTURE (C AND D) SUBJECTED TO NA1-DIRECTED FISH UNDER 480 NM EPIFLUORESCENCE MICROSCOPY (A AND C) AND BRIGHT-FIELD MICROSCOPY (B AND D).

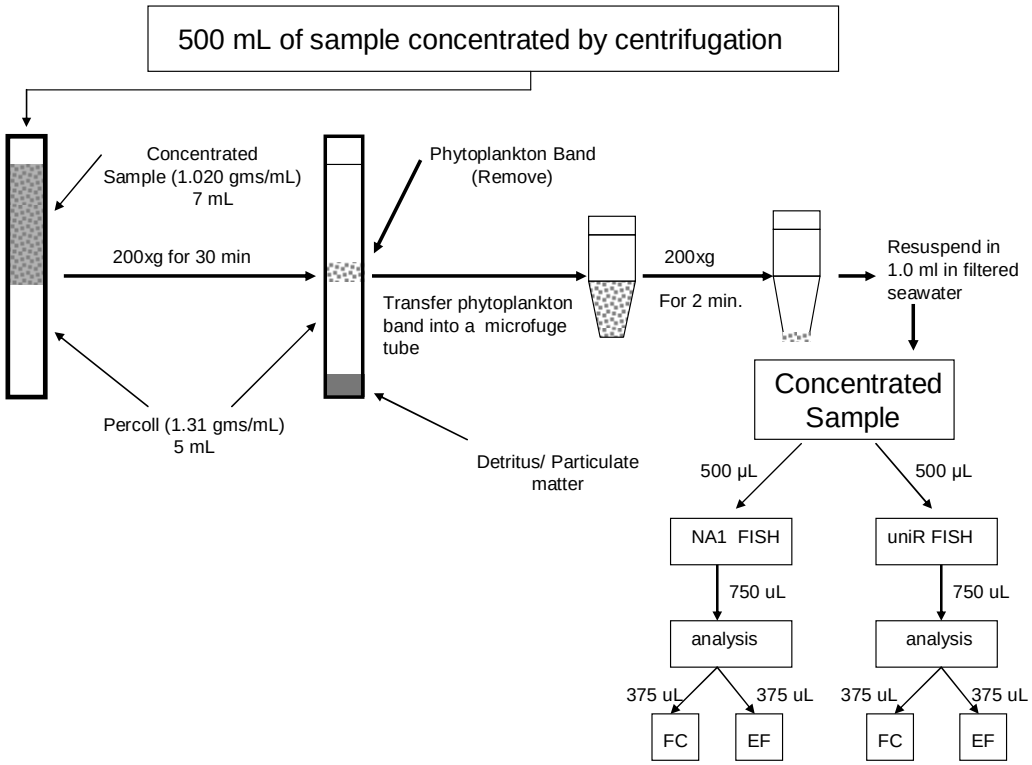


The NA1 FITC labeled probe specific for *Alexandrium* North American ribotype shows cross reactivity with *Prorocentrum micans* and *Heterocapsa triquetra* (A and B). Single cells of *Alexandrium catenella* subjected to NA1-directed FISH and observed under epifluorescence microscopy show different morphologies depending on cell orientation. The *Alexandrium* dorsal or ventral profile cells appear as green fluorescent

spheres, while viewed from the side (lateral view) *A. catenella* appear as fluorescent spheres with transverse dark notches (C and D)

Analysis of NA1-directed flow cytometry of laboratory cultures indicated cross reactivity between *Alexandrium catenella* and *Prorocentrum micans*. Therefore, NA1-directed fluorescence alone is insufficient to distinguish between these genera. With the use of a chlorophyll a parameter (FL-3), sequential logical gating as described in methods increased specificity to acceptable levels (Altman, 1994). *Prorocentrum* (and *Heterocapsa*) have differing FSC and SSC properties as well as differing chlorophyll a fluorescence signatures than *Alexandrium*. Sequential logical gating used in flow cytometry reduces misidentification is use due to cross reactivity of the NA1 probe compared to other fluorescence detection systems such as epifluorescent microscopy.

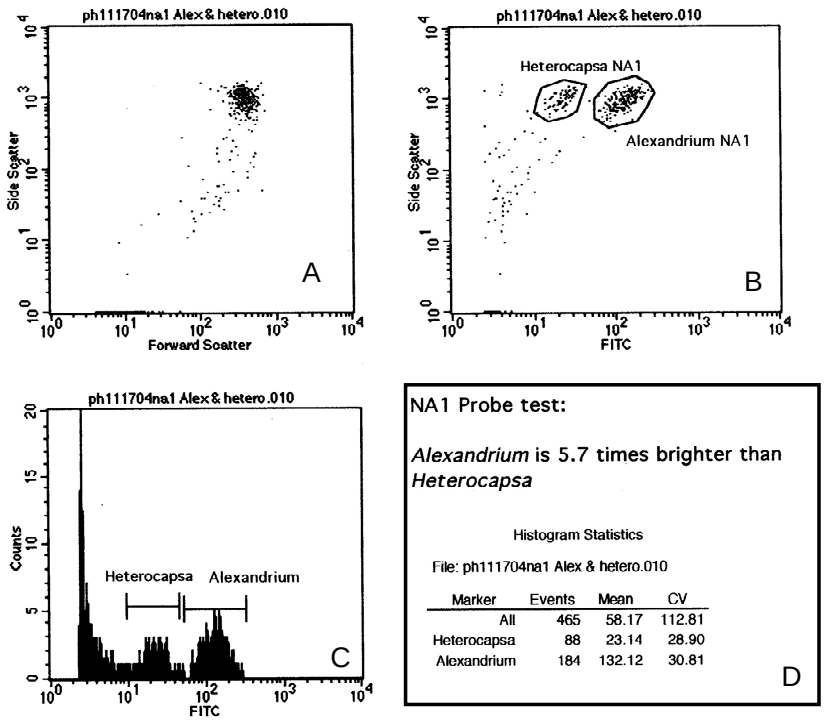
APPENDIX K. FLOW DIAGRAM OF ENVIRONMENTAL SAMPLE CONCENTRATION FOR FISH PROBE HYBRIDIZATION FOLLOWED BY FLOW CYTOMETRY (FC) AND EPIFLUORESCENT MICROSCOPY (EF).



From 500 mL of environmental sample, differential centrifugation is used to concentrate the sample to 7 mL. This sample will ultimately represent two 250 mL aliquots to be analyzed, each hybridization equaling a environmental volume of 250 mL. This was done to ensure that the analysis between the two probed samples had consistent populations of cells between them. Following differential centrifugation, rate-zonal centrifugation was done to separate the phytoplankton from the detritus and other debris. Following concentration the sample was equally split for separate hybridizations with

NA1 and uniR FITC labeled probes. After hybridization the samples were once again equally split for separate analysis by both epifluorescent microscopy and flow cytometry. Although the hybridized samples were resuspended in 375 μL of 5x SSC, only 300 μL of the sample was actually analyzed. This was done to ensure that sample analyzed by flow cytometry could collect data for five minutes at 60 μL min^{-1} without the possibility of sample and data loss (pipette error) due to insufficient volume of interrogated sample applied to the cytometer. In the final analysis, the environmental sample interrogated by flow cytometry was equivalent to a volume of 125 mL for each probe used.

APPENDIX L. FLOW CYTOMETRY DOT PLOTS AND A HISTOGRAM OF A SAMPLE PREPARED FROM PURE LABORATORY CULTURES OF *ALEXANDRIUM CATENELLA* AND *HETEROCAPSA TRIQUETRA* MIXED TOGETHER THEN SUBJECTED TO NA1-DIRECTED FISH.

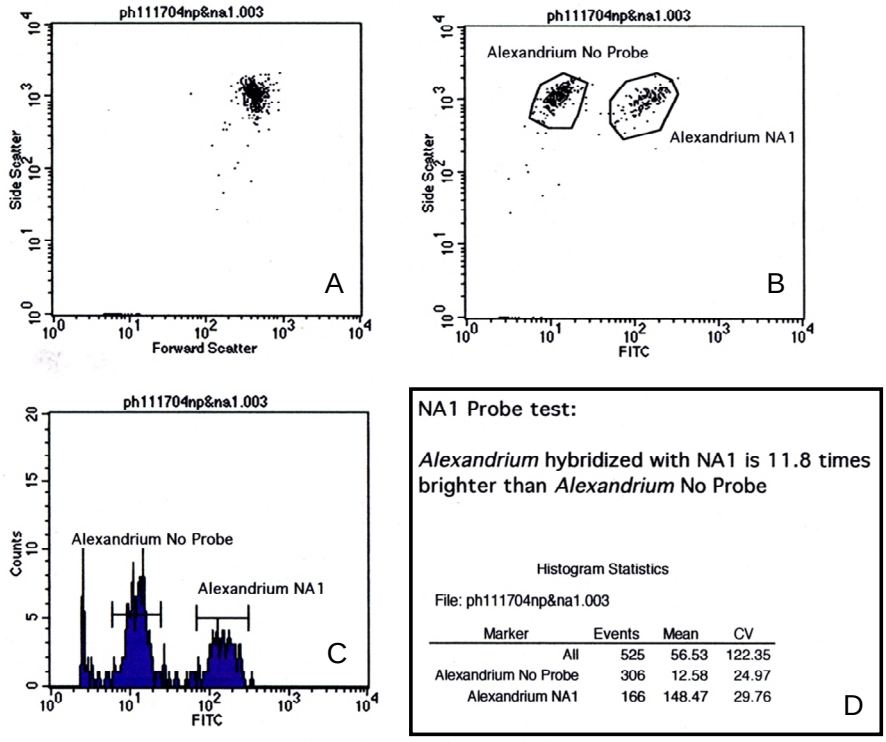


Alexandrium subjected to NA1-directed FISH and *Heterocapsa triquetra* subjected to NA1-directed FISH are indistinguishable from each other with respect to forward scatter vs. side scatter (dot-plot A). *Alexandrium* subjected to NA1-directed FISH has a 5.7 times greater mean fluorescence value than *Heterocapsa* subjected to NA1-directed FISH (dot-plot B and box D).

Properties of forward scatter and side scatter of *Alexandrium* and *Heterocapsa* which have been subjected to NA1-directed FISH cannot resolve between the two

separate populations. To resolve the two populations, the FL-1 (FITC) detector was used for specific species enumeration within this sample. Since the FITC labeled probe hybridized in a specific species manner, accurate enumeration of the target species was possible. This species-specific probe is homologous to ribosomal RNA of that species (*Alexandrium*), and thus will not hybridize to non-homologous rRNA of other species (*Heterocapsa*) within that sample.

APPENDIX M. FLOW CYTOMETRY DOT PLOTS AND HISTOGRAM SHOWING A COMPARISON OF DIFFERENTIAL FLUORESCENCE BETWEEN *ALEXANDRIUM* SUBJECTED TO NA1-DIRECTED FISH AND *ALEXANDRIUM* SUBJECTED TO NO PROBE**.**

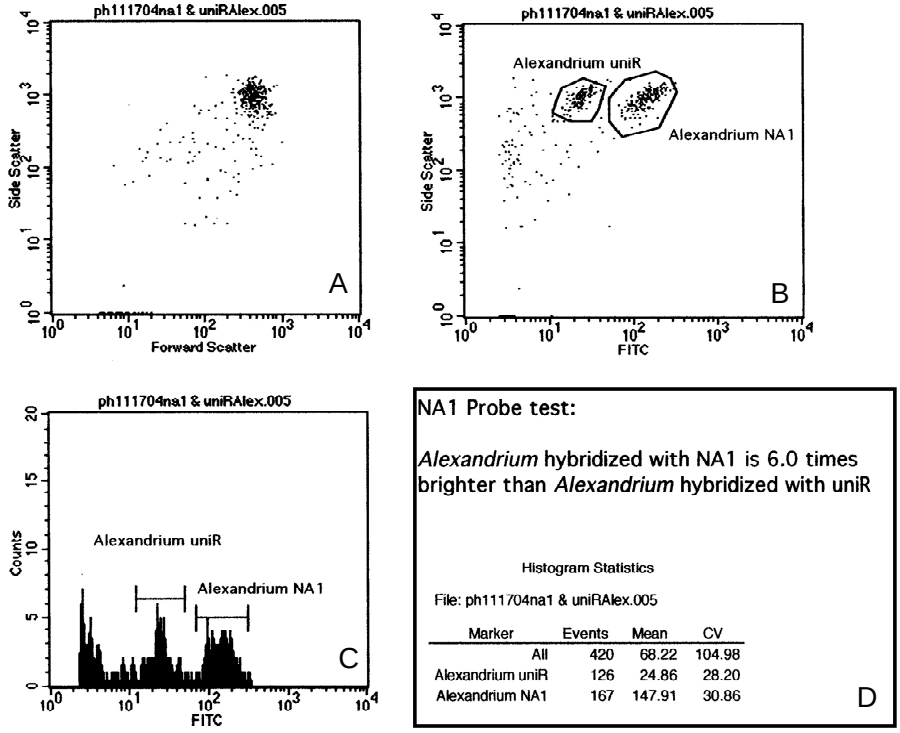


Laboratory cultured *Alexandrium* subjected to NA1-directed FISH has an 11.8 times greater fluorescence value than *Alexandrium* not subjected to NA1-directed FISH and enumerated by flow cytometry (dot-plot B and D).

Alexandrium subjected to NA1-directed FISH shows no difference in forward scatter and side scatter signatures while showing great disparity between the *Alexandrium* subjected to NA1-directed FISH and the *Alexandrium* not subjected to NA1-directed FISH with respect to FL1 (FITC) fluorescence. This amplified the FITC fluorescence

signal by labeled cells is caused by NA1 binding specifically to *Alexandrium catenella*, was compared to natural FL1 fluorescence in fixed cells. In the case of analyzing unfixed (Non-FISH) natural samples, the intrinsic FL1 fluorescence of *Alexandrium* show little difference to other phytoplankters in the same sample. The NA1-directed FISH procedure allowed for increased, uniform fluorescence in *Alexandrium* cells that enabled us to in define a region (increased signal to noise ratio) for the purposes setting sequential logical gates.

APPENDIX N. FLOW CYTOMETRY DOT PLOTS AND HISTOGRAM SHOWING A COMPARISON OF DIFFERENTIAL FLUORESCENCE BETWEEN *ALEXANDRIUM* SUBJECTED TO NA1-DIRECTED FISH AND *ALEXANDRIUM* SUBJECTED TO UNIR-DIRECTED FISH.



The *Alexandrium* subjected to NA1-directed FISH has a 6.0 times greater mean fluorescence value than *Alexandrium* subjected to uniR negative control directed FISH using flow cytometry (dot-plot B and box D).

In this study, the uniR probe acted as a universal control. The sequence of the probe is based on prokaryote ribosomal DNA sequences and functions as a control for non-specific probe binding. *Alexandrium* cells in one sample were subjected to NA1-directed FISH, and another was stained with uniR-directed FISH. When mixed and

analyzed by flow cytometry these two samples showed no difference in forward scatter and side scatter values and are indistinguishable (Fig. 13A). They are easily distinguishable when the FITC detector was employed (Fig 13 B and C.)

APPENDIX O. SENSITIVITY AND SPECIFICITY RESULTS FOR ALL 253 SAMPLES FROM MONTEREY BAY AND ELKHORN SLOUGH AS DETERMINED BY NA1-DIRECTED FLOW CYTOMETRY FISH (TEST) AND EPIFLUORESCENCE MICROSCOPY FISH (DEFINITIVE) DATA.

		Epifluorescence Microscopy		
		Pos	Neg	total
Flow Cytometry	Pos	74	18	92
	Neg	22	139	161
	Total	96	157	253

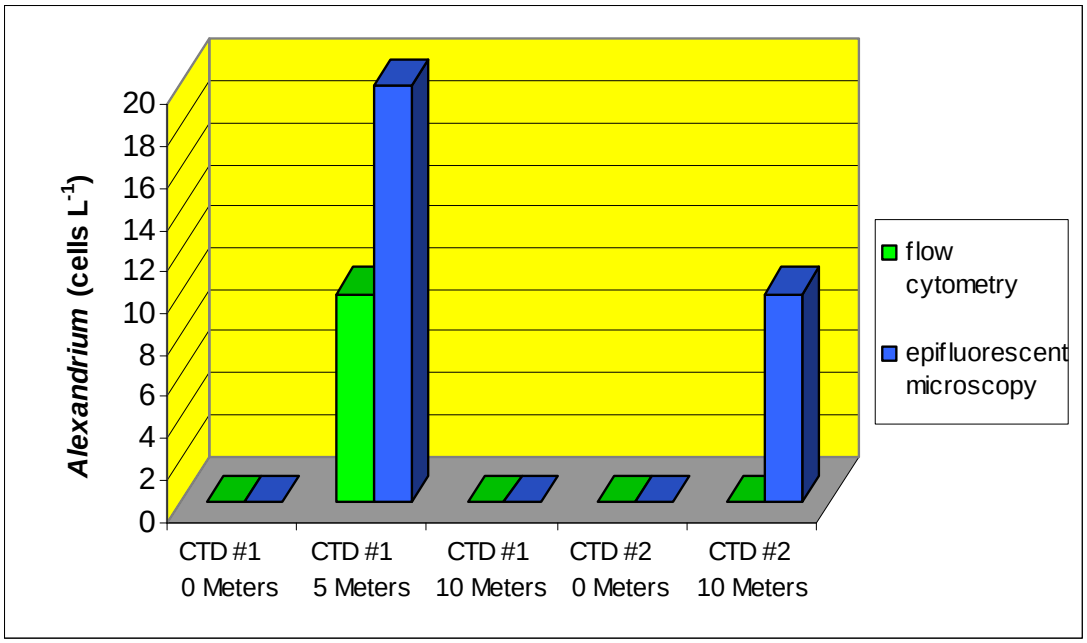
Sensitivity = 77.1% N=253 Specificity = 88.5%

Alexandrium was not demonstrated in 55% of the 253 samples from Elkhorn Slough and Monterey Bay, either by flow cytometry or epifluorescent microscopy. *Alexandrium* cells were detected in 114 of the 253 samples (45%) by either flow cytometry or epifluorescent microscopy. Of the 114 samples positive for *Alexandrium* for either flow cytometry or epifluorescent microscopy, in 74 positive samples (65%) containing *Alexandrium* were detected both by flow cytometry and epifluorescent microscopy.

In all the samples, specificity was higher than sensitivity, possibly due to majority of the samples having very few (one or two) detected *Alexandrium* in them in relation to more numerous other phytoplankton in the same sample. The lower sensitivity value may be caused by a low signal to noise ratio in sample consisting of a sparse population of *Alexandrium* in a specific sample (one or two *Alexandrium* cells in a sample), there was better agreement in the flow cytometry data and the epifluorescent data in samples

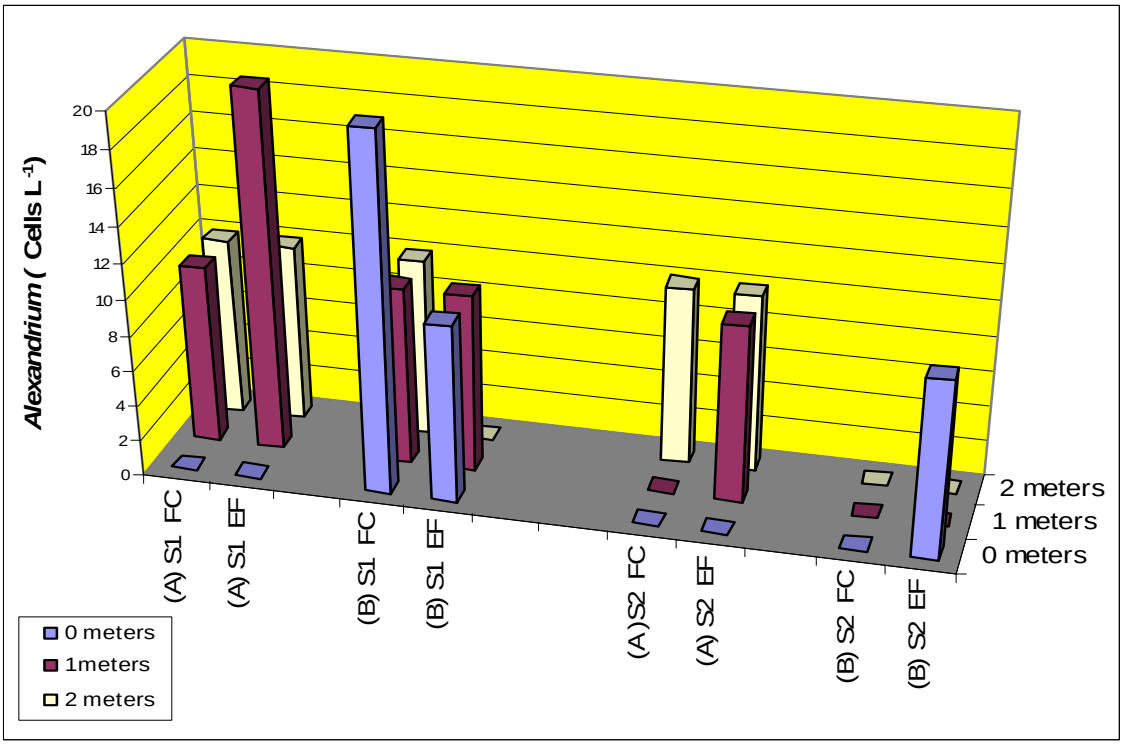
with higher numbers of *Alexandrium* detected. The decreased specificity value may be caused by a low signal to noise ratio in sample consisting the presence of phytoplankters other than *Alexandrium* non-specifically binding to the NA1 probe during FISH, this signal to noise problem can be minimized with the use of sequential logical gating. In Elkhorn Slough it is expected not to find *Alexandrium* in the more inland stations; although in very few cases flow cytometry did detect one or two *Alexandrium* cells, where epifluorescent microscopy did not, these detected events may be attributed to noise caused by non-specific binding of the NA1 probe to other species.

APPENDIX P. COMPARISON OF NA1-DIRECTED FISH FLOW CYTOMETRY AND EPIFLUORESCENT MICROSCOPY RESULTS OF SAMPLES FROM CTD VERTICAL SAMPLES COLLECTED AT TWO SITES IN MONTEREY BAY (SEE FIG. 1A).



Monterey Bay samples taken from a cruise on the R/V Point Sur indicate near sub-surface populations of *Alexandrium* in very low concentrations. Low concentrations of *Alexandrium* (10 -20 cells L^{-1}) was observed at 5 and 10 meters by epifluorescence microscopy and flow cytometry while no *Alexandrium* was detected at the surface; this may be due to high irradiance at the surface driving *Alexandrium* into slightly deeper water due to photo-saturation at the surface.

APPENDIX Q. COMPARISON OF NA1-DIRECTED FISH FLOW CYTOMETRY AND EPIFLUORESCENT MICROSCOPY RESULTS OF SAMPLES FROM VERTICAL SAMPLING AT ELKHORN SLOUGH STATION 2 (S1) AND STATION 3 (S2) (SIMULTANEOUS 0M, 1M AND 2M DEPTH SAMPLE ACQUISITION) ON TWO DIFFERENT DATES. (A= 21 OCTOBER 2008, B= 16 NOVEMBER 2008).



Alexandrium was observed at all three depths (0, 1M and 2M) at both stations sampled on two different dates. In some samples *Alexandrium* was not detected at the surface, but was detected at 1 and 2 meters. More *Alexandrium* was detected at station #3 than at station #2 and on both dates by flow cytometry and epifluorescent microscopy of NA1-directed FISH samples.

These data indicate that the use of vertical sampling (and not just surface sampling) for detection of a specific phytoplankton species may yield a more complete

understanding of the trace concentrations at a given sampling site (Grisselson, 2002). With the use of only surface samples instead of near surface vertical sampling, the single surface sample may yield low or inaccurate results.

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