UAF LIBRARIES
10022465248

PARALYTIC SHELLFISH POISONING:

THE RELATIONSHIP BETWEEN *ALEXANDRIUM* ABUNDANCE AND

PSP TOXINS ON KODIAK ISLAND, ALASKA

By

Julie A. Matweyou

RECOMMENDED:

Advisory Committee Chair

Head, Graduate Program in Marine Science and Limnology

APPROVED:

Dean, School of Fisheries and Ocean Sciences

Dean of the Graduate School

12-03-03

Date

PARALYTIC SHELLFISH POISONING:

THE RELATIONSHIP BETWEEN *ALEXANDRIUM* ABUNDANCE AND PSP TOXINS ON KODIAK ISLAND, ALASKA

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Julie A. Matweyou, B.S.

ALASKA
RA
1242
S52
M38
2003

Fairbanks, Alaska

May 2003

RASMUSON LIBRARY
UNIVERSITY OF ALASKA-FAIRBAN

ABSTRACT

Paralytic shellfish poisoning (PSP) events have severe negative impacts on Alaska commercial shellfish fisheries as well as recreational and subsistence harvests. This study, designed to improve existing PSP monitoring programs, involved the use of a rapid sandwich hybridization assay to detect and quantify the relative abundance of *Alexandrium catenella* based on species-specific LSU rRNA targeted oligonucleotide probes. Blue mussel (*Mytilus edulis*) toxicity, expressed as saxitoxin equivalents, was determined using the ^{3}H-Saxitoxin receptor binding assay. Shellfish toxicity was relatively low in both 2000 and 2001 compared to historically high values on Kodiak, but exhibited pronounced late spring and late summer peaks, in both years at four to seven sampling sites. Temporal and spatial variability in shellfish toxicity among sites, seasons, and years suggested dynamic, and possibly unpredictable, *Alexandrium* bloom events. Importantly, DNA probe data revealed a strong association between *Alexandrium* abundance and shellfish toxicity. The results also demonstrated that increases in *Alexandrium* abundance preceded elevated toxin levels in shellfish, indicating that this assay may prove useful as a monitoring tool to predict toxic events in shellfish before they are harvested. Water column nutrients and climate data were evaluated to determine if bloom-triggering mechanisms could be identified.

Table of Contents

List of Figures

List of Tables

Acknowledgments

I would like to thank my major advisor Dr. Gerry Plumley for his support and guidance throughout this degree process. I was given a lot of freedom in this project, freedom to learn from my mistakes and to learn about myself as I worked through this degree.

I also want to thank my committee members Drs. Dean Stockwell, Susan Hills, and Robert Foy for their help. Each offered valuable advice and guidance in their areas of expertise. I extend a special thanks to Dr. Susan Henrichs who helped me in the early stages of writing and was a constant source of support.

Funding for this research was provided by Alaska Science and Technology Foundation. Support in the form of tuition assistance and travel funding was also provided by Alaska Sea Grant and School of Fisheries and Ocean Sciences, UAF.

I dedicate this thesis to my niece, Ella Rose Matweyou, who has been in my thoughts daily since I came to Alaska. She has been my motivation and strength, as well as a wonderful research technician when she came to Kodiak during my field seasons. She battled the wind and rain to help collect samples and brought joy to my heart. For now, I leave her the honor of being the first in our family to obtain a Ph.D. (if she so wishes!). In the time that it has taken me to receive this degree, she has grown leaps and bounds, and hopefully has learned some lessons in strength and perseverance from her 'scientist' aunt in Alaska. Good luck Ella, on everything you set out to do in life! All my love!

I would also like to thank the rest of my family, particularly my mother Bernadette Juscak, my sister Lisa Matweyou, and my aunt Judy Carrell, for their support and encouragement. Many thanks to my friends in Fairbanks, Hua, Wendy and Jen and my friends in Kodiak, Art, Jeff, Jenny, Grif, Ginny, Verna, Gidget and many others. I will always have a special place in my heart for Kodiak and the peace I found on the water, in my kayak, paddling and contemplating life.

Chapter 1
General Introduction
Paralytic Shellfish Poisoning:
Phytoplankton Monitoring as a Tool for Predicting Shellfish Toxicity

1.1 Thesis Overview

The work presented in this thesis revolves around development of a monitoring program to detect paralytic shellfish poisoning (PSP) events in Alaska. New technologies that rely on DNA probes were used to monitor the abundance of *Alexandrium* spp. in seawater collected near Kodiak Island. The potentially negative impacts of *Alexandrium* were ascertained by measuring the levels of PSP toxins in a sentinel species, the mussel *Mytilus edulis*, and the level of toxins in the water column. Nutrient concentrations and hydrographic and meteorological data were measured to help explain *Alexandrium* bloom dynamics.

1.2 Global Issues

On a global scale harmful algal blooms (HABs) appear to have been steadily increasing over the past few decades in frequency, intensity and geographic distribution (Anderson 1989; Smayda 1990; Hallegraeff 1993). Hallegraeff (1993) suggests possible explanations for this trend including human-related nutrient enrichment selecting for HAB species (Lam and Ho 1989; Okaichi 1989), dispersal of HAB species via ship ballast water (Hallegraeff et al. 1988; Hallegraeff et al. 1990) and shellfish seeding activity, increased utilization of coastal waters for aquaculture (Shumway 1990), and long term climate trends (Ebbesmeyer et al. 1995), i.e., global warming. Although it has been argued that perhaps the increase is due merely to increased scientific scrutiny, improved detection methods and better communications, it is generally accepted that HABs are on the rise (Anderson 1989). The costs are medical, economic, and social.

Of the several types of HAB events (Table 1.1), Alaska has reported low levels of domoic acid in shellfish and in the water column (Horner et al. 1997). However, human illnesses associated with domoic acid poisoning have not been reported on the West Coast of North America (Wekell 2001). The largest HAB-related problem in Alaska is paralytic shellfish poisoning (Horner et al. 1997). This thesis work was directed at PSP events in Alaska.

Table 1.1. Major trends in HAB events (modified from Baker 2001).

TOXIC HARMFUL ALGAL BLOOMS			
EVENT	**CAUSATIVE SPECIES**	**TOXIN (if applicable)**	**MODE OF ACTION**
Ciguatera Fish Poisoning (CFP)	*Gambierdiscus toxicus* *Prorocentrum* spp. *Ostreopsis* spp. *Coolia monotis* *Amphidinium* spp.	ciguatoxin maitotoxin scaritoxin gambiertoxin	Binds to the Na^+ / Ca^{++} channels promoting ion influx
Diarrhetic Shellfish Poisoning (DSP)	*Dinophysis* spp. *Prorocentrum lima*	okadaic acid dinophysistoxins pectenotoxins yessotoxins	Inhibits proteins (phosphatase 1 and 2a) that control sodium secretion in intestine
Neurotoxic Shellfish Poisoning (NSP)	*Karenia brevis*	brevetoxins	Binds to Na^+ channel promoting ion influx
Paralytic Shellfish Poisoning (PSP)	*Alexandrium* spp. *Gymnodinium catenatum* *Pyrodinium bahamense* Red algae Cyanobacteria	saxitoxins	Binds to Na^+ channel and blocks ion influx
Amnesic Shellfish Poisoning (ASP)	*Pseudo-nitzschia* spp. *Nitzschia actydrophila* *Amphora coffaeiformis* Three red algal species	domoic acid	Over-stimulates neurons and keeps Ca^{++} channels open promoting ion influx
	Pfeisteria piscicida	toxin has not been confirmed	Suspect toxin active only in presence of fish; considered ambush predator
NONTOXIC HARMFUL ALGAL BLOOMS			
	Gonyaulax polygramma *Noctiluca scintillans* *Scrippsiella trochoidea* *Trichodesmium erythraeum* *Ceratium furca* *Mesodinium rubrum* *Aureococcus anophagefferens* *Aureoumbra lagunensis*		Causes damage from oxygen depletion and high H_2S/NH_3 due to accumulation of high biomass; affecting fish and invertebrates
	Chaeteceros convolutes *Heterosigma akashiwo* *Gymnodinium mikimotoi* *Chrysochromulina polylepis* *Prymnesium* spp. *Chattonella antiqua*		Mechanical damage or production of hemolytic substances that clog fish gills

1.3 PSP Events in Alaska

Paralytic shellfish poisoning (PSP) has long been recognized in the state of Alaska. Written records date back to 1799 when about one hundred Aleut hunters became sick and died after consuming mussels they found near Sitka Island (Halstead 1965). The Alaska Natives responded to the illness by inducing vomiting, indicating they were aware of PSP and had a traditional remedy (Fortuine 1996). Subsistence and recreational harvests of shellfish continue to be important to many Alaskans today. From 1973 through 1994 the Alaska Division of Public Health documented 66 outbreaks of PSP involving 143 people from coastal communities around the state (Gessner 1996). Cases were reported in all months except November and December during this time period, and a variety of shellfish were implicated in these illnesses. Between 1995 and 2000 at least 51 additional illnesses, have been documented, and many cases are thought to go unreported (Trainer 2002). Gessner and Middaugh (1995) were unable to find an association between race, age or sex of the victim and PSP illness, suggesting that all persons are at equal risk from consumption of shellfish taken from uncertified beaches.

Commercially, the loss of revenue due to PSP toxins has been extensive, affecting both the crab and clam fisheries (Ralonde 2001). Crab processing and handling have been changed from a whole, live product to a sectioned, cooked one due to PSP toxins found in crab viscera. Once a growing industry, the Alaska clam industry today is virtually nonexistent due to the destruction of the market by PSP contaminated product in the 1940's. It remains perhaps "the largest untapped fisheries resource in the United States" (Neve and Reichardt 1984). The difficulties, expenses and fear of contamination make developing a viable shellfish market a financial risk. The Alaska Department of Environmental Conservation (ADEC), located in Palmer, AK, operates a testing program that requires commercially harvested shellfish to comply with strict, tiered, lot sampling (RaLonde 2001). Under this program, commercial operations pay for the collection, shipping and holding of their shellfish, an added cost above the direct economic impact from toxic events.

1.4 *Alexandrium*, the Causative Agent of PSP Events in Alaska

In the North Pacific, PSP is caused by dinoflagellates of the genus *Alexandrium*, primarily *Alexandrium catenella* (Prakash and Taylor 1966; Price et al. 1991; Horner et al. 1997). The genus *Alexandrium* has 29 species, with at least 8 – 10 toxic species, depending on classification

(*A. acatenalla, A. catenella, A. cohorticula, A. fundyense, A. ostenfeldii, A. minutum, A. tamarense and A. tamiyavanichi)*. The genus name has changed as new species and morphological traits have been identified. *Alexandrium, Protogonyaulax, Gessnerium, Pyrodinium, Goniodoma* in part and *Gonyaulax* in part, have all been used synonymously (Steidinger1993). The genus name *Alexandrium* was agreed upon at the 4th International Conference on Toxic Marine Phytoplankton (Steidinger and Moestrup 1990) under the classification proposed by Balech (1985).

Dinoflagellates of the genus *Alexandrium* produce several saxitoxin congeners as secondary cell byproducts (Cembella 1998). Saxitoxin is a neurotoxin that acts as a sodium channel blocker, preventing the uptake of Na^+, thus stopping the flow of nerve impulses (Strichartz and Castle 1990). Saxitoxin is typically accumulated in filter feeding shellfish that are relatively immune to the toxin, and is transferred through the food chain by secondary consumers (Hall et al. 1990). Symptoms in humans include tingling and/or numbness in the lips and extremities, nausea, dizziness, shortness of breath, and in extreme cases, paralysis and death (Meyer et al. 1928). The toxins are water-soluble and will pass from the system without causing permanent damage if victims are kept alive during the stages of respiratory paralysis. The toxins can also be passed through the pelagic food web via zooplankton and forage fishes (Haya et. al 1990; Turner et al. 2000), ultimately affecting upper trophic levels; i.e., fish, seabirds, and marine mammals (Geraci et al. 1989).

The complexity of the *Alexandrium* life cycle, altering between an asexual and a sexual stage, enables cells to persist through unfavorable conditions as well as to thrive in a spectrum of habitats and hydrographic regimes. Anderson (1998) reviewed the life cycle. The vegetative cells are able to divide rapidly during favorable conditions. Under nutrient stress, typically limitation in nitrogen or phosphorous (Anderson et al. 1984), gametes (isogamous or anisogamous depending on species (Turpin et al. 1978; Anderson 1980)) are formed, and fuse to form a zygote. The swimming zygote (planozygote) becomes a dormant, resting cyst (hypnozygote) that can survive in sediments for years (Cannon 1993). After an obligatory period of dormancy, the cyst will enter quiescence, and germinate under favorable temperatures (Anderson1980), light, salinity, and oxygen conditions (Cannon 1993). Although nutrient stress induces cyst formation, nutrient concentrations do not appear to affect germination (Cannon

1993). It is also unclear if cyst germination is completely dependent on environmental triggers or if, at least in some cases, an endogenous annual clock is involved. Both strategies may occur (Anderson and Keafer 1987) making the life cycle of *Alexandrium* even more complex and, presumably, the organism better suited for survival.

Under unfavorable conditions (i.e., mechanical shock or sudden change in temperature and salinity) vegetative cells can also enter into a temporary resting stage, or pellicle cyst. Motility can be reestablished when conditions again become favorable, thus allowing cells to withstand short-term environmental fluctuations (Anderson 1998). Cyst formation can make cells difficult to identify microscopically because morphological traits are altered.

Few studies have been conducted on the ecology, oceanography, distribution, and/or abundance of *Alexandrium* in Alaska. Plankton surveys conducted in May 1948, over a small region of southeast (SE) Alaska, found only an occasional organism that resembled *Alexandrium* (Schantz and Magnusson 1964). These authors also paraphrased unpublished results from surveys conducted in the summer of 1949 by L. Foster and H. Sommer in collaboration with the Fishery Products Research Laboratory, who also identified *Alexandrium* in SE Alaska but only at exceedingly low densities. *Alexandrium* has been identified in Alaska in two Ph.D. dissertations (Neal 1967; Hall 1982) and one M.S. thesis (Chang 1971) but data are sparse since the surveys were a minor component of each study. Neal (1967) demonstrated a positive correlation between mussel toxicity and *Alexandrium* abundance (then referred to as *Gonyaulax*) in SE Alaska, but only for three of the five sites studied. *Alexandrium* has also been reported during isolated sampling events, by Meyers and Hilliard (1955) near False Pass and Zimmerman and McMahon (1976) in SE Alaska, and observed by scientists, fishermen, and interested individuals (e.g., R. RaLonde (Univ. Alaska Fairbanks), pers. comm.; R. Horner (Univ. Washington), pers. comm.).

1.5 Other Possible Causes of PSP Events in Alaska

Because *Alexandrium* abundance and distribution data for Alaska are limited, a recurring theme is that shellfish toxicity and *Alexandrium* abundance are not correlated (Schantz and Magnusson 1964; Hall 1982). Shellfish toxicity has been reported as geographically patchy and difficult to explain based on the presumed distribution of *Alexandrium* (Schantz and Magnusson 1964).

Dinoflagellates have been linked to PSP events in marine environments since the 1930s (Sommer et al. 1937). However, recent observations in freshwater systems have also convincingly demonstrated PSP events caused by cyanobacteria (Carmichael 1997 et al.). These freshwater PSP events occur in several places within the U.S. (Sawyer et al. 1968; Carmichael et al.1997), in Portugal (Pereira et al. 2000), and in Australia (Humpage et al. 1993). The role of cyanobacteria in toxin production in marine environments remains poorly understood, and was a cause for concern when this study was initiated (summer 1999).

Another cause for concern at the onset of this study related to speculation that eubacteria also synthesized PSP toxins, including saxitoxin congeners (Doucette and Trick 1995; Plumley et al. 1999). Recent work has cast doubt on the idea that eubacteria synthesize saxitoxins or other toxins that block sodium channels (Baker et al. 2003), but, as was the case for cyanobacteria, the idea that organisms other than dinoflagellates synthesize saxitoxins impacted the experimental design of this project.

1.6 Nucleotide Probes as Tools for Identification of HAB Species

Many HAB species, particularly those associated with PSP events, have been difficult to study for a number of reasons. Life cycles of the cells in question are often complex, requiring long-term monitoring to understand bloom events. The species of interest often represent a minor component of the overall planktonic assemblage, necessitating high-density sampling; and these species are sometimes very difficult to identify, even by a trained taxonomist (Anderson 1995). Morphologically similar strains, sometimes toxic and non-toxic varieties, are nearly impossible to identify using light microscopy, and while electron microscopy may be useful, it is also time consuming and is not a reasonable choice for studies requiring intensive routine analysis. Research has turned to molecular probes for rapid and accurate identification and enumeration of HAB species (Anderson 1995).

Based on the Watson and Crick model of a double helical DNA structure, we know that hybridization of a given sequence in one chain is compatible with only one sequence in the opposite chain, or with RNA transcribed from that DNA (reviewed in Alberts et al. 1994). A non-conserved region of DNA/RNA can be identified and a probe can be developed to target and hybridize with this sequence segment. Depending on the desired level of resolution, probes can

be developed for identification at the genus, species and even sub-species levels (Anderson 1995). An oligonucleotide probe, typically consisting of 15-50 nucleotides, is just one type of nucleic acid probe that has become an easily synthesized, commercially available, inexpensive tool (Anderson 1995; Scholin 1998). These probes can be designed to target either DNA or RNA, but because the amount of rRNA per cell is high (Delong et al. 1989), these naturally occurring molecules make rRNA-targeted probes desirable.

A large nucleotide database does not exist for HAB species, therefore identifying a unique base pair sequence can be difficult. Not only is it necessary to find a unique sequence in the organism of interest, it is also necessary to know the DNA makeup of closely related and/or co-occurring algae (Anderson 1995). Proper hybridization conditions, the three-dimensional structure of the target and the tendency of the probe to bind to itself are some of the other considerations in developing a probe (Scholin 1998).

Scholin et al. (1994) sequenced rRNA from several *Alexandrium* species and found that the large subunit (LSU) rDNA sequences provided fine-scale species and population resolution. From this sequencing work, five distinct ribotypes were identified that subdivided the *tamarense/catenella/ fundyense* species complex and were named with reference to their geographic origin. The North American (NA1) probe used in this present study was developed from the work of Scholin (1994).

Nucleic acid probes are currently being applied with whole cell or homogenate formats (Scholin 1998). Whole cell hybridization employs the use of intact (whole) target cells throughout the hybridization process (Scholin et al. 1997; Miller and Scholin 1998). Cells are concentrated and chemically preserved. The chosen fixative must be effective in making the cell wall and inner membranes permeable without destroying the intact cell, thus allowing the fluorescently labeled oligonucleotide probe access to target molecules (Miller and Scholin 2000; Scholin et al. 1996). Once the hybridization is complete, cells can be detected and quantified, usually either by epifluorescence microscopy or flow cytometry (Anderson 1995; Lange et al. 1996; Scholin et al. 1996). The advantage of the whole cell method is the identification of the labeled target cells for cell shape and morphology (epifluorescence microscopy) or cell size (flow cytometry).

Sandwich hybridization uses cell homogenates rather than intact cells (Scholin 1998). Concentrated cells are lysed in a lysis buffer, thus liberating targeted molecules (Scholin et al. 1997; 1999). Probes can often be applied to the unpurified lysate (cell homogenate). In studies reported here, the hybridization was carried out with two distinct probes. The first captured the target sequence (rRNA) and a second signal probe attached to a separate sequence on the target, thus forming a "sandwich" ensuring a highly specific response (Scholin 1998). Through a series of enzymatic reactions, a color signal is produced when target molecules are present. This color change may be determined visually or by reading the optical density (e.g., with a microplate reader). Reaction intensity (color development) must be correlated with corresponding epifluorescence, light, or electron microscopic cell counts (e.g., Scholin et al. 1997). The method employed in this thesis work has been automated and is in beta testing with Saigene Corporation (Seattle WA).

The sandwich hybridization assay significantly reduces misidentification and microscope time (Scholin 1998). The assay is highly sensitive and permits identification during periods of low *Alexandrium* (< 50 cells/L) abundance (C. Scholin (Monterey Bay Aquarium Research Institute, (MBARI),) pers. comm.). The sandwich hybridization assay offers a rapid approach to phytoplankton monitoring.

1.7 Monitoring PSP Events

Screening for and monitoring PSP events is difficult. Commercial sale of shellfish in the U.S. requires a FDA-mandated procedure. Shellfish are harvested and samples submitted for mouse bioassay. This screening protocol has been proven to be safe and reliable, but it is also labor-intensive and time-consuming. Several states use the mouse bioassay for routine monitoring of beaches that are frequented by recreational harvesters (Nishitani and Chew 1988; Shumway et al. 1988; Price et al. 1991). However, shellfish monitoring via mouse bioassay provides data on current toxicity levels, and cannot be used as a predictive tool to forecast future toxicity levels. To predict toxic events, researchers have turned to plankton monitoring. The premise behind this approach is that toxic blooms of algae will appear in the water column, shellfish will consume the algae, and exhibit toxicity levels proportional to the number and toxicity of algae in the water. For PSP events, several studies have demonstrated a close association between certain *Alexandrium* species and the toxicity of blue mussel, *Mytilus edulis* (Nishitani and Chew 1984;

Jeon et al. 1996). Based on relationships between toxic algae and shellfish toxins, phytoplankton monitoring programs are now being used in several parts of the world and have proven to be an effective "first line" strategy for estimating shellfish toxicity (Ono et al. 1996; Trusewich et al. 1996; Rhodes et al. 2001).

The correlation between *Alexandrium* abundance and shellfish toxicity does not hold for all shellfish. Clams, for instance, accumulate PSP toxins and store them for years (Price et al. 1991). In contrast, mussels have proven to be excellent sentinel species of water column toxicity because of their ability to accumulate extremely high levels of toxins and subsequently depurate the toxins at fairly rapid rates (on the order of weeks) once the toxic algae disappear from their food source (Price et al. 1991). In other words, mussels are excellent integrators of "current events" in the water column.

1.8 Objectives

This study, funded by the Alaska Science and Technology Foundation, had two major objectives: (1) to describe *Alexandrium* abundance in relation to shellfish toxicity over time and space in the NE Kodiak Island vicinity (Figure 1.1) and (2) to evaluate the sandwich hybridization (SH) probe assay as a means to detect and quantify *Alexandrium*. Specifically, my goal was to determine if the sandwich hybridization assay was a useful tool in detecting and quantifying *Alexandrium* abundance in the water column and if the information from this assay could be used to predict PSP toxin events in mussels. A longer-term goal was to help implement a DNA probe-based assay as part of a buoy-deployed system that would provide useful information to recreational, commercial, and subsistence shellfish harvesters in Alaska. A minor objective was (3) to describe local environmental parameters in relation to *Alexandrium* abundance.

The data from my thesis project are presented in the following four chapters. These chapters are inter-related and cover many of the same points, each with a unique focus that highlights different elements of the overall project. Chapter 2 includes my overall sampling strategies as well as general strengths and weaknesses of the sandwich hybridization assay. The chapter addresses some of the difficulties I had in running the SH assay and incorporates a comprehensive Materials and Methods section pertaining to water sampling and processing that will be referred to in subsequent chapters. In Chapter 3, the most complete data from summer studies conducted in

2000 and 2001 were combined into a short, publishable version (submitted to the Xth International Conference on Harmful Algae) of results from the sandwich hybridization assay and associations with water and mussel toxicity. This chapter provides data that generally support the use of the SH assay in a monitoring program. Chapter 4 includes a more comprehensive presentation of data from the sandwich hybridization assays and mussel toxicity data including all samples from the summers of 1999, 2000, and 2001. Data presented in Chapter 4 support the overall conclusions reached in Chapter 3. However, the data in Chapter 4 are discussed within the context of an oceanographic model that explains dynamics of PSP events in the Gulf of Maine. Chapter 5 includes nutrient and hydrographic data from one of the Kodiak Island sampling sites during 2000 and 2001. Although the sampling was only local and not designed to be comprehensive, the data are evaluated in the context of whether environmental "triggers" (e.g., rainfall, nutrients, temperature) can be identified that serve to predict blooms of *Alexandrium* on Kodiak Island.

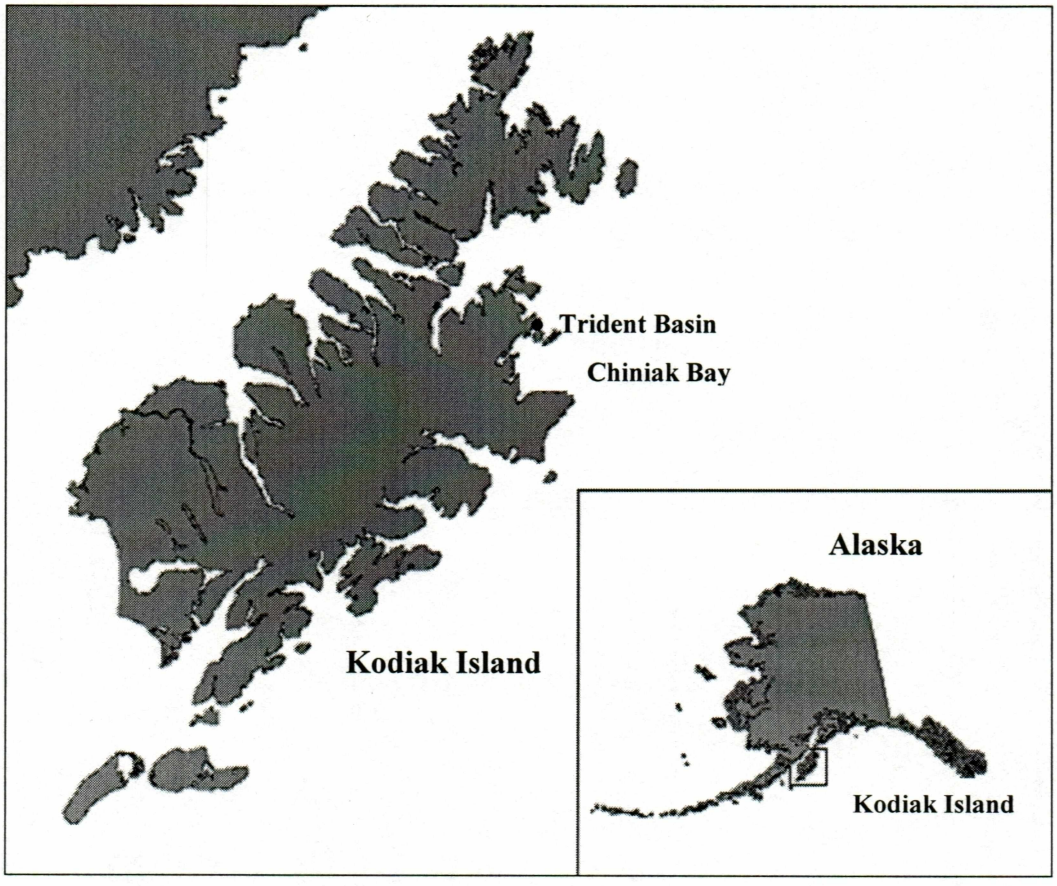

Figure 1.1. Map showing location of Kodiak Island in relation to the Alaska coastline. The study was conducted in the NE Kodiak Island vicinity, with a focus on Trident Basin in Chiniak Bay.

1.9 References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D., 1994. Molecular Biology of the Cell. 3rd ed. Garland Publishing, New York.

Anderson, D.M., 1980. Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae) hypnozygotes. J. Phycol. 16, 66-172.

Anderson, D.M., 1989. Toxic algal blooms and red tides: a global perspective. In: Okaichi, T., Anderson, D.M., Nemoto, T. (Eds.), Red Tides: Biology Environmental Science and Toxicology. Elsevier, New York, pp. 11-16.

Anderson, D.M., 1995. Identification of harmful algal species using molecular probes: an emerging perspective. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou C. (Eds.), Harmful Marine Algal Blooms. Technique et Documentation, Lavoisier, Intercept Ltd., pp. 3-13.

Anderson, D.M., 1998. Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 29-48.

Anderson, D.M., Keafer, B.A. 1987. An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature 325, 616-617.

Anderson, D.M., Kulis, D.M., Binder, B.J., 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures. J. Phycol. 20, 418-425.

Baker, T.R. 2001. Saxitoxins: Role of Prokaryotes. M.S. Thesis. Univ. Alaska.

Baker, T.R., Doucette, G.J., Powell, C.L., Boyer, G.L., Plumley, F.G., 2003. GTX_4 Imposters: Characterization of fluorescent compounds synthesized by *Pseudomonas stutzeri* SF/PS and P*seudomonas/Alteromonas* PTB-1, symbionts of saxitoxin-producing *Alexandrium* spp. Toxicon 41, 339-347.

Balech, E., 1985. The genus *Alexandrium* or *Gonyaulax* of the tamarensis group. In: Anderson, D.M., White, A.W., Baden, D.G. (Eds.), Toxic dinoflagellates. Elsevier, New York, pp. 33-38.

Cannon, J., 1993. Germination of the toxic dinoflagellate, *Alexandrium minutum*, from sediments in the Port River, South Australia. In: Smayda, T.J., Shimizu Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, Amsterdam, pp. 103-107.

Carmichael, W.W., Evans, W.R., Yin, Q.Q., Bell, P., Moczydlowski, E., 1997. Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov. App. Environ. Microbiol. 63, 3104-3110.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 381-403.

Chang, J.C., 1971. An Ecological Study of Butter Clams (*Saxidomus giganteus*) in Southeastern Alaska. M.S. Thesis. Univ. Alaska.

Delong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360-1364.

Doucette, G.J., Trick, C.G., 1995. Characterization of bacteria associated with different isolates of *Alexandrium tamarense*. In: Lassus, P. Arzul, G. Erard, E. Gentien, P. Marcaillou C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Paris, pp. 33-38.

Ebbesmeyer, C.C., Chiang, R., Copping, A., Erickson, G.M., Horner, R., Ingraham, W.J., Nishitani, L., 1995. Decadal covariations of Sequim Bay paralytic shellfish poisoning (PSP) with selected Pacific Northwest environmental parameters. In: Proc. Puget Sound Research '95. Puget Sound Water Qual. Auth. pp. 415-421.

Fortuine, R., 1996. Paralytic shellfish poisoning in the North Pacific: Two historical accounts and implications for today. In: Alaska's Marine Resources. Univ. Alaska Fairbanks. Marine Advisory Program. 8 (2), 18-19.

Geraci, J.R., Anderson, D.M., Timperi, R.J., St. Aubin, D.J., Early, G.A., Prescott, J.H., Mayo, C.A., 1989. Humpback whales (*Megaptera novaeangliae*) fatally poisoned by dinoflagellate toxin. Can. J. Fish. Aquat. Sci. 46, 1895-1898.

Gessner, B.D., Middaugh, J.P., 1995. Paralytic Shellfish Poisoning in Alaska: a 20 year retrospective analysis. Am. J. Epidemiol. 141, 766-770.

Gessner, B., 1996. Epidemiology of paralytic shellfish poisoning outbreaks in Alaska. In: Alaska's Marine Resources. Univ. Alaska Fairbanks. Marine Advisory Program. 8 (2), 16-17.

Hall, S., 1982. Toxins and Toxicity of *Protogonyaulax* from the Northeast Pacific. Ph.D. Thesis, Univ. Alaska.

Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., Reichardt, P.B., 1990. The Saxitoxins: Sources, Chemistry, and Pharmacology. In: Hall, S., Strichartz, G. (Eds.), Marine Toxins: Origin, Structure, and Molecular Pharmacology. American Chemical Society, Washington, DC, pp. 29-65.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 70-99.

Hallegraeff, G.M., Steffensen, D.A., Wetherbee, R., 1988. Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. J. Plank. Res.10, 533-541.

Hallegraeff, G.M., Bolch, C.J., Bryan, J., Koerbrin, B., 1990. Microalgal spores in ship's ballast water: a danger to aquaculture. In: Graneli, E., Sundstrom, B., Edler L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton, Elsevier, New York, pp. 475-480.

Halstead, B.W., 1965. Poisonous and Venomous Marine Animals, vol. 1. U.S. Printing Office, Washington, DC. 994 pp.

Haya, K., Martin, J.L, Waiwood, B.A., Burridge, L.E., Hungerford, J.M., Zitko, V., 1990. Identification of paralytic shellfish toxins in mackerel from Southwest Bay of Fundy, Canada. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 350-355.

Horner, R.A., Garrison, D.L., Plumley, F.G., 1997. Harmful algal blooms and red tide problems on the U.S. west coast. Limnol. Oceanogr. 42, 1076-1088.

Humpage, A.R., Rositano, J., Baker, P.D., Nicholson, B.C., Steffensen, D.A., 1993. Paralytic shellfish poisons from freshwater blue-green algae. Med. J. Australia 159, 423.

Jeon, J., Han, M.S., Lee, H.O., 1996. Paralytic shellfish toxins in the mussels and *Alexandrium tamarense* (Dinoflagellate) from Gamraepo, Korea in 1989. In: Yasumoto, T., Oshima Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 65-68.

Lam, C.W.Y., Ho, K.C., 1989. Red tides in Tolo Harbour, Hong Kong. In: Okaichi, R.T., Anderson, D.M., Nemoto, T. (Eds.), Red Tides: Biology Environmental Science and Toxicology. Elsevier, New York, pp. 49-52.

Lange, M., Guillou, L., Valuot, D., Simon, N., Armann, R.I., Ludwig, W., Medlin, L.K., 1996. Identification of the class Prymneiophyceae and the genus Phaeocystis with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. J. Phycol. 32, 858-868.

Meyers, H.F., Hilliard, M.S., 1955. Shellfish poisoning episode in False Pass, Alaska. Pub. Health Rept. U.S. 70, 419-420.

Meyer, K.F., Sommer, H., Schoenholz, P., 1928. Mussel poisoning. J. Prevent. Med. 2, 365-394.

Miller, P.E., Scholin, C.A., 1998. Identification and enumeration of cultured and wild *Pseudo nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J. Phycol. 34, 371-382.

Miller, P.E., Scholin, C.A., 2000. On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridization: sample fixation and stability. J. Phycol. 36, 238-250.

Neal, R.A., 1967. Fluctuations in the Levels of Paralytic Shellfish Toxin in Four Species of Lamellibranch Molluscs near Ketchikan, Alaska, 1963-1965. Ph.D. Dissertation, Univ. Washington.

Neve, R.A., Reichardt, P.B., 1984. Alaska's shellfish industry. In: Ragelis, E.P. (Ed.), Seafood Toxins, American Chemical Society, pp. 53-58.

Nishitani, L., Chew, K.K., 1984. Recent developments in paralytic shellfish poisoning research. Aquaculture 39, 317-329.

Nishitani, L., Chew, K.K., 1988. PSP toxins in the Pacific coast states: monitoring programs and effects on bivalve industries. J. Shell. Res. 7, 653-669.

Okaichi, T., 1989. Red tide problems in the Seto Inland Sea, Japan. In: Okaichi, R.T., Anderson, D.M., Nemoto, T. (Eds.), Red Tides: Biology Environmental Science and Toxicology. Elsevier, New York, pp. 137-142.

Ono, C., Yoshimatsu, S., Matsuoka, S., 1996. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 23-25.

Pereira, P., Onodera, H., Andrinolo, D., Franca, S., Araujo, F., Lagos, N., Oshima, Y., 2000. Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aquae*, isolated from Montargil reservoir, Portugal. Toxicon 38, 1689-1702.

Plumley, F.G., Wei, Z., Toivanen, T.B., Doucette, G.J., Franca, S., 1999. Tn5 mutagenesis of Pseudomonas stutzeri SF/PS, a bacterium associated with *Alexandrium lusitanicum* (Dinophyceae) and paralytic shellfish poisoning. J. Phycol. 35, 1390-1396.

Prakash, A., Taylor, F.J.R., 1966. A "red water" bloom of *Gonyaulax acatenella* in the Strait of Georgia and its relation to paralytic shellfish toxicity. J. Fish. Res. BD. Canada, 23, 1265-1270.

Price, D.W., Kizer, K.W., Hansgen, K.H., 1991. California's paralytic shellfish poisoning prevention program, 1927-89. J. Shellfish Res. 10, 119-145.

RaLonde, R., 2001. Harmful algal blooms: the economic consequences for Alaska. In: Harmful Algal Blooms on the North American West Coast. Univ. Alaska Sea Grant. AK-SG-0105. pp. 43-51.

Rhodes, L.L., Mackenzie, A.L., Kaspar, H.F., Todd, K.E., 2001. Harmful algae and mariculture in New Zealand. J. Mar. Sci. 58, 398-403.

Sawyer, P.J., Gentile, J.H., Sasner, J.J., 1968. Demonstration of a toxin in *Aphanizomenon flos aquae* (L.) Ralfs. Can J. Microbiol. 14, 1199-1204.

Schantz, E.J., Magnusson, H.W., 1964. Observations on the origin of the paralytic poison in Alaska butter clams. J. Protozool. 11, 239-242.

Scholin, C.A., 1998. Development of nucleic acid probe-based diagnostics for identifying and enumerating harmful algal bloom species. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 337-349.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30, 999-1011.

Scholin, C., Miller, P., Buck, K., Chavez, F., Cangelosi, G., Haydock, P., Howard, J., Harris, P., 1996. DNA probe-based detection of harmful algal species using *Pseudo-nitzschia* species as models. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 439-442.

Scholin, C.A., Miller, P.E., Buck, K.R., Chavez, F.P., Harris, P., Haydock, P., Howard, J., Cangelosi, G., 1997. Detection and quantification of *Psuedo-nistzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol. Oceanogr. 42, 1265-1272.

Scholin, C.A., Marin III, R., Miller, P.E., Doucette, G.J, Powell, C.L., Haydock, P., Howard, J., Ray, J., 1999. DNA probes and a receptor binding assay for detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J. Phycol. 35, 1356-1367.

Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. J. World Aqua. Soc. 21, 65-104.

Shumway, S.E., Sherman-Caswell, S., Hurst, J.W., 1988. Paralytic shellfish poisoning in Maine: monitoring a monster. J. Shell. Res. 7, 643-652.

Sommer, H., Whedon, W.F., Kofoid, C.A., Stohler, R., 1937. Relation of paralytic shellfish poison to certain plankton organisms of the genus *Gonyaulax*. Arch. Pathol 24, 537-559.

Smayda, T.J., 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 29-40.

Steidinger, K.A., Moestrup, O., 1990. The taxonomy of *Gonyaulax, Pyrodinium Alexandrium, Gessnerium, Protogonyaulax*, and *Goniodoma*. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 522-523.

Steidinger, K.A., 1993. Some taxonomic and biologic aspects of toxic dinoflagellates. In: Falconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Water, Academic Press, London, pp.1-18.

Strichartz, G., Castle, N., 1990. Pharmacology of marine toxins: effects on membrane channels. In: Hall, S., Strichartz, G. (Eds.), Marine Toxins: Origin, Structure, and Molecular Pharmacology. American Chemical Society, Washington, DC, pp. 2-20.

Trainer, V.L. 2002. Harmful Algal blooms on the U.S. West Coast. PICES Scientific. Report. No. 23 (2002).

Trusewich, B., Sim, J., Busby, P., Hughes, C., 1996. Management of Marine Biotoxins in New Zealand. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 27-30.

Turner, J.T., Doucette, G.J., Powell, C.L., Kulis, D.M., Keafer, B.A., Anderson, D.M., 2000. Accumulation of red tide toxins in larger size fractions of zooplankton assemblages from Massachusetts Bay, USA. Mar. Ecol. Prog. Ser. 203, 95-107.

Turpin, D.H., Dobel, P.E.R., Taylor, F.J.R., 1978. Sexuality and cyst formation in Pacific strains of the toxic dinoflagellate *Gonyaulax tamarensis.* J. Phycol. 14:235-238.

Wekell, J., 2001. West coast harmful algal blooms: the moving target. In: Harmful Algal Blooms on the North American West Coast. Univ. Alaska Sea Grant. AK-SG-0105. pp. 19- 29.

Zimmerman, S.T., McMahon, R.S., 1976. Paralytic shellfish poisoning in Tenakee, Southeastern Alaska: a possible cause. Fisheries Bull. (U.S.) 74, 679-680.

Chapter 2
Evaluation of the Sandwich Hybridization Assay and Sampling Method

2.1 Introduction

Alaska has a long-standing history of paralytic shellfish poisoning (Chapter 1). Unlike many other states plagued by this problem, Alaska, with an immense coastline and dispersed population, has been unable to routinely screen for PSP toxins (Nishitani and Chew 1988). Most PSP toxin data have been collected either sporadically after outbreaks of illness or semi-regularly for commercial harvesters. Improved monitoring in the State of Alaska is needed. Researchers worldwide have turned to plankton monitoring as a first-line strategy for estimating shellfish toxicity (Ono et al. 1996, Trusewich et al. 1996). This study focuses on a newly developed nucleotide probe assay (Saigene Corporation, Seattle, WA) as a tool to detect HAB species and to monitor the State's coastline more effectively.

The overall objective of this study was to determine if an association exists between *Alexandrium* spp. abundance, as measured by the sandwich hybridization (SH) probe assay, and mussel toxicity (i.e., can the probes be used to predict toxin events?). To answer this question it was first necessary to determine the accuracy with which the probe assay assesses *Alexandrium* abundance in field-collected samples. Measuring rRNA in environmental samples is a new field. The SH assay has been primarily used for the detection of *Psuedo-nistzschia* (Scholin et al. 1997; Miller and Scholin 1998; Scholin et al. 1999) and the format has only recently been applied to target *Alexandrium* spp. Because this study was part of a beta test of the SH assay for *Alexandrium* in field situations there were many unknowns, such as how many samples needed to be analyzed, how many replicates were needed per sample, how the probe would work in a field situation, and what was the best way to collect phytoplankton samples.

For the SH assay to be useful for public safety or in a commercial setting, it must be reliable and easy to use, and routine sampling must be practical. This chapter addresses these concerns in implementing a field-monitoring program.

2.2 Materials and Methods

2.2.1 Water collection

Water samples were collected in Trident Basin (Figure 2.1) located in Chiniak Bay, Kodiak Island, Alaska, USA (57° 47' N 152° 24' W). Trident Basin is approximately 27 m in depth and is relatively well mixed (E. Munk, NOAA, pers. comm.), with three entrances into Chiniak Bay. Water temperatures ranged from 7 – 13° C during the sampling period.

During the 2000 field season (May-Nov), net samples were collected weekly using a 20 μm mesh plankton net constructed for quantitative collection (Aquatic Research Instruments, Lemhi, ID; 20 cm X 80 cm). The net was towed vertically from a depth of 5 m, except for samples collected prior to 6/11/00, when the sampling depth was 1.5 m. The calculated, sampled volume was 157 L (5 m) or 48 L (1.5m). Plankton in the net were washed into the cod end and the recovered volume recorded. During periods of low phytoplankton abundance, as determined by visual examination of the concentrated water sample, the net was deployed twice for a total sample of 314 L. Niskin bottle casts were conducted periodically throughout the 2000 field season. Approximately 11 L of seawater were collected from discrete depths of 1, 2, 5, 10, and 17 m with a 5 L Niskin bottle lowered twice to each depth. (A 5 L bottle was used and was found to over-collect, resulting in approximately 5.5 L water collected). The seawater from each depth was combined in the field and passed through the 20 μm plankton net for concentration of the plankton. Water samples were stored on ice in darkness for transport to the laboratory.

During the 2001 field season (May-Oct), water samples were collected twice weekly. Net samples were 5 m vertical tows, as described above. For Niskin bottle samples, approximately 11 L of seawater were collected at 1, 3, 5, 10 and 17 m with a 5 L Niskin bottle lowered twice to each depth. Seawater from each depth was combined in the lab, 10 L were concentrated using 20 μm Nitex mesh screens, rinsed with filtered seawater, and collected as a concentrated sample in a known volume.

From the concentrated phytoplankton samples, sub-samples were filtered for epifluorescence whole cell (WC) counts and the sandwich hybridization (SH) assay. The data from each assay were combined to generate calibration curves for the SH assay.

2.2.2 *Whole cell*

Microscopic identification of *Alexandrium* spp. was by fluorescence *in situ* whole cell (WC) hybridization using a LSU rRNA-targeted probe. Oligonucleotides, synthesized with a fluorescein label attached to the 5' end (Oligos Etc., Eugene OR), were provided by Dr. C. Scholin (MBARI). Probes consisted of a positive and negative control and a probe specific to *Alexandrium* spp. The positive control (uniC) was a small subunit (SSU)-targeted, universally conserved sequence; the negative control (uniR) was the reverse of uniC. The North American (NA1) probe, with rRNA complement sequence (5'-3') AGTGCAACACTCCCACCA, targeted the LSU rRNA of the *Alexandrium tamarense/catenella/fundyense* species complex, North American ribotype. Details of the sequence analysis have been published (Scholin et al. 1994). Probes were resuspended in TE buffer to attain working concentrations of 200 ng/μL. Aliquots from the net samples (2000) and one of the Niskin bottle depth samples (2001) were filtered onto 25 mm Isopore membrane filters (1.2 μm pore size) using a 15 ml self-contained filter apparatus (Scholin et al. 1997; Miller and Scholin 1998) and processed for whole cell probing (Miller and Scholin 1998). Cells on the filter were fixed with an EtOH fixative (Miller and Scholin 2000), hybridized with the fluorescein-labeled probe at 45° C, and viewed with an epifluorescence microscope.

2.2.3 *Sandwich hybridization*

Aliquots for SH were filtered in duplicate onto 25 mm Durapore (Millipore) membrane filters (0.65 μm pore size) using vacuum filtration. The filters were stored at –70° C (for up to six months) until the assay was run. Assay protocols followed Scholin et al. (1999), utilizing the NA1 LSU rRNA oligonucleotide (Scholin et al. 1994). All reagents and equipment for hybridization were provided by Saigene Corporation. (Seattle, WA). The protocol called for lysis of cells on each filter with 250 μL of 3 M lysis buffer at 85° C. The lysate was passed through a syringe-driven filter unit (0.45 μm) and loaded in triplicate into the preloaded NA1 reagent assay plates. Plate conformation is described in Scholin et al. (1999). The plates were processed on the Sample Hybridization Processor at 30° C. The processor unit was semi-automated, pre-programmed to process through the hybridization steps specified by Saigene Corporation. The resultant colorimetric signal was read on an EI 800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT), at 650 nm.

Triplicate OD readings at 650 nm were averaged for each replicate. The standard curve equation derived from the WC calibration (described below) was applied to the triplicate average to determine the number of cells/250 µL lysate. Cell numbers were calculated to units of cells/L using the following calculation:

$$\frac{\#\,\text{cells}}{250\,\mu\text{L lysate}} * \frac{1000\,\mu\text{L lysis}}{\text{sample on SH filter (mL)}} * \frac{\text{conc. sample (mL)}}{\text{unconc. sample (L)}} = \frac{\#\,\text{cells}}{\text{L}}$$

The cell densities determined from replicate filters were averaged to obtain one data point for each sample. The standard error between replicates was determined and plotted on the depth profiles of *Alexandrium* abundance. In most cases, two replicates were used (i.e., n=2). However, when water samples were also used for creation of the standard curve (see below), 4-10 replicates were used (n>2).

Alexandrium abundance data from net samples were plotted over time in units of cells/L. *Alexandrium* abundance from the Niskin bottle collection at 1, 3, 5, 10 and 17 m was depth-averaged; cell numbers were integrated through the water column to determine cell abundance/m^2 then divided by the depth of the sampled water column (16 m) to give units of cell/L. Depth-averaged *Alexandrium* abundance was plotted over time.

2.2.4 Construction of sandwich hybridization (SH) standard curve

Whole cell counts were performed periodically (approximately every two weeks) throughout the field seasons for calibration of the sandwich hybridization assay. Typically 1-2 mL from the concentrated sample were filtered in replicate. The uniC positive control was run to verify overall hybridization conditions while the uniR negative control provided a check for autofluorescence. Between 2-3 (2000) and 3-5 (2001) filters were labeled with the NA1 probe for enumeration of target cells. The entire filter was counted twice and results averaged. At the time of filtration, between 4 and 10 filters with varying volumes were also collected for sandwich hybridization. A standard curve relating mean optical density (OD) readings from the SH assay and the mean WC counts for the same sample was determined by standard linear regression analysis (Scholin et al. 1997). The negative control OD values were averaged and set as the y intercept. Field data collected for calibration purposes throughout the summer were plotted as one curve. For comparison, a standard curve was obtained with cultured strains of *Alexandrium* spp., KOA 08 and KOA 30 (2000) and KOA 10 (2001), isolated from water and sediments

collected near Kodiak in March 2000 (S. Hall, US Food and Drug Administration, Washington D.C.). Cultures were grown in f/2' at 12° C on a 14:10 L:D cycle with periodic (every 20-30 day) dilutions. Algal culture growth stages were not determined for this study.

In the creation of the standard curve, small amounts of water were filtered for SH. If there were insufficient cells on the filter for detection, OD readings fell below the y intercept, resulting in a negative cell number after calculations. These numbers were included in the regression but were deleted from the final mean and standard error calculations for that depth (described above).

2.2.5 Estimation of error in the SH assay

The average coefficient of variation (CV) was determined from a subset of 174 SH samples. These samples where taken during the first part of the 2001 field season and represent samples with true replicate filters (i.e., the same volume of water was filtered for each replicate filter). Midseason modification of the protocol used to collect replicate filters (i.e., different volumes of concentrated water were filtered for each replicate filter) made it necessary to estimate error based only on the 174-member subset. Average CV was determined among triplicate OD readings and between replicate filters and an estimated error term was determined.

2.3 Results

2.3.1 Evaluation of the SH assay

Data from eight sampling dates in 2000 (Appendix 1. Table A.1a) and fourteen sampling dates in 2001 (Appendix 1. Table A.1b) were used to construct the field calibration curves. Cell counts averaged 67 cells per filter ±9 SD (2000) and 62 cells per filter ±8 SD (2001).

The average coefficient of variation (CV) among the 174 sample subset was 14% and ranged from 0% to 65% (Appendix 1. Table A.2). Of the total 468 field samples analyzed, 83% had a coefficient of variation equal to or less than 20%, which was the accepted CV in this study. Neither anomalously high nor unusually low OD values (fliers/dropouts; defined by ODs that when included in the triplicate average gave CV > 20%) were discarded in the data reported here. In some cases, the anomalous values were easily identified, but for 40 of the 468 total samples, it was not possible to determine which triplicate reading to eliminate.

Two filters (replicates) were analyzed for each water sample collected. The variability between the average OD readings for the replicate filters from the 174-member subset of samples was estimated by calculating the average CV, which was 12%. The replicate error for all samples is shown diagrammatically as SE bars for *Alexandrium* depth profiles (Appendix 1. Figure A.1).

The overall error for the SH assay includes error associated with triplicate OD readings (14%) and variability in replicate filters (12%). If this error is additive, the total error would be 26%. However a portion of the replicate variation is due to the variation in the triplicate well readings. If the six OD readings for each sample (e.g., 2 filters X 3 wells) are averaged, the total CV is 18%.

The sensitivity of the assay kit varied depending on plate manufacture between years. Therefore, a calibration curve was established for each lot of plates, i.e., each field season. The r^2 values of the standard field curves generated for 2000 and 2001 were 0.67 and 0.62, respectively (Figures 2.2 and 2.3). Additional curves were generated from cultured *Alexandrium*, KOA 30 and KOA 08 (2000; Figure 2.2) and KOA 10 (2001; Figure 2.3). The slopes of the curves between culture/culture and culture/field (2000) and culture/field (2001) were distinctly different, with a three-fold difference seen between the upper and lower extremes.

The field data from each season were combined to obtain an "averaged" regression equation for each year (Figure 2.2 and 2.3). An averaged regression was calculated because there was substantial variation in the slopes of the individual standard curves generated within each field season, as shown for 2001 in Figure 2.4, with no clear week-to-week pattern.

To investigate the changes in slope (Figure 2.4) throughout the season, the numerical value of the slopes from the 2001 calibrations were plotted over time versus cell numbers obtained from the whole cell counts. The calibration slopes appear to be inversely associated with cell numbers (r^2 = 0.30; Figure 2.5).

Both WC and SH assays were performed on the same water samples for each of the 14 field calibrations in 2001 (Figure 2.3). These samples provided a ready means of assessing the validity of using a single field standard curve instead of multiple weekly standard curves. A theoretically

perfect field standard curve would generate *Alexandrium* abundance data from the SH assay that perfectly matched the WC counts. The data in Figure 2.6 show the relationship between *Alexandrium* abundance obtained by epifluorescence microscopy and the SH assay results calculated from the field curve in 2001. The coefficient of determination (r^2) between the two methods was 0.47. Aside from the first three data points the trends were very similar, and if these points are removed from the regression analysis the $r^2 = 0.79$. On average, the difference between the two techniques was ~ 2 fold (differences range from 0.65 to 6.96X). In most cases the SH assay overestimated *Alexandrium* abundance relative to the WC assay.

2.3.2 Comparison of water sampling methods

For the following comparison of water collection methods, cell densities were calculated from the field calibration curves for 2000 and 2001. Water samples were collected with either a net or a Niskin bottle. Cell densities obtained from the two collection methods were compared. Niskin cell densities were consistently higher than the net densities, although the factor by which they varied was not constant. Niskin data from 2000 (Figure 2.7) were on average 6.5X greater than the net data (range 1.1 to 13.1), while 2001 data (Figure 2.8) were 4.4X greater (range 1.2 to 9.3). The coefficients of determination between the two techniques for 2000 and 2001 were 0.38 and 0.76, respectively.

2.4 Discussion

2.4.1 Evaluation of the SH assay

The results presented in this chapter indicated that SH assay could be used in a semi-quantitative manner to estimate *Alexandrium* abundance in water samples from Kodiak Island, Alaska. Overall, the SH assay plates provided triplicate results that were judged to be acceptable, with 83% of the samples yielding CV values of 20% or less. In some cases, there was considerable variation between triplicate OD readings for a single sample. Triplicate variation can occur due to uneven heat across the plate during hybridization, plate/prong manufacture problems, or loading/pipetting error. The plate manufacturer accepts a variation of 10% across the plate (R. Gordan (Saigene Corporation) pers. comm.). In addition, the manufacturer notes a possible defect in prong build in positions 1, 6, 7, 12. A mold defect is thought to cause an increase or decrease in surface area on the prong at these positions. This potential defect would affect the amount of capture probe applied to the prong, which in turn would lead to anomalous OD values.

In this study 86% of the samples with CV >20% were in at least one of the specified well positions. However, samples with high CV were not deleted, due to the difficulty in determining the "true" OD for many of the samples. Saigene Corporation does not recommend excluding these values unless the triplicate CV is over 15% and the inconsistencies correspond to the suspect positions. To determine the effect of including these points, the data were plotted both with and without the anomalous values corresponding to suspect well positions. There was little difference in cell concentrations after integration of the depth samples (data not shown). In addition, the high coefficients of variation were not a problem specific to high or low cell densities, as high variability was found occasionally throughout the sampling season regardless of *Alexandrium* abundance. I conclude that the variation in the triplicate well readings was not a major source of concern with the study design in 2001, primarily because multiple samples (i.e., from five discrete depths) were taken for each time point and integrated to derive a single cell density value.

Average variability between replicate filters for the 174-member data set was 12%. Some possible explanations for this replicate variability are subsampling error, storage effects, day-to-day variation in plate runs, and the 10% error designed into the assay by Saigene Corporation. Further research is needed to determine the exact cause of this variability.

For this study, variation associated with replicate filters and for triplicate well readings were separated so that both plate variation and replicate differences could be addressed. However because of the mid-season modifications to protocols used to collect replicate filters it was impossible to directly compare the six OD values, except for the 174-member subset. This modification complicated the determination of the overall error in the SH assay for the entire study. However, as described in section *2.3.1*, the combined error (triplicate and replicate error) was unlikely to be less than 18% or greater than 26%.

Another problem encountered in this study pertained to calibration of the SH assay. The probe signal varied from week to week when field samples were used for calibration, and between field samples and cells grown in culture, presumably due to fluctuations in the rRNA content of the target cells. It is known that the rRNA cell quota can vary depending on growth and environmental conditions (Anderson et al. 1999; Delong et al. 1989). The standard curves

(Figures 2.2 and 2.3) relating WC counts to SH optical density revealed a threefold difference between the average rRNA content of natural *Alexandrium* populations relative to the rRNA content in cultured cells (2001), and between average rRNA content of different cultures (2000). In a similar study using the SH assay to target *Heterosigma akashiwo*, Tyrell et al. (2001), found that the SH assay signal varied by a factor of approximately two throughout the growth cycle of the cell. The variation increased further during late stationary phase, when batch cultures were dying. In contrast, my greatest discrepancies between WC counts and SH data (Figure 2.6) occurred early in the year, at the beginning of the *Alexandrium* bloom, when rRNA content might reasonably be expected to be maximal. Although the growth stages of the cultures were not determined in this study, it is reasonable to expect that the cultures were at different growth phases (e.g., log phase, early stationary phase, etc.) and that cells in the field would be at various stages of growth, giving varying OD signals relative to cell numbers.

These differences in the calibration slopes, when data from each week were separately plotted (Figure 2.4), suggested that the rRNA content of cells in nature fluctuated during the summer field season. However, it appears that WC densities were inversely related to the slopes from the calibration curves calculated in 2001(Figure 2.5). Weekly results generated during periods with low *Alexandrium* cell densities resulted in a steeper slope of the WC vs. SH curve, i.e., low *Alexandrium* cell abundance correlated with steep curves. More study is needed to determine whether these data reflect a biological trend or problems with the assays.

Additional explanations for the differences seen between calibration slopes include complications with the whole cell assay. In studies with *Pseudo-nitzschia* (Miller et al. 2002), WC fluorescence intensity decreased under nitrogen-limited conditions. Results from SH assays also showed reduced signals, but not to the extent expected based on the WC observations. It is possible that severely reduced WC signal hindered accurate cell counts of *Alexandrium*, while the SH assay still detected rRNA present in the cell-free homogenate. Support for this hypothesis is evident from our 2001 KOA 10 culture. The majority of cells on the WC filters were brightly labeled. However, there were also background cells exhibiting extremely weak labeling, which were not included in the cell counts. Presumably these cells were not labeled due to very low RNA content. If similar, weakly-labeled cells also occurred in natural samples, these cells would not be included in the WC count, but rRNA from these cells would be included in the extracted

sample and measured in the SH assay. Problems can also arise when large quantities of water are needed for the assays (i.e., when *Alexandrium* abundance is low). Intact *Alexandrium* cells could have been obscured under debris or non-target cells, again hindering WC enumeration while having insignificant effects on the SH assay. Finally, recently consumed *Alexandrium* cells would still provide intact rRNA in the guts of consumers, but would not have been detected by microscopy.

In other studies using the SH technology (Scholin et al.1999; Tyrell et al. 2001), standard curves relating OD readings from SH assays to WC counts have been established using cultured cells. The standard curve was then applied to field samples. However, this study focused on field calibration, with culture work for comparison. In choosing to calibrate with field samples I encountered difficulties that would have been absent or reduced when working with cultured cells. One of the main difficulties of performing both the WC and SH assays was the requirement that the number of *Alexandrium* cells on the filters needed to be within certain quantifiable ranges. Filters with too many *Alexandrium* cells were difficult to count microscopically and would fall outside the upper bounds of the SH assay (~ 600 cells/250 μL lysate) due to probe depletion. In contrast, filters with too few cells cause statistical problems with WC counts and would fall below the limits of detection of the SH assay (~10 cells/250 μL lysate). For each sample, preliminary *Alexandrium* density estimates were based on visual light microscope scans prior to filtration. However, in periods of low to medium cell density, cells could not be identified in this manner. It was frequently necessary to estimate the amount of water to be filtered, sometimes resulting in overloading (2000) or, due to over-compensation, underloading (2001). In both years there were problems in plate/prong production by the manufacturer and in calibration of plates/prongs in the field. As a result, WC and SH assays were not run simultaneously with sampling, making mid-season adjustments in sampling strategies impossible. A key issue for continued use of the SH assay is ready availability of plates/prongs and a well-trained workforce to troubleshoot technical problems.

Despite the problems encountered in calibrating the two techniques, I found that SH assay results tracked the WC counts with relatively high fidelity (Figure 2.6). On average the differences between the two techniques was ~ 2 fold, with the SH assay often overestimating cell densities compared to the WC assay. Scholin et al. (1997), in comparing both techniques, found that

abundance data determined by the SH and WC assays varied by a factor of two (on average) with SH often yielding higher cell densities. Scholin et al. (1997) suggested that differences in rRNA content of target cells, the differences between a single fluor-labeled probe in the WC assay versus a double label in the SH assay, as well as possible cross-reactions with non-target species were possible explanations for the variability in probe signal and the systematic overestimate by the SH assay. Recent studies have found excellent agreement between WC and SH estimates during some sampling periods, but have also found that SH estimates were 2-50X higher than WC estimates at other times (Anderson et al. 2002). Scholin et al. (1999) also observed detectable SH signals that were not corroborated by the WC assay. Reasons for these discrepancies are not clear. However, effects of zooplankton grazing (i.e., *Alexandrium* present in zooplankton would not be counted by the WC assay but would contribute signal to the SH assay due to cell lysis), potential physical damage to cells during WC probing, and the above-mentioned problems with whole cell probing and SH plate builds, may contribute to the disparate results between the two methods. Because the WC and SH probe formats produce different results (cell numbers and optical density readings) we cannot yet rely on molecular probe work alone. For high-resolution quantitative studies, physical samples must be preserved for traditional microscopic analysis.

2.4.2 Comparison of water sampling methods

In many open ocean studies a bucket sample is an easy and quick method to collect surface seawater samples. However, the freshwater lens frequently encountered in coastal Kodiak waters necessitated the use of either plankton nets or Niskin bottles for sampling. Additionally, as *Alexandrium* can inhabit a range of depths, it was necessary to collect a more comprehensive water sample rather than just a surface sample.

For quantitative studies, a Niskin bottle that gathers a known quantity of whole seawater is needed. However, bottles are cumbersome to use and greatly increase the number of samples that must be collected to estimate the entire water column. I sampled five depths with Niskin bottles to obtain data equivalent to one net sample. Another limitation of Niskin bottles is that HAB species are often concentrated in a thin depth stratum. In the case of dinoflagellates, which can swim vertically to obtain light and nutrients, the ideal sampling depth to collect these organisms varies and a bloom could be missed with Niskin bottles.

Plankton nets are easy to use and permit rapid sampling throughout the water column. The limitations, however, are that hand-held nets, as used in this study, must be used at shallower depths, and even then they can become clogged and consequently 'push' water. The net was constructed for quantitative collection but could not be fitted with a flow meter; therefore an accurate calculation of the volume of water collected was not possible. Niskin cell densities were consistently higher than the net densities, presumably due to this inability to accurately determine the volume collected. I found that the relationship between net and Niskin samples was not consistent throughout the sampling season. This was probably due to changes in the phytoplankton community assemblage throughout the summer. Although algal community composition assessments were not a part of this project, clogging was most problematic when smaller plankton species were abundant, perhaps because they lodged easily in the 20 μm net.

Despite its limitations, I concluded that the net is the preferred collection method for general plankton monitoring of HAB events associated with toxic shellfish. The net method is fast, easy, and, most importantly, provided *Alexandrium* abundance data that proved to be an excellent proxy for shellfish toxicity (data presented in subsequent chapters). However, I recommend taking multiple net samples for each collection period to compensate for the sometimes variable SH results.

2.5 Summary

The SH assay proved to be a useful proxy for *Alexandrium* abundance, though there was quantifiable error associated with the assay. Importantly, the SH assay results generally tracked WC counts throughout the 2001 season. The SH assay is rapid (compared to cell counts) and relatively easy to use, making it an excellent tool for gathering real time abundance data.

Several factors must be evaluated further before the SH assay is fully deployed. In addition to the variation in assay runs, the calibration of the assay proved to be an important source of concern, as the calculated *Alexandrium* abundance results depend on the slope of the calibration curve, which varied throughout the season. More work is needed to better understand the discrepancies between the WC and SH assays. It is also important that the end user of the probe understands both the power and the limitations of the techniques. Ultimately, the questions being asked are

crucial to deciding on the appropriate use and interpretation of the results. If the end user is looking for absolute cell numbers, more work is needed to develop an understanding of both the WC and SH probe assays, and perhaps traditional microscopy still needs to be conducted. However, if absolute cell numbers are not critical and the end user is looking for a powerful tool to assess trends in HAB species abundance, the SH assay is suitable.

Researchers and developers of molecular probes are working to improve probe specificity and application. Saigene Corporation is in the beta testing phase of this SH assay development and is working to improve their product and eliminate quality control issues. The company represents an important connection between probe development and potential end users.

Harmful algal blooms are difficult to study. The organisms are often present in low numbers in a mixed population, they are often patchy and ephemeral, and require high-density sampling over long periods of time. Molecular probes can help narrow the search, reduce monitoring time, location and type. Overall, this report provides compelling evidence that DNA probe chemistry can be used to estimate the abundance of a HAB species.

Many of the problems outlined in this chapter have been encountered in other laboratories studying HAB events and were discussed at the X^{th} International Conference on Harmful Algae (St. Pete Beach, Florida, USA, October 2002). Molecular probes offer great promise, but much work remains before they can be used routinely.

2.6 Acknowledgements

I thank C. Scholin and R. Marin III at MBARI, and J. Ray at Saigene Corporation, for their assistance and guidance with the WC and SH assays.

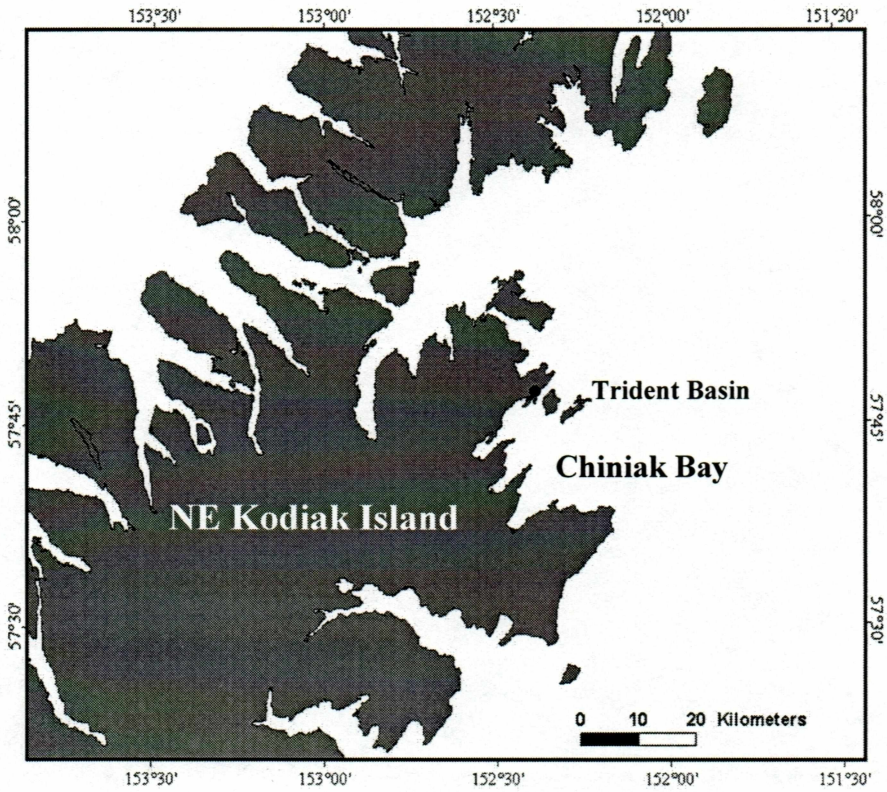

Figure 2.1. Map of the study site, Trident Basin, located in Chiniak Bay in the NE Kodiak Island region.

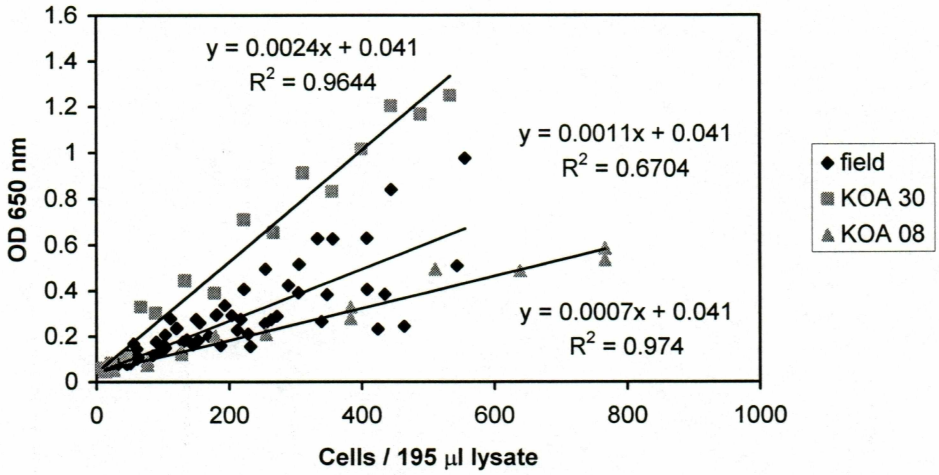

Figure 2.2. Standard curves derived from the 2000 field and culture data. Triplicate SH optical density readings at 650 nm were plotted relative to mean WC counts. The y-intercept was set at the average negative control of the assay for 2000. Standard curve equations were determined by linear regression analysis.

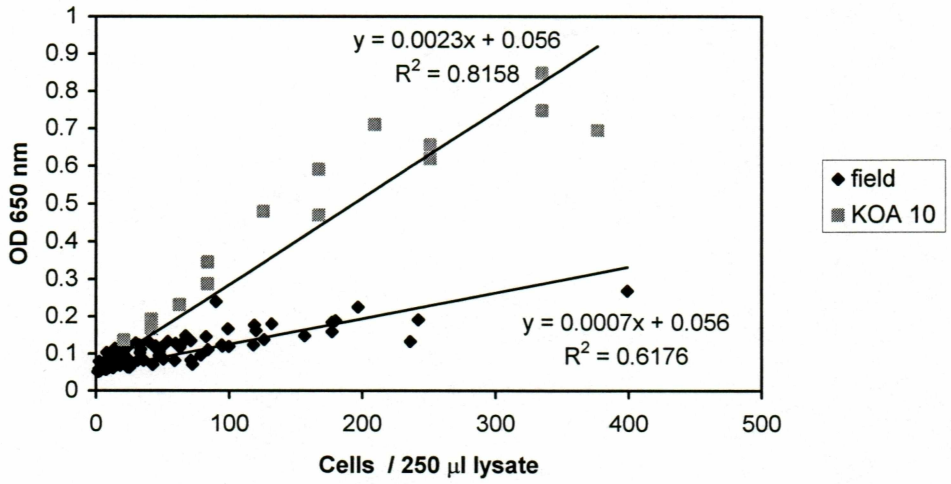

Figure 2.3. Standard curves derived from 2001 field and culture data. Triplicate SH optical density readings at 650 nm were plotted relative to mean WC counts. The y-intercept was set at the average negative control of the assay for 2001. Standard curve equations were determined by linear regression analysis.

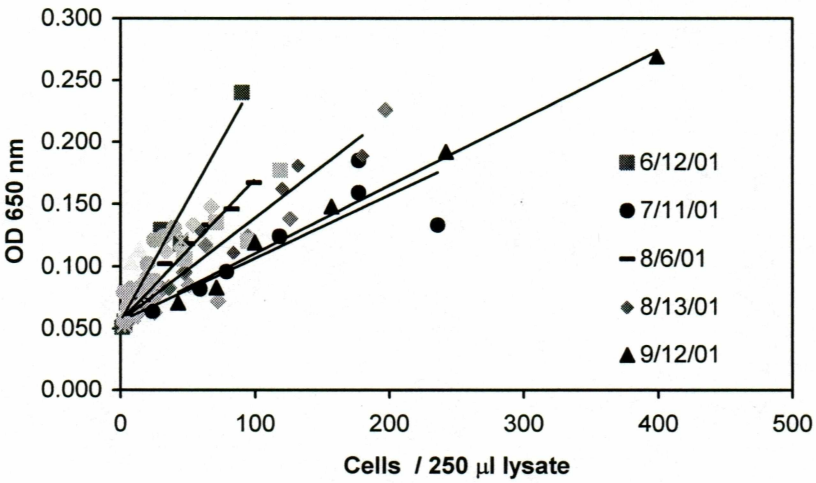

Figure 2.4. Plot of selected calibration curves that were generated as part of the field calibration of the SH assay in 2001. The trend lines are plotted for 5 of the 14 calibrations to demonstrate the differences in the slopes seen throughout the season. Symbols not identified in the legend represent data points that were included in the average field regression (Figure 2.3) but were not selected in this plot as one of the 5 depicted trend lines.

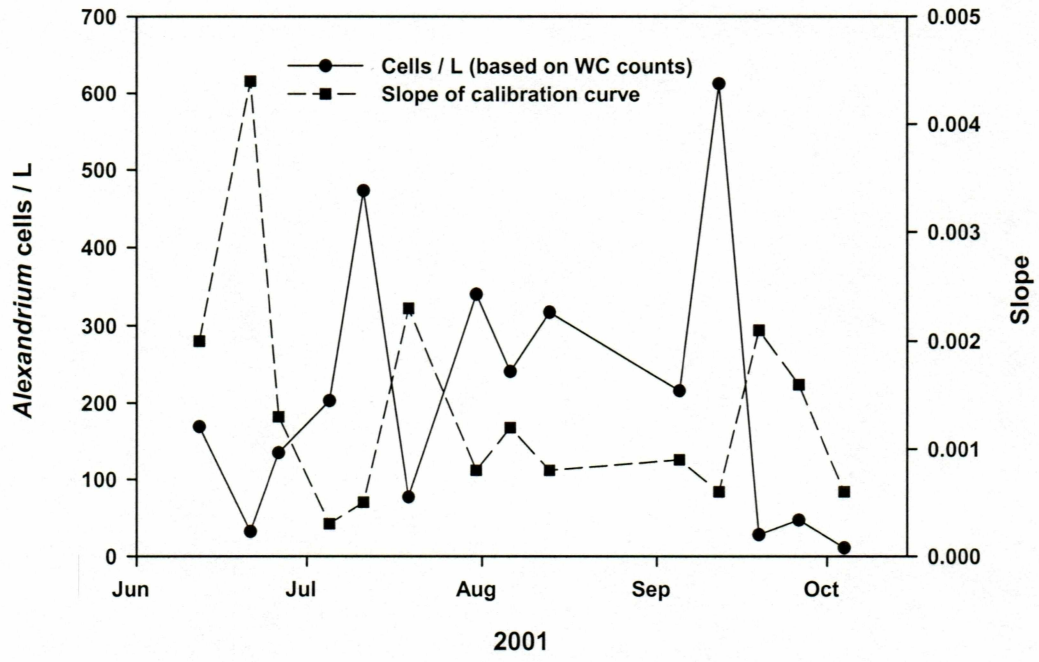

Figure 2.5. Cell densities based on WC counts (Appendix 1) and the slope of the calibration curve for that count (Figure 2.4), throughout the 2001 field season.

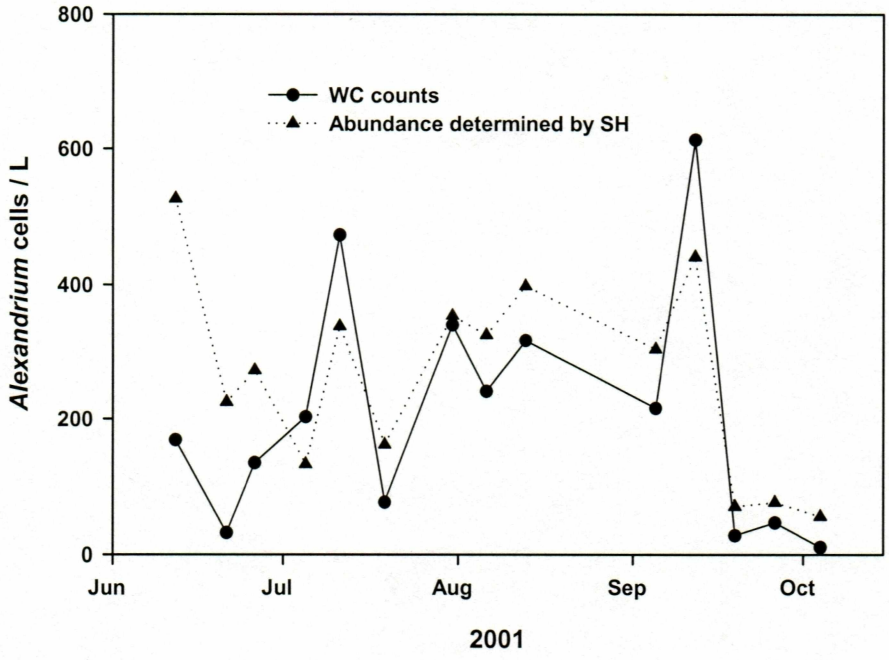

Figure 2.6. *Alexandrium* cell abundance determined by the WC and SH assays.

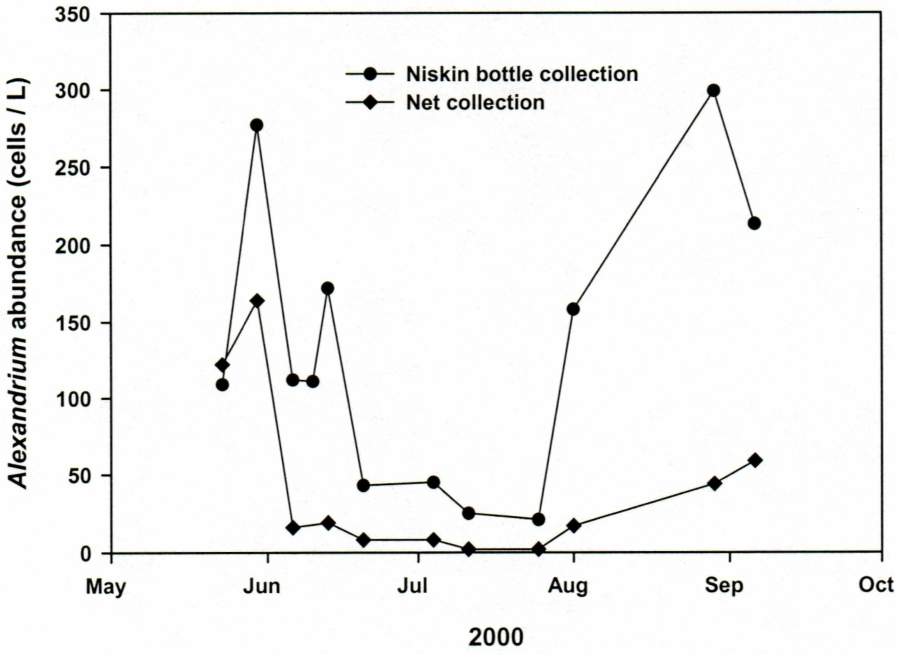

Figure 2.7. *Alexandrium* abundance from Niskin and net collections throughout the 2000 field season.

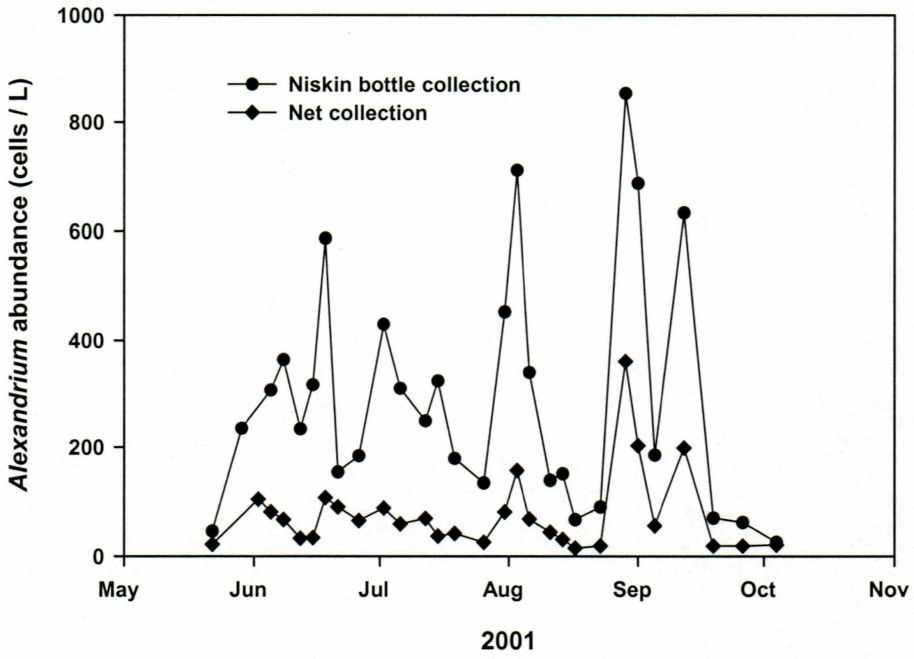

Figure 2.8. *Alexandrium* abundance from Niskin and net collections throughout the 2001 field season.

2.7 References

Anderson, D.M., Kulis, D.M., Keafer B.A., 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: variability in labeling intensity with physiological condition. J. Phycol. 35, 870-883.

Anderson, D.M., Keafer, B.A., Kulis, D.M., Connell, L., Scholin, C.A., 2002. Application of molecular probes in studies of *Alexandrium* in the Gulf of Maine: successes and problem areas. In: Xth International Conference on Harmful Algae Book of Abstracts, pp. 14.

Delong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360-1364.

Miller, P.E., Scholin, C.A., 1998. Identification and enumeration of cultured and wild *Pseudo nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J. Phycol. 34, 371-382.

Miller, P.E., Scholin, C.A., 2000. On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridization: sample fixation and stability. J. Phycol. 36, 238-250.

Miller, P., Marin III, R., Tyrell, J., Scholin, C., Goldman, J., Doucette, G., Powell, C., 2002. Variations in reactivity of rRNA-targeted probes and toxicity for *Pseudo-nitzschia multiseries* grown in nitrogen- and silicate-limited continuous cultures. In: Xth International Conference on Harmful Algae Book of Abstracts, pp.198.

Nishitani, L., Chew, K.K., 1988. PSP toxins in the Pacific coast states: monitoring programs and effects on bivalve industries. J. Shell. Res. 7, 653-669.

Ono, C., Yoshimatsu, S., Matsuoka, S., 1996. Monitoring system of harmful and toxic phytoplankton in Kagawa Prefecture, Japan. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.) Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, p. 23-25.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group and strain specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30, 999-1011.

Scholin, C.A., Miller, P.E., Buck, K.R., Chavez, F.P., Harris, P., Haydock, P., Howard, J., Cangelosi, G., 1997. Detection and quantification of *Psuedo-nistzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol. Oceanogr. 42, 1265-1272.

Scholin, C.A., Marin III, R., Miller, P.E., Doucette, G.J, Powell, C.L., Haydock, P., Howard, J., Ray, J. 1999. DNA probes and a receptor binding assay for detection of *Psuedo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J. Phycol. 35, 1356-1367.

Trusewich, B., Sim, J., Busby, P., Hughes, C., 1996. Management of Marine Biotoxins in New Zealand. In: Yasumoto, T., Oshima, Y. Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 27-30.

Tyrell, J.V., Bergquist, P.R., Bergquist, P.L., Scholin, C.A., 2001. Detection and enumeration of *Heterosigma akashiwo* and *Fibrocapsa japonica* (Raphidophyceae) using rRNA-targeted oligonucleotide probes. Phycologia. 40, 457-467.

Chapter 3

Use of *Alexandrium* rRNA Targeted Probes to Predict Paralytic Shellfish Poisoning Events on Kodiak Island, Alaska*

Abstract

Alexandrium spp. abundance was ascertained at a near-shore site on Kodiak Island, Alaska, during the summers of 2000 and 2001 using a sandwich hybridization assay and species-specific LSU rRNA targeted oligonucleotide probes. Absorption values from the sandwich hybridization assay were fitted to standard curves based either on whole cell microscopic counts of *Alexandrium* or to cultured cells isolated from the same location. The r^2 values for the standard curves were 0.62 and 0.82 for the natural samples and cultured cells, respectively. The rRNA cell quota of cultured cells of *Alexandrium* was approximately 3X greater than that of natural populations. *Alexandrium* abundance exhibited two distinct peaks (>400 cells/L) in 2000, one in May/June and a second in August/September, both of which lasted approximately 2 weeks. *Alexandrium* abundance in 2001 was much more sporadic, with 4-5 peaks, each lasting only 3-7 days. In 2001, *Alexandrium* abundance was correlated with water column toxicity as determined via a ^{3}H-Saxitoxin receptor-binding assay ($r^2 = 0.71$). Toxicity averaged 13.7 pg STX equivalents per cell throughout 2001. Importantly, DNA probe data revealed a strong association between *Alexandrium* abundance and blue mussel (*Mytilus edulis*) toxicity in both 2000 and 2001. The results also demonstrated that increases in *Alexandrium* abundance preceded elevated toxin levels in shellfish suggesting that this method could prove useful as a monitoring tool to predict toxic events in shellfish before they are harvested.

---

*Prepared for submission under the same title with authors Julie A. Matweyou, Dean A. Stockwell, Christopher A. Scholin, Sherwood Hall, Vera L. Trainer, Jason D. Ray, Terry E. Whitledge, Amy R. Childers, and F. Gerald Plumley in the Proceedings of the Xth International Conference on Harmful Algae.

3.1 Introduction

Paralytic shellfish poisoning (PSP) in Alaska is due to *Alexandrium* spp. *A. catenella* has been identified as the primary species responsible for PSP in the Pacific (Nishitani and Chew 1984; Price et al. 1991) but at least five other *Alexandrium* species have been identified in the NW Pacific (Scholin et al. 1994; Taylor and Horner 1994). Some of the highest toxicity levels worldwide have been recorded in Alaska, with values in excess of 20,000 μg of saxitoxin per 100 g shellfish (RaLonde 1996).

Monitoring PSP events in Alaska and elsewhere is problematic. Phytoplankton monitoring programs that estimate the abundance of *Alexandrium* in the water column have proven to be an effective "first line" strategy for estimating shellfish toxicity in Japan (Ono et al. 1996) and New Zealand (Trusewich et al. 1996). A crucial question that must be addressed in a phytoplankton-monitoring program is whether phytoplankton abundance accurately predicts shellfish toxicity. A significant correlation has been shown to exist between the abundance of certain *Alexandrium* species and the toxicity of blue mussel, *Mytilus edulis* (Nishitani and Chew 1984; Jeon et al. 1996). This mussel is common in Alaska and can serve as a sentinel species because of its ability to accumulate extremely high levels of toxins as well as to depurate those toxins at a fairly rapid rate.

Here we report results of a combined study using a rapid sandwich hybridization probe assay (Scholin et al. 1999) to detect and quantify the abundance of *Alexandrium* during the summers of 2000 and 2001 on Kodiak Island, Alaska. Water column and shellfish toxicity were estimated with a receptor-binding assay (Trainer and Poli 2000) and evaluated in relation to the *Alexandrium* abundance.

3.2 Materials and Methods

Water and mussel samples were collected in Trident Basin located in Chiniak Bay, Kodiak Island, Alaska, USA (57° 47' N 152° 24' W). Trident Basin has an average depth of 27 m and is relatively well mixed (E. Munk, NOAA, pers. comm.) with three entrances into Chiniak Bay. Water temperatures ranged from 7 – 13° C during the sampling period.

3.2.1 Water collection

Water samples were collected from a dock where the water depth was 17 m, every 3-14 days from May 9 to Nov 11 in 2000 and from May 22 to October 5 in 2001. Samples were collected near high tide during daylight hours. In 2000, a 20 μm mesh plankton net constructed for quantitative collection (Aquatic Research Instruments, 20 cm X 80 cm) was towed vertically from a depth of 5 m to the surface. Cells were concentrated in the cod end to an approximate volume of 250 mL. Collection using a Niskin bottle was performed periodically throughout the summer for calibration. In 2001 water samples were collected from discreet depths (1,3,5,10, and 17 m) with a 5 L Niskin bottle deployed twice at each depth. Ten L were concentrated through 20 μm Nitex mesh, rinsed with filtered seawater, and collected as a concentrated sample of approximately 100-150 mL. For both 2000 and 2001, sub-samples were filtered for epifluorescence whole cell (WC) counts and the sandwich hybridization (SH) assay. In 2001, sub-samples were also collected to determine water column toxicity.

3.2.2 Sandwich hybridization

Aliquots for SH were filtered from each sample in duplicate onto 25 mm Durapore (Millipore) membrane filters (0.65 μm) using vacuum filtration. The assay protocol followed Scholin et al. (1999), utilizing the *Alexandrium* NA1 LSU rRNA targeted oligonucleotide probe (Scholin et al. 1994). All reagents and equipment for hybridization were provided by Saigene Corporation (Seattle, WA). Filters were stored at –70° C (for up to six months) until the assays were run. Color was read on an EI 800 Universal Microplate Reader, Bio-Tek Instruments, Inc. (Winooski, VT) at 650 nm. Triplicate optical density (OD) readings were averaged for each replicate, and *Alexandrium* abundance estimated from a standard curve (see below). Results from duplicate samples were averaged. In 2000, a single estimate of *Alexandrium* abundance was obtained for each sample, after correction (see below) from the plankton net sample. In 2001, water column estimates of *Alexandrium* abundance were based on a depth-weighted average of abundance data from each discreet depth sample. Side-by-side comparisons of plankton net samples and Niskin bottle samples in 2000 and again in 2001 clearly demonstrated that the plankton net samples underestimated *Alexandrium* abundance, presumably due to clogging and "water pushing" effects. Based on a correction factor generated during 2000, the 2000 *Alexandrium* abundance data were corrected using a multiplier of 6.5 (Matweyou 2003; Chapter 2).

3.2.3 Alexandrium whole cell counts – construction of standard curve

Alexandrium whole cell (WC) counts were performed approximately once a week throughout the 2001 field season for calibration of the SH assay. Aliquots from one of the depth samples were filtered in replicate, labeled with the NA1 probe and processed for epifluorescence microscopy as described (Miller and Scholin 1998). The entire filter was counted twice and results averaged. For every sample used for the WC assay, 4-10 filters with varying cell densities were collected and run through the SH assay as described above. A standard curve relating WC and SH was determined by regression analysis. Field data collected throughout the season from the WC assay and the corresponding SH assays were combined to generate a single standard curve. All data reflecting *Alexandrium* abundance reported in this study were obtained by utilizing this "field" standard curve. For comparative purposes, a second standard curve was obtained with a cultured strain of *Alexandrium*, KOA 10, isolated from water/sediments collected from Kodiak in March 2000. KOA 10 was grown in f/2' at 12°C on a 14:10 L:D cycle with periodic (once every 20-30 days) dilutions.

3.2.4 Water column and mussel toxicity

Aliquots of concentrated seawater were filtered onto 47 mm Millipore HA filters (0.45 μm) for determination of water column toxicity. Approximately 40 mussels were collected weekly from a beach close to the water collection site. Mussels were selected with an average shucked wet mass of 3-5grams per mussel. Mussel toxins were extracted according to Association of Analytical Communities (AOAC) protocol (AOAC 1995). Both mussel and water column toxins were analyzed with the receptor-binding assay (Trainer and Poli 2000).

3.3 Results

3.3.1 Reproducibility of SH assay

Variations between triplicate OD readings were determined from a subset of 174 samples. The average coefficient of variation among these samples was 14% with a range from 0%-65%. Of the total 468 field samples processed with the SH assay, 83% of the samples run in triplicate on the assay plate had a coefficient of variation equal to or less than 20%, which was the accepted CV of this study. "Fliers" and "dropout" OD values were not discarded form the data reported here. In most cases, the anomalous value was clear, but for other samples, the three triplicate values for the SH assay were so disparate that it was not possible to determine which one(s) to

eliminate. Variation was also found between replicates. Total variation is estimated to be between 18 and 26% (Matweyou 2003; Chapter 2).

3.3.2 Calibration of SH assay

SH optical density values were plotted relative to WC epifluorescence *Alexandrium* cell counts from samples collected throughout the summer of 2001, to create a standard curve of field data (Figure 3.1; r^2=0.62). A second standard curve was generated from cultured *Alexandrium* (KOA 10; Figure 3.1; $r^2 = 0.82$). The slope of the culture curve (0.0023) was 3X that of the field curve (0.0007).

Figure 3.2 shows the relationship between *Alexandrium* abundance obtained by epifluorescence microscopy and the SH assay results calculated from the field curve. The coefficient of determination between the two methods was 0.47 based on linear regression analysis. Aside from the first three data points, the trends were very similar. On average the difference between the two techniques was ~ 2 fold (differences range from 0.65 to 6.96X). In most cases the SH assay overestimated *Alexandrium* abundance relative to the WC assay.

3.3.3 Seasonal trends in Alexandrium abundance

The abundance of *Alexandrium* in the water column for each sampling period was calculated from the optical density readings obtained from the SH assay using the field fitted regression line. Cell densities in 2000 (calculated from a standard curve generated in 2000 and adjusted by a factor of 6.5) ranged from near zero to 1100 cells/L with two distinct peaks in late May and late August (Figure 3.3). During the summer of 2001, *Alexandrium* abundance ranged from near zero to about 850 cells/ L (Figure 3.4). *Alexandrium* densities fluctuated throughout the summer with five peaks of 400 cells/L or greater, occurring in June, July, August, and twice in September.

3.3.4 Relationship between Alexandrium abundance and water column and mussel toxicity

Alexandrium abundance was strongly correlated with water column toxicity in 2001 ($r^2 = 0.71$ based on linear regression analysis). Statistically, the relationship between *Alexandrium* abundance and mussel toxicity was moderate to weak (r^2= 0.44, 2001; r^2= 0.10, 2000). However, from inspection of Figures 3.3 and 3.4, *Alexandrium* abundance and mussel toxicity appear to be positively related. Water column toxicity fluctuated with changes in cell densities (Figure 3.5),

while mussel toxicity typically exhibited a slight lag (approximately one week). The relationship between *Alexandrium* abundance and shellfish toxicity was seen most clearly in the 2000 Trident Basin data (Figure 3.3), which showed two distinct peaks and the general trend of increased *Alexandrium* abundance followed by, or at least was closely linked to, a rise in mussel toxicity.

3.4 Discussion

The SH assay proved useful as a semi-quantitative method to estimate *Alexandrium* abundance in water samples from Kodiak Island, Alaska. Abundance estimates from the SH assay were positively correlated with water column toxicity and, with a lag incorporated, shellfish toxicity. Overall, the SH assay is rapid, easy to use and has potential to be an excellent tool in gathering real-time *Alexandrium* abundance data and as a "first line" in estimating shellfish toxicity.

Despite the overall positive results reported here, several factors must be evaluated further before the SH assay is fully deployed. The probe signal varied from week to week, presumably due to fluctuations in the rRNA content of the target cells. The rRNA cell quota varies with environmental conditions that impact growth rates (Anderson et al. 1999; Delong et al. 1989). Our standard curves (Figure 3.1) relating WC data to SH optical density revealed a threefold difference between the average rRNA content of natural populations of *Alexandrium* relative to rRNA levels of cells grown in culture We saw similar differences in the slopes of data points when data from each week were separately plotted (Matweyou 2003; Chapter 2), suggesting that the rRNA content of cells in nature fluctuated during the summer field season. In a similar study using the SH assay to target *Heterosigma akashiwo*, Tyrell et al. (2001), found that the SH assay signal varied by a factor of approximately two throughout the growth cycle of the cell. The variation increased further during the late stationary phase when batch cultures were dying. In contrast, our greatest discrepancies between WC counts and SH data (Figure 3.2) occurred early in the year, at the beginning of the *Alexandrium* bloom, when rRNA quotas might reasonably be expected to be maximal.

Whole cell microscopic assays and SH assays tracked relative *Alexandrium* abundance, but the SH assay typically overestimated cell densities compared to the whole cell assay (Figure 3.2). Scholin et al. (1997), in comparing both techniques, found that abundance data determined by the SH and WC assays varied by a factor of 2 (on average) with SH often yielding higher cell

densities. Additional explanations for the variability in probe signal and the systematic overestimate by the SH assay include differences in rRNA content of target cells, as mentioned above, the differences between a single fluor-labeled probe in the whole cell assay versus a double label in the SH assay, as well possible cross reactions with non-target species (Scholin et al. 1997). Recent reports also present the effects of zooplankton grazing on the disparate results.

Additional problems encountered in implementing the SH probe assay on Kodiak have been described (Matweyou 2003; Chapter 2) and will be outlined here briefly. We encountered difficulties in obtaining representative samples from the water column using a plankton net or a Niskin bottle, in loading the appropriate amount of cells onto the filter for the SH assay, and in running the assay without a working positive control.

Despite the problems in obtaining absolute cell numbers, patterns in water column toxicity coincided well with relative *Alexandrium* abundance (Figure 3.5). Average toxicity was 13.66 pg STX equivalents per cell (range 2.0 – 44.8), well within the expected range (Cembella 1998). We did not see large scale seasonal differences in cellular toxicity and were not surprised by fluctuations in cellular toxin levels, because numerous studies (Hamasaki et al. 2001, Cho and Lee 2001; Anderson et al. 1990) have shown that toxicity per cell can vary with environmental and growth conditions. Mussel toxicity, in most cases, lagged slightly behind *Alexandrium* abundance, as expected due to mussel feeding and uptake kinetics. Throughout most of the 2001 study period, shellfish toxicity remained low, at or below the safe consumption level (80 µg STX equivalents/ 100 g). Three small *Alexandrium* blooms with relative densities of 550-800 cells/L, corresponded to increased toxicity above the quarantine level. In the 2000 season, two blooms had cell densities between 600-1100 cells/L. It appears from these data that *Alexandrium* cell densities of about 600 cells/ L or greater are reason for concern for mussel toxicity in this area.

3.5 Conclusion

The SH assay was found to be a good proxy for *Alexandrium* abundance and a useful tool in PSP monitoring studies where high-density sampling and routine analysis are necessary. In locations such as Alaska, where the coastline is extensive and blooms episodic, the SH assay would be useful as a first line strategy in monitoring PSP events. In a commercial setting, the assay may be

used to predict a toxic event in the water column before shellfish are harvested, helping to eliminate product waste due to contamination.

For more rigorous scientific studies, an accurate estimate of *Alexandrium* cell abundance requires that a complete standard curve be generated at each sampling point, and that this curve be generated with natural populations of *Alexandrium.* Clearly, this approach is not practical for extensive, routine monitoring, as microscopic cell counts are tedious and time consuming. The variations in rRNA content appear to be sufficiently small that the SH assay can be used more conveniently to rapidly detect *Alexandrium*, with the caveat that the cell density estimates may vary by a factor of approximately three.

3.6 Acknowledgements

This work was supported by the Alaska Science & Technology Foundation and by Alaska Sea Grant.

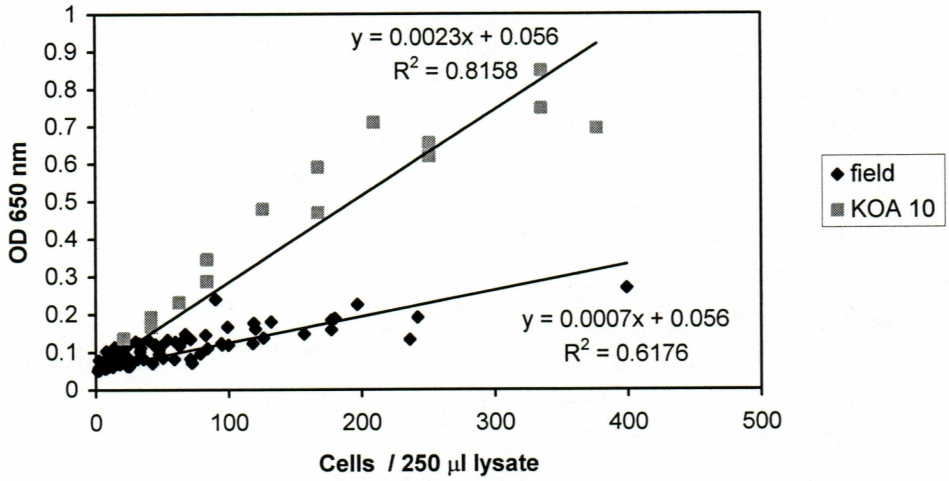

Figure 3.1. Standard curves derived from the 2001 field and culture data.

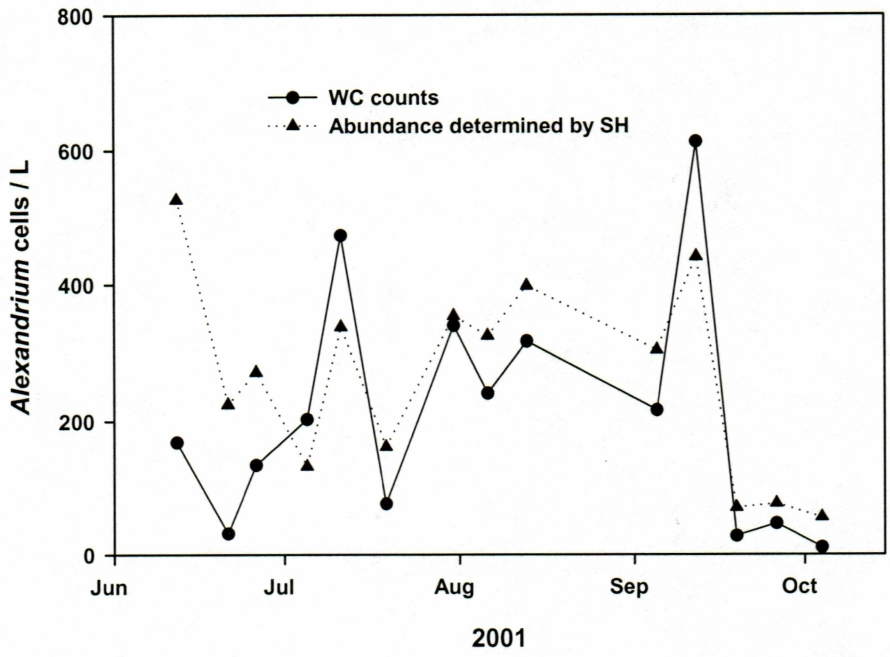

Figure 3.2. *Alexandrium* cell abundance determined by WC and SH assays.

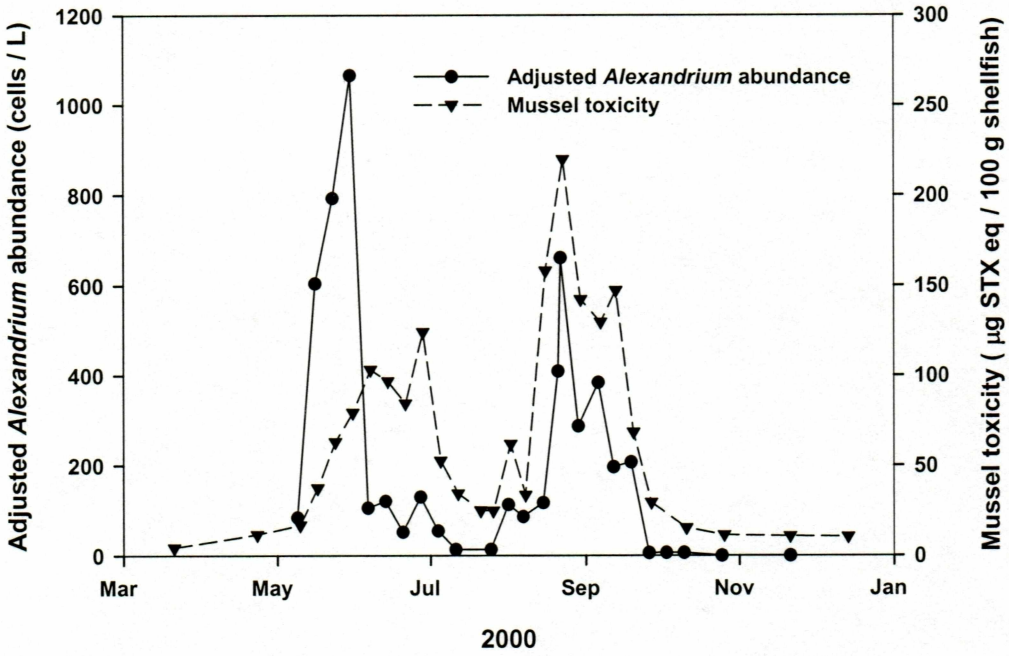

Figure 3.3. Adjusted *Alexandrium* abundance and mussel toxicity at Trident Basin 2000. Samples were collected using a net and data were corrected by a factor of 6.5 to compensate for the differences between net and Niskin collection.

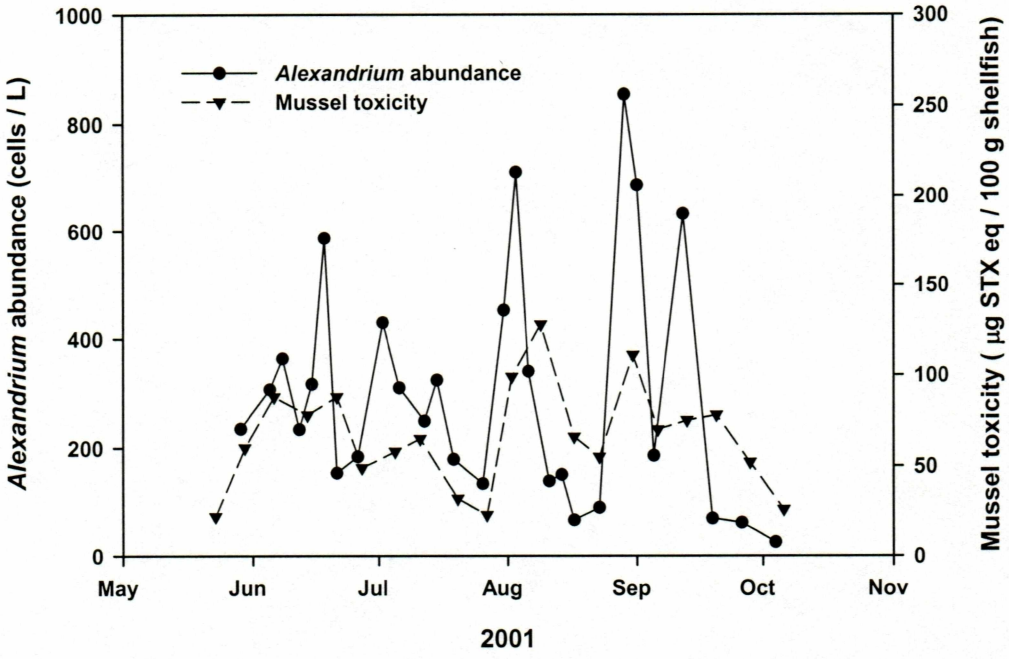

Figure 3.4. *Alexandrium* abundance and mussel toxicity at Trident Basin 2001.

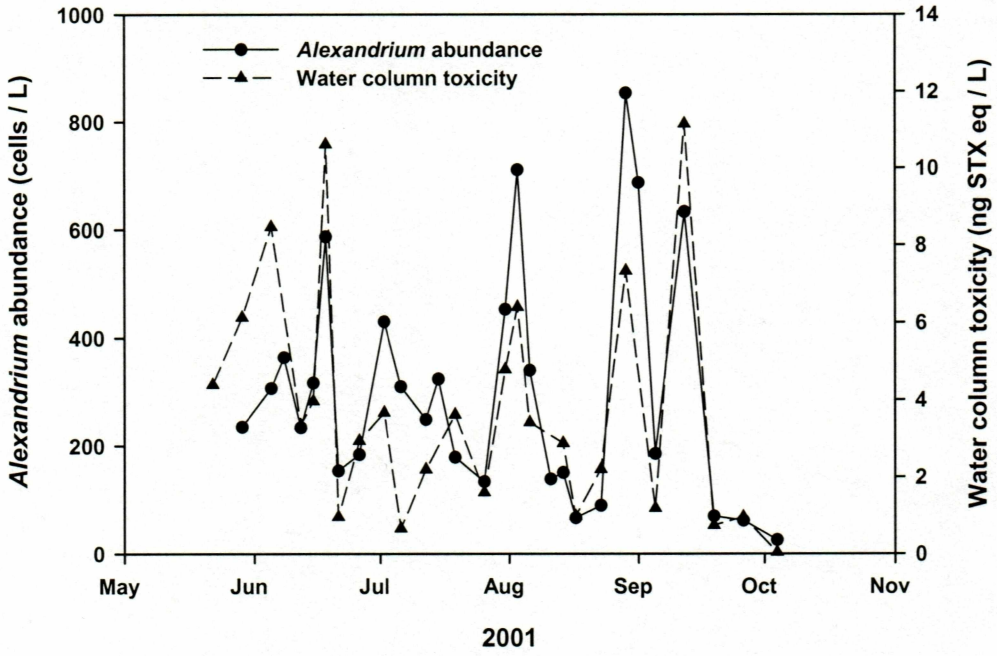

Figure 3.5. *Alexandrium* abundance and water column toxicity at Trident Basin 2001.

3.7 References

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104, 511-524.

Anderson, D.M., Kulis, D.M., Keafer, B.A., 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: variability in labeling intensity with physiological condition. J. Phycol. 35, 870-883.

AOAC 1995. Official Methods of Analysis, 16th ed., Cunniff, P. (Ed.), AOAC International, Arlington. pp 46-49.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.) Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 381-403.

Cho, E.S., Lee, H.J., 2001. Thecal plates, toxin content and growth of five clones of *Alexandrium tamarense* (Dinophyceae) isolated from Jinhae Bay, Korea. Phycologia. 40, 435-439.

Delong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360-1364.

Hamasaki, K., Horie, M., Tokimutsu, S., Toda, T., Taguchi, S., 2001. Variability in toxicity of the dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions. J. Plank. Res. 23, 271-278.

Jeon, J., Han, M.S., Lee, H.O., 1996. Paralytic shellfish toxins in the mussels and *Alexandrium tamarense* (Dinoflagellate) from Gamraepo, Korea in 1989. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.) Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 65-68.

Matweyou, J.A., 2003. Paralytic Shellfish Poisoning: The relationship between *Alexandrium* abundance and PSP toxin on Kodiak Island, AK. M.S. Thesis, Univ. Alaska.

Miller, P.E., Scholin, C.A., 1998. Identification and enumeration of cultured and wild *Pseudo-Nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J. Phycol. 34, 371-382.

Nishitani, L., Chew, K.K., 1984. Recent Developments in Paralytic Shellfish Poisoning Research. Aquaculture 39, 317-329.

Ono, C., Yoshimatsu, S., Matsuoka, S., 1996. Monitoring system of harmful and toxic phytoplankton in Kagawa Prefecture, Japan. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 23-25.

Price, D.W., Kizer, K.W., Hansgen, K.H., 1991. California's paralytic shellfish poisoning prevention program, 1927-89. J. Shellfish Res. 10, 119-145.

RaLonde, R., 1996. Paralytic Shellfish Poisoning: The Alaska Problem. In: Alaska's Marine Resources. Univ. Alaska Fairbanks. Marine Advisory Program. 8 (2), 1-7.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group and strain specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30, 999-1011.

Scholin, C.A., Miller, P.E., Buck, K.R., Chavez, F.P., Harris, P., Haydock, P., Howard, J., Cangelosi, G., 1997. Detection and quantification of *Psuedo-nistzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol. Oceanogr. 42, 1265-1272.

Scholin, C.A., Marin III, R., Miller, P.E., Doucette, G.J, Powell, C.L., Haydock, P., Howard, J., Ray, J., 1999. DNA probes and a receptor binding assay for detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. J. Phycol. 35, 1356-1367.

Taylor, F.J.R., Horner, R.A., 1994. Red tides and other problems with harmful algal blooms in the Pacific Northwest coastal waters. In: Review of the marine environment and biota of Strait of Georgia, Puget Sound and Juan de Fuca Strait. Can. Tech. Rep. Fish. Aquat. Sci. 1948. pp. 175-186.

Trainer, L.V., Poli, M.A., 2000. Assays for dinoflagellate toxins, specifically brevetoxin, ciguatoxin, and saxitoxin. In: Rochat, H., Martin-Eauclaire, M.-F. (Eds.), Methods and Tools in Biosciences and Medicine Animal Toxins. Birkhauser Verlag Basel/Switzerland, pp. 1-19.

Trusewich, B., Sim, J., Busby, P., Hughes, C., 1996. Management of Marine Biotoxins in New Zealand. In: Yasumoto, T., Oshima, Y. and Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp. 27-30.

Tyrell, J.V., Bergquist, P.R., Bergquist, P.L., Scholin, C.A., 2001. Detection and enumeration of *Heterosigma akashiwo* and *Fibrocapsa japonica* (Raphidophyceae) using rRNA-targeted oligonucleotide probes. Phycologia 40, 457-467.

Chapter 4
Spatial and Temporal Patterns of PSP Events on Kodiak Island, AK

4.1 Introduction

Paralytic shellfish poisoning (PSP) events in Alaska are thought to be caused exclusively by dinoflagellates of the genus *Alexandrium* (Horner et al. 1997). These algae produce several saxitoxin congeners as secondary cell by-products that act as neurotoxins by blocking sodium channels. PSP events in Alaska have been little studied, but available data clearly indicate that toxin production and growth of *Alexandrium* are erratic and patchy throughout the summer, with considerable variation on both temporal and spatial scales (Schantz and Magnusson 1964; Neal 1967; Hall 1982). The sporadic nature of PSP events in Alaska seemingly mirror those reported elsewhere around the world.

PSP events are often seasonal and recurrent. However the toxicity episodes are variable in magnitude, frequency and duration (Anderson 1997). Rapid, transient toxin events are common, with mussel toxicity rising from undetectable to above quarantine levels within a few days and decreasing shortly afterwards (on the scale of weeks) depending on the abundance of toxic plankton (Shumway et al. 1988; Bricelj et al. 1990). Two hypotheses have been postulated to explain harmful algal outbreaks: 1) rapid *in situ* growth of the causative organism and 2) physical oceanographic processes that concentrate plankton.

A model of *Alexandrium* growth and PSP toxin production is currently being developed for the Gulf of Maine (GOM; Franks and Anderson 1992 a, 1992b; Anderson 1995, 1997). The GOM model represents the first comprehensive attempt to describe PSP toxin production within an integrated oceanographic context that includes biological, chemical and physical oceanography. While many components of the GOM model remain unresolved, this has been the most extensive study conducted pertaining to the oceanography of *Alexandrium*. This chapter includes an initial attempt to relate the spatial and temporal PSP data from Kodiak Island, Alaska, to the processes incorporated within the GOM model. In essence, the Kodiak data are used to formulate a hypothesis about the role of physical oceanographic processes in bloom dynamics in the Kodiak Island area, analogous to the GOM model.

4.2 Materials and Methods

4.2.1 General sampling strategies

Water samples and mussels were collected from several sites in the NE Kodiak Island vicinity from 1999-2001 (Figure 4.1; Table 4.1). Water samples were collected using a 20 μm plankton net (2000) towed vertically from a depth of 5 m and a 5 L Niskin bottle (2001) deployed at depths of 1, 3, 5, 10 and 17 m. *Alexandrium* abundance was determined using the sandwich hybridization assay described in Chapter 2. Values from 2000 were adjusted for differences in cell densities obtained using the Niskin bottle and the net (Chapter 2 and 3).

4.2.2 Water column toxicity

Subsamples of concentrated seawater (2001) were filtered for water column toxicity onto 47 mm Millipore HA filters (0.45 μm) and stored at –70° C until analyzed. Filters were cut into pieces, placed in 15 mL Falcon tubes, extracted in 2 mL distilled H_20 with sonication (Branson Sonifier 450; duty cycle 50/output 5 for 1 min) and centrifuged. Extracts were analyzed by the receptor binding assay (RBA; Trainer and Poli 2000), following protocols established at the NOAA Biotoxin Lab in Seattle, WA. Extracts with initial RBA signals outside the linear portion of the standard curve (see below) were analyzed with appropriate dilutions.

The RBA measures competitive binding of ^{3}H-STX (saxitoxin) and sample STX to sodium channels in rat brain membranes. Samples with low STX provide high ^{3}H signals. A standard curve was generated from a stock solution of STX, with upper and lower STX limits of 536 and 0 ng/mL, respectively. Each assay, conducted in triplicate, consisted of 400 μL sample, 500 μl ^{3}H-STX, and 100 μL rat membrane preparation. Assay tubes were mixed on a vortex mixer and incubated on ice for 1 hr. After incubation, samples were collected onto GF/C glass fiber filters under vacuum and rinsed with wash buffer. Filters were loaded into scintillation vials with 10 mL Ecolume scintillation cocktail (ICN Biomedicals, Irvine, CA) and left overnight before ^{3}H was measured with a scintillation counter. Triplicate values were averaged; if the average gave a CV > 20% the anomalous reading was eliminated. Bound ^{3}H-SXT (measured in average disintegrations per minute (DPM) per sample) was plotted as the log of STX. Sample STX was interpolated from the standard curve and converted to STX equivalents in ng/mL of concentrated water sample.

Table 4.1. Water and mussel collection sites in the NE Kodiak region. The table includes location sites, with date and frequency of sampling.

Site location	1999			2000			2001		
	Dates	Frequency	n	Dates	Frequency	n	Dates	Frequency	n
Anton Larsen	7/6-8/24	1X/wk (M)	9	5/10-11/21[a]	1X/wk[b] (M, WA) (N)	27	5/23-9/28	1X/wk[b] (M)	21
Middle Bay	7/6-8/24	1X/wk (M)	6	5/10-11/21	1X/wk (M)	8	5/23-9/14	1X/wk (M)	16
Monashka	7/6-8/24	1X/wk (M)	5	5/10-9/27	2X/mo (M)	12	5/23-9/28	1X/wk[b] (M)	17
Pasagshak	7/6-8/24	1X/wk (M)	4	5/10-9/15	2X/mo (M)	9			
Pier 2	7/6-8/24	1X/wk (M)	8	5/10-11/21	1X/wk[b] (M, WA) (N)	25			
Thumbs Up	7/6-8/24	1X/wk (M)	8	5/10-9/15	2X/mo (M)	5			
Trident Basin	7/6-8/24	1X/wk (M)	8	5/10-11/21[a]	1X/wk[b] (M, WA) (N)	27	5/23-10/6	1X/wk (M, WA, WT) (NB)	24

M, mussel; WA, water column *Alexandrium*; WT, water column toxicity

N, net; NB, Niskin bottle

[a] Monthly collections extended through the winter of 2000/2001, with weekly collections resuming in the spring of 2001.

[b] Frequency of sampling was reduced during the last 2-4 weeks of collection.

Water column toxicity for each sampling period was depth-averaged from depths of 1, 3, 5, 10, and 17 m (as described in Chapter 2) to give STX/L for the water column.

4.2.3 Mussel toxicity

Mussel sample collection sites are given in Table 4.1. Each sample consisted of approximately 40 mussels. Mussels were selected with an average shucked wet mass between 3-5 grams per mussel. Mussel toxins were extracted according to AOAC protocols (AOAC 1995). Briefly, 100 g of shellfish meat was homogenized and boiled in 100 mL of 0.1 N HCl for five minutes with the pH maintained between 2 and 4 at all times. Upon cooling the total volume was brought to 200 mL with 0.1 N HCl and the pH adjusted below 3 with 5 N HCl for storage at –70° C. The extracts were analyzed for total toxins using the receptor binding assay (Trainer and Poli 2000). Extracts with RBA signals outside the linear portion of the standard curve were reanalyzed until the appropriate dilution was obtained.

4.3 Results

4.3.1 Spatial and temporal differences in mussel toxicity

Mussel toxicity was at moderately high levels (> 200 µg toxin/100 gm shellfish meat) at 5 of the 7 test sites when sampling was initiated in early July 1999 (Figure 4.2a). Mussels from all sites except Middle Bay exhibited toxicity above the quarantine level (80 µg toxin/100 gm shellfish meat) by late July. Toxicity decreased through August and was near zero at all sites when sampling was terminated in late August. Pasagshak and Monashka, with high values of 1200 and 800 µg SXT equivalent /100 g shellfish, respectively, were the most toxic sites tested.

Mussel toxicity was near zero in the spring of 2000 (Figure 4.2b) when sampling was resumed, but began to increase in early May at two sites, Monashka and Pasagshak. Increases were seen at the other sites by mid to late May. These periods of increased toxicity generally lasted 6 weeks, with all sites decreasing to near zero toxicity in late July. A second rise in toxicity was seen in mid to late August, again lasting about 6 weeks before dropping to near zero by the beginning of October. Five out of seven sites exhibited the highest toxicity during the initial peak in May. However, toxicity at Trident Basin and Pier 2 was highest during the second peak in August. As in 1999, Pasagshak and Monashka again exhibited the highest toxin concentrations, with 385 and 353 µg SXT equivalent /100 g shellfish, respectively.

In 2001 (Figure 4.2c), toxicity levels were near zero during the spring. The initial rise in mussel toxicity was slightly later than in 2000 with increases seen in late May at two sites, Monashka and Trident Basin. A second peak was seen in the beginning of August at the same two sites with toxicity dropping after three weeks. A third peak in September occurred at three sites, Monashka, Trident Basin and Anton Larsen. Toxicity levels fell to near zero by October. Middle Bay did not exhibit elevated toxicity in 2001.

Overall, mussel samples collected during the three field seasons showed similar timing of increased toxicity at the different locations, but considerable variation in toxicity levels among sites. The timing, frequency and potency of toxicity events were not consistent among sampling years. Initiation of toxicity events in 2001 occurred 1-2 weeks later than the 2000 season and the events were more sporadic. Peak mussel toxicity generally decreased over the three-year study. Monashka Bay, which was one of the most toxic sites and which was sampled all three years, went from a high of 800 μg STX equivalents/100 g shellfish in 1999 to approximately 350 and 300 μg STX equivalents/ 100 g shellfish in 2000 and 2001, respectively.

Two sites, Anton Larsen and Trident Basin (Figure 4.3) were sampled monthly throughout the winter of 2000. Mussel toxicity remained below 10 μg STX equivalents/ 100 g shellfish from October 2000 to May 2001.

4.3.2 Relationship between Alexandrium abundance and mussel toxicity (2000 and 2001)

Alexandrium abundance was clearly associated with mussel toxicity in 2000 (Figure 4.4) and 2001 (Figure 4.5). However, peaks in mussel toxicity tended to occur a few days to a week after *Alexandrium* peaks, and the magnitude of the mussel toxicity did not relate consistently to *Alexandrium* cell densities. Hence, statistically, the relationship was significant only for 2001 (r^2= 0.10, 2000; r^2= 0.44, 2001; based on linear regression analysis). However, from visual inspection of Figures 4.4 (2000) and 4.5 (2001), *Alexandrium* abundance and mussel toxicity are positively related. Increased *Alexandrium* abundance was followed by, or closely associated with, mussel toxicity. The relationship between *Alexandrium* abundance and shellfish toxicity was seen most clearly in the 2000 Trident Basin data (Figure 4.4a), which had two distinct peaks and a general trend of increased *Alexandrium* abundance accompanied by a rise in mussel toxicity.

Alexandrium cell densities from Trident Basin in 2000 ranged from near zero to 1100 cells/L. Two distinct peaks in late May and late August, with 1100 cells/L and 600 cells/L, respectively, (Trident Basin) were associated with mussel toxicity levels above the quarantine limit. Trident Basin exhibited higher mussel toxicity in the second part of the summer even though fewer cells were detected in this second bloom. These two distinct *Alexandrium* blooms, and corresponding mussel toxicity peaks, also occurred at the other two collection sites (Anton Larsen and Pier 2). Although the timing of events among the three sites coincided, cell densities and toxin levels varied.

During the summer of 2001, *Alexandrium* abundance at Trident Basin ranged from near zero to about 850 cells/L (Figure 4.5). *Alexandrium* densities fluctuated throughout the summer with five peaks, in June, July, August, and two in September. The peaks in June, August and early September, with densities of 550-800 cells/L, corresponded to mussel toxicities above the quarantine level.

4.3.3 Relationship between Alexandrium abundance and water toxicity (2001)

Water column toxicity was strongly correlated with *Alexandrium* abundance ($r^2 = 0.71$; based on linear regression analysis). Toxicity fluctuated with changes in *Alexandrium* cell densities with no observable lag (Figure 4.6), as expected. Average toxicity was 13.66 pg STX equivalents per cell (range 2.0 – 44.8)

4.4 Discussion

4.4.1 Data overview

We observed large variations in mussel toxicity among sites during each field season as well as substantial year-to-year variation in mussel toxicity at the same sites. However, similarities in the timing of toxicity events were seen among sites within each sampling season.

Alexandrium abundance patterns mirrored water column toxicity (Figure 4.6). Average *Alexandrium* toxicity was 13.66 pg STX equivalents per cell, well within the expected range (0-400) recorded from other sites (Cembella 1998). Large-scale seasonal differences in cellular toxicity for *Alexandrium* were not evident, a surprising result as numerous studies have shown

that toxicity per cell can vary with environmental conditions and growth phase (Anderson et al. 1990; Cho and Lee 2001; Hamasaki et al. 2001).

Mussel toxicity, in most cases, lagged slightly behind *Alexandrium* abundance (Figures 4.4 and 4.5), as expected due to mussel feeding and uptake kinetics. The 2000 season exhibited two blooms with cell densities between 600-1100 cells/L at Trident Basin. Two caveats exist concerning the 2000 *Alexandrium* abundance data. First, cell densities were underestimated due to the use of plankton nets for sample collection. Second, the plankton net either caught on the shallow bottom (Anton Larsen) or was towed horizontally due to strong currents (Pier 2), with unknown effects on the cell density estimates. However the data generally supported our overall conclusions that *Alexandrium* abundance and mussel toxicity exhibited similar timing between sites.

The sampling strategy was modified in 2001. Water was collected with Niskin bottles and efforts were focused on Trident Basin. Throughout most of the 2001 study period, shellfish toxicity remained at or below safe consumption levels. Three small *Alexandrium* blooms with densities of 550-800 cells/L corresponded to toxicity above the quarantine level. It appears from these data that *Alexandrium* cell densities of about 600 cells/L or greater are reason for concern about PSP toxins in mussels on Kodiak Island.

4.4.2 Gulf of Maine model of PSP

One of the most extensive studies of *Alexandrium* bloom dynamics is being carried out in the Gulf of Maine. Five separate habitats within the Gulf of Maine (40° 15' N to 45° 30' N; from the Bay of Fundy south to Connecticut), each with its own mechanical system driving bloom events, have been characterized (Anderson 1997). One of the habitats, designated the Western Maine region, extends from Penobscot Bay to Massachusetts Bay and is controlled by a combination of buoyancy plume advection and alongshore winds. Franks and Anderson (1992a and b) determined that the temporal and spatial patterns of shellfish toxicity along the coast were consistent with buoyancy-driven, alongshore transport as opposed to *in situ* growth. *Alexandrium* cells were trapped in the freshwater buoyant plume and carried south. The plume behavior was influenced by the volume of freshwater outflow, as well as alongshore winds. Downwelling-favorable winds from the northeast trapped the plume onshore and accelerated the plume

southward. Upwelling-favorable winds slowed the plume and moved the cells offshore. Timing and toxin intensity south of the plume were dependent on the strength and speed of the plume. Other habitats in the region are likely to be controlled by a combination of these physical processes, as well as tidal mixing and tidal fronts (Anderson 1997).

The inoculation dynamics in the western GOM are still unclear (Anderson 2002). Local cyst germination is believed to take place in some coastal areas (Anderson 1997). However, recent findings suggest that germination of cysts also takes place offshore, where recently germinated vegetative cells are entrained during periods of strong upwelling-favorable winds, then carried shoreward and concentrated when winds become downwelling-favorable (Anderson 2002; McGillicuddy et al. 2002). Other studies have linked toxic dinoflagellate blooms with upwelling relaxation in Spain (Fraga et al.1988) and California (Price et al. 1991). These are further evidence of large-scale physical processes affecting bloom dynamics.

4.4.3 Application of the GOM model to Kodiak PSP data

Few physical oceanographic data exist for the Kodiak Island region. The Alaska Coastal Current (ACC) primarily flows into Cook Inlet and Shelikof Strait, with little influence on the eastern side of Kodiak Island (Sobey1980; Stabeno et al. 2001). The Alaska Current follows the continental shelf, intensifying as it becomes a western boundary current (called the Alaska Stream west of 150° W), and continues along the shelf break east of Kodiak Island (mean baroclinic transport $12x10^6$ m^3/sec; Sobey 1980). Net alongshore flow on the Kodiak Island shelf is comparatively small. However, mean speeds can be substantial (30 cm/sec) (Sobey 1980). The flows are often complex due to the existence of deep troughs off the east coast of the island (Reed and Schumacher 1986). Freshwater runoff from Kodiak Island is not large compared to the mainland and the majority of the freshwater advected by the ACC is believed to pass Kodiak via Shelikof Strait (Sobey 1980). Therefore the continental shelf east of Kodiak is probably not heavily influenced by the buoyancy driven currents described by Royer (1979) in the northern Gulf of Alaska (Sobey 1980). Tidal mixing and wind-induced mixing occur on the shallow banks of the shelf (<100 m), however, waters remain stratified over the troughs (Sobey 1980). Drift cards released on Portlock Bank (NE of Kodiak and East of Afognak Islands) in June 1976 were recovered primarily along the east coast of Kodiak and Afognak, in the straits between the islands, and partly along Shelikof Strait on the western side of the island (Burbank 1977). This

physical environment, along with the demonstrated importance of advective processes in the GOM and the temporal and spatial patterns of toxicity observed near Kodiak, suggest a hypothesis that could help form the basis for future work. The fact that bloom timing in this study was relatively consistent among sites within a year suggests that the system is affected by large-scale advective processes.

Location of bloom inoculation events is beyond the scope of this study. Vegetative *Alexandrium* cells could be advected into the area via the Alaska Current or cyst resuspension could be initiated by tidal mixing on the shallow banks. Sexual stages of dinoflagellates have been found to exist throughout a bloom and not just at the end of a bloom, as previously thought (Elbrächter 2002). Cysts may therefore be found wherever vegetative cells persist. Cysts were found in sediment samples collected from Monashka and Middle Bay in March 2000 (isolated and cultured by S. Hall, US Food and Drug Administration, Washington D.C.). Based on the drift card data (Burbank 1977), an inoculation event of either type (advected vegetative cells or resuspended cysts) to the northeast of Kodiak Island would be carried into Marmot and Chiniak Bays and could potentially affect much of the northeastern coastline of Kodiak. This flow pattern includes all sampling sites in this study except Pasagshak (located in Ugak Bay) and could account for spatial toxicity patterns found in this study. Transport of the drift cards to the eastern shores of Afognak and Kodiak was presumed to be direct (Burbank 1977). Monashka would be the first site affected by this flow pattern. Monashka typically exhibited increased toxicity slightly before the other sites and possibly retained high levels of toxins due to the shape of the bay. Toxicity in Anton Larsen, located in Marmot Bay, typically lagged a few days behind the other sites. Middle Bay was not always affected by toxin events, suggesting more complex dynamics regulating flow in this area. Pasagshak cannot be explained by this suggested flow pattern. However it is similar in shape to Monashka, which may account for *Alexandrium* cells being trapped within the bay. Wind speeds and direction are most likely influential in dictating local bloom patterns, and would be an additional parameter to consider in future studies.

A severe outbreak of PSP illness in 1994 was related to the consumption of mussels from beaches along the eastern coast of Kodiak Island (Chiniak Bay, Monashka Bay and Sitkalidak Strait). Although shellfish from the entire eastern shoreline of Kodiak were not tested, the bloom presumably stretched along much of the eastern side of the island, as Sitkalidak Strait is

approximately 54 air miles south of the other sites. This is further evidence in support of the hypothesis that large-scale advective processes are important for PSP events in the Kodiak Island vicinity.

4.4.4 Monitoring: Future studies

Recent advances in technology have changed many protocols used to detect harmful algal blooms. For instance, molecular probes have facilitated the identification and enumeration of the causative organisms. Near real-time data collection is in the foreseeable future. A buoy- or dockside-deployed Environmental Sample Processor (ESP) has been developed by Dr. Chris Scholin at the Monterey Bay Aquarium Research Institute (MBARI) to gather real time data (Scholin et al. 2002). The fully automated instrument collects and concentrates water samples, extracts nucleic acids, and uses molecular probes to estimate *Alexandrium* abundance. The ESP was designed to simultaneously test for other potentially harmful organisms. Positioning of the instrument buoy is important for early warning of invasive blooms. Where should ESPs be positioned in Alaska? Initial areas to be considered should have a long-standing history of PSP toxicity and either a high population density or high frequency of shellfish harvesting (e.g., Clam Gulch in Kachemak Bay). Kodiak Island, specifically the NE Kodiak area, has a history of PSP toxicity and is densely populated. Based on the spatial patterns established in this study and the limited physical oceanographic data for the area, outer Marmot Bay (latitude 58° N 152°W) could be an ideal location for placement of an ESP. If waters from this region are found to have high densities of *Alexandrium*, currents could carry the bloom to most of eastern Kodiak Island. Future shipboard study is needed to identify *Alexandrium* cells in this area, and a study combining inshore testing and latitudinal transects are needed to understand inoculation dynamics.

4.5 Acknowledgements

We thank Dr. Vera Trainer and Keri Baugh for help with the receptor binding assay, which was conducted at the NOAA Biotoxin Lab in Seattle, WA. We thank Stacey Studebaker for assistance in sample collection and mussel toxin extractions throughout the winter of 2000/2001. Shellfish collection permits were issued by Alaska Department of Fish and Game.

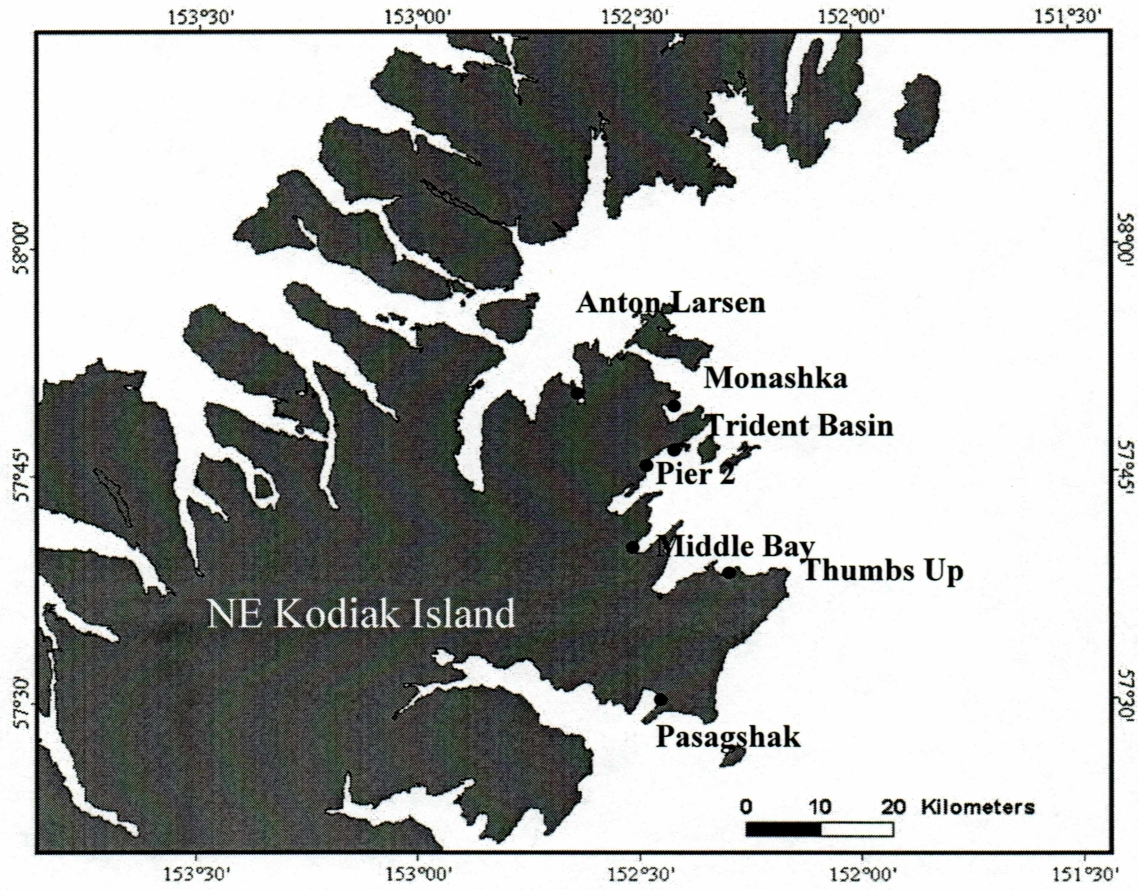

Figure 4.1. Map showing study sites in the NE Kodiak Island area. Water and mussel samples were collected from Trident Basin, Pier 2 and Anton Larsen (2000) and Trident Basin (2001). The other sites were sampled for mussels only.

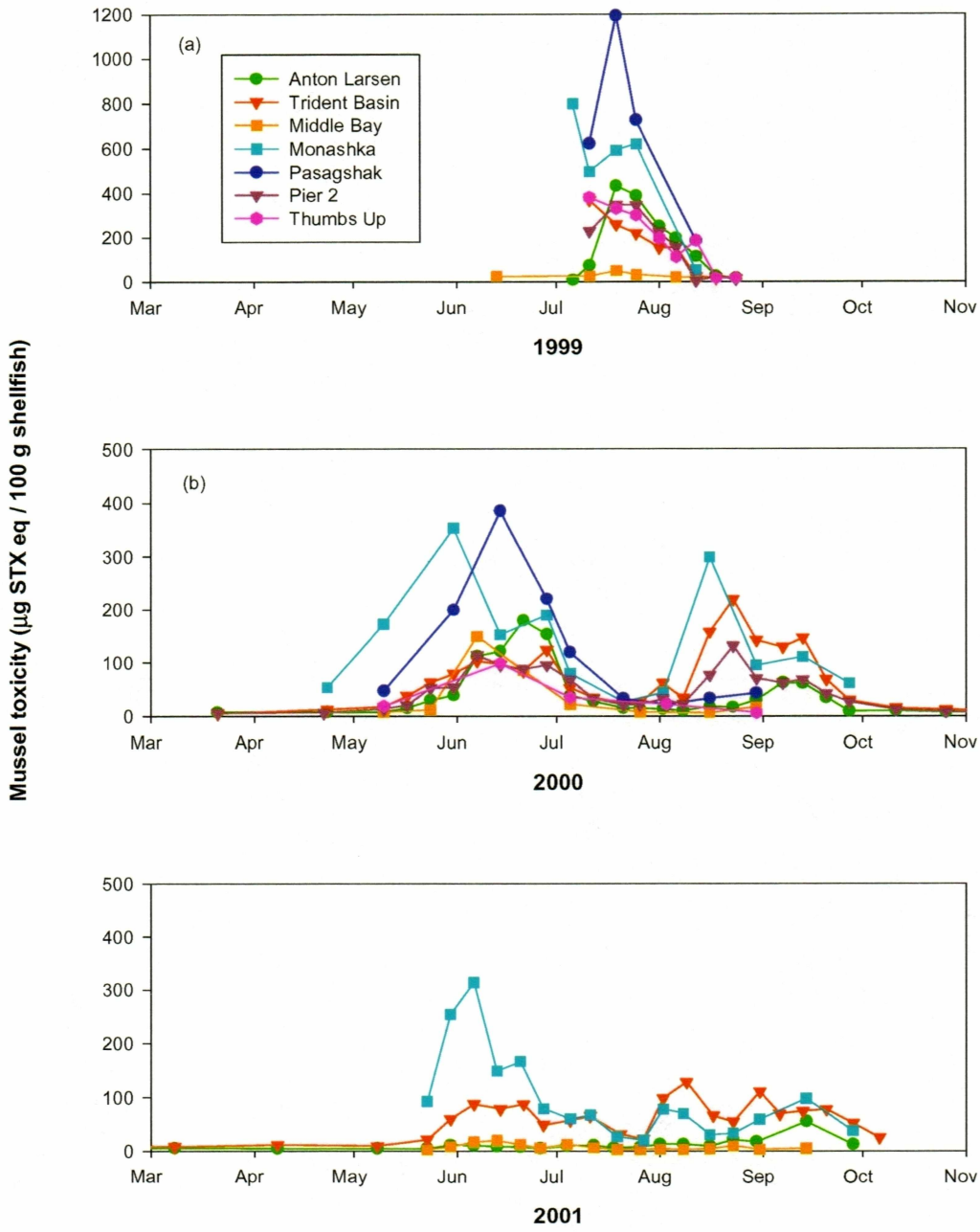

Figure 4.2. Toxicity of mussels collected at various sites in the NE Kodiak Island vicinity for (a) 1999, (b) 2000 and (c) 2001.

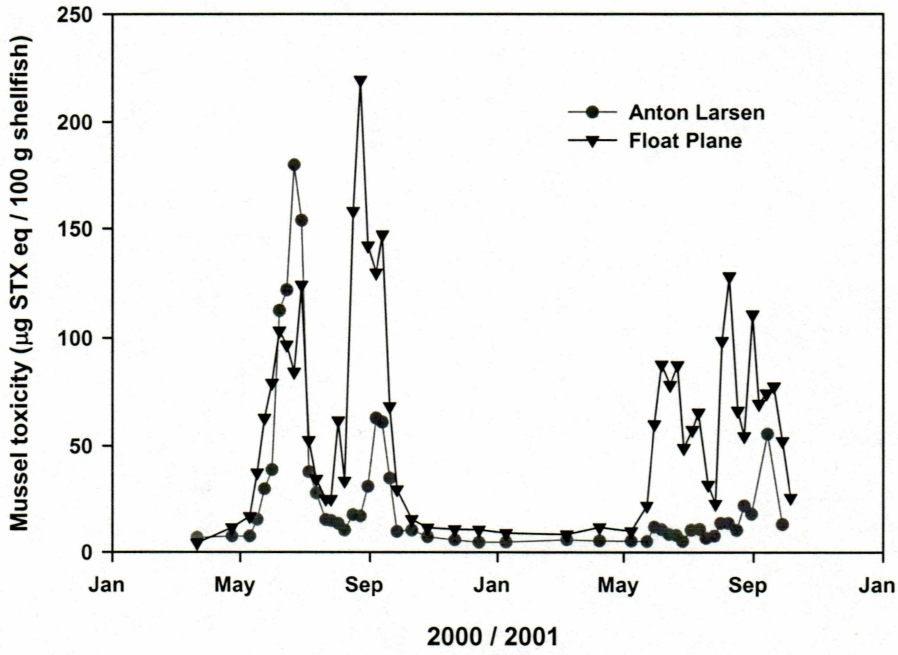

Figure 4.3. Toxicity of mussels collected at two sites on Kodiak Island that were sampled continuously from March 2000 to October 2001.

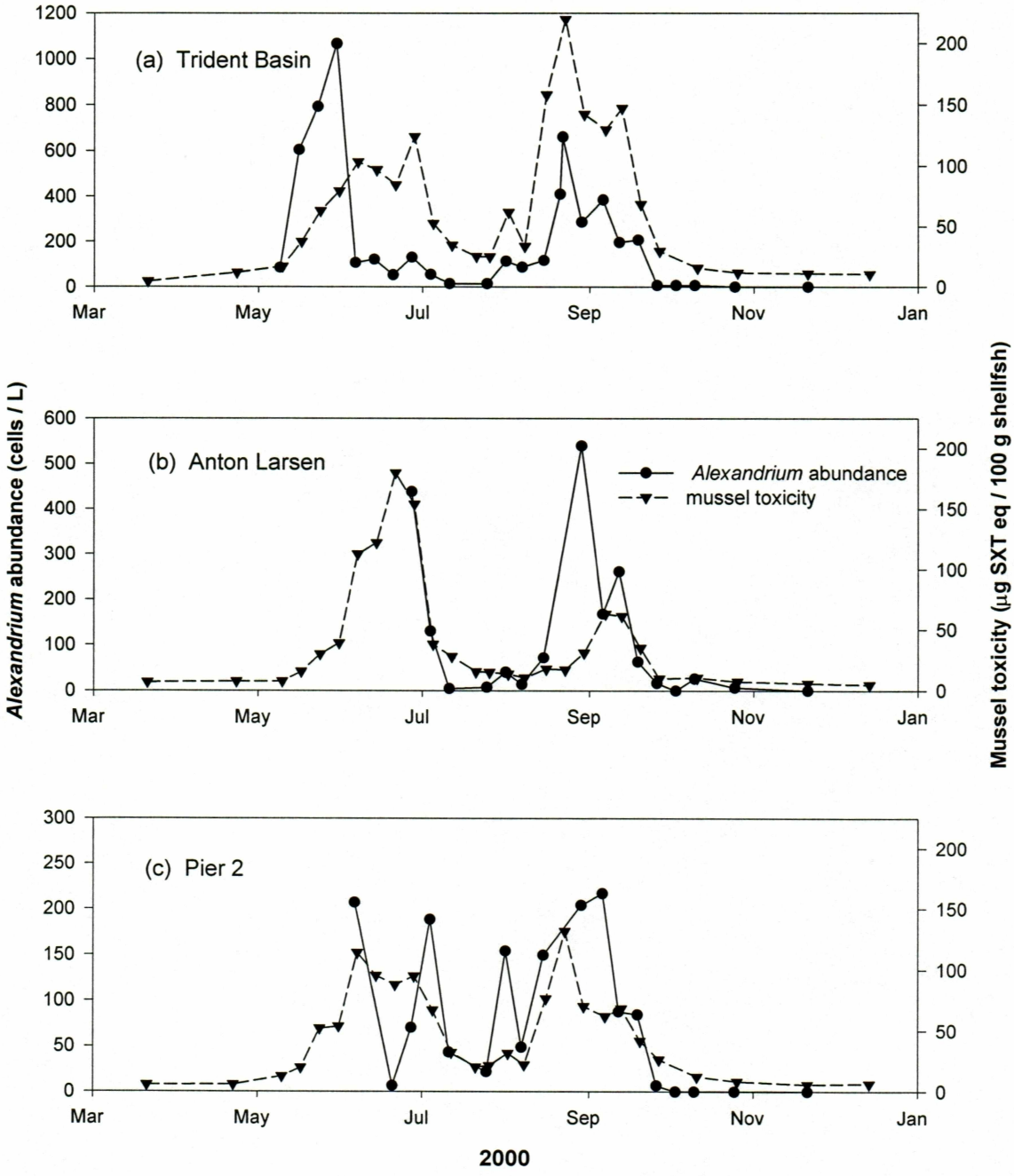

Figure 4.4. *Alexandrium* abundance and mussel toxicity at three sites in 2000 (a) Trident Basin, (b) Anton Larsen and (c) Pier 2.

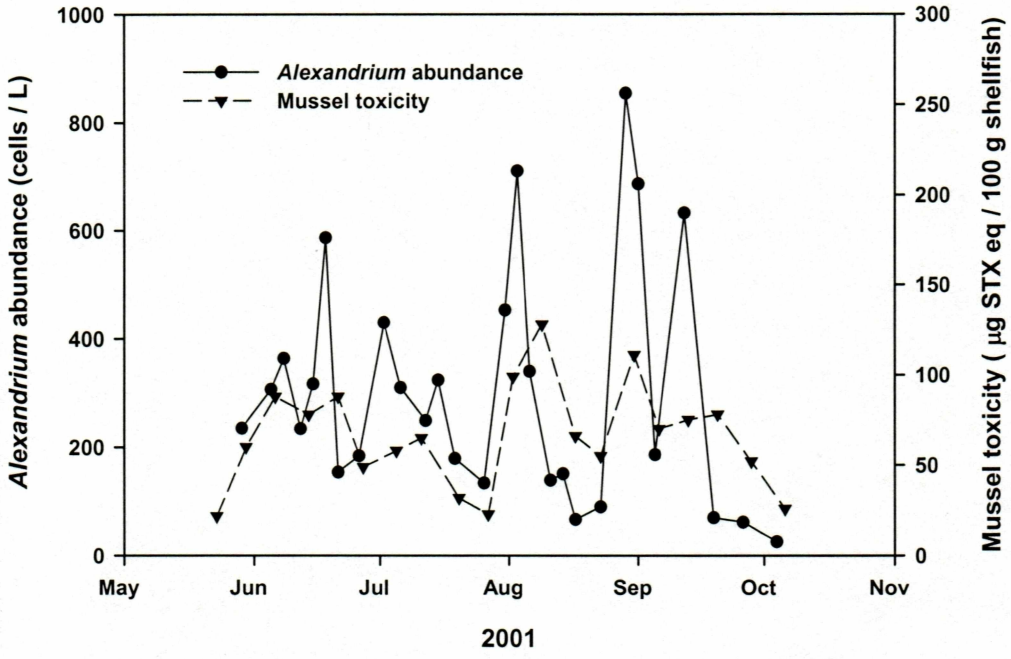

Figure 4.5. *Alexandrium* abundance and mussel toxicity at Trident Basin 2001.

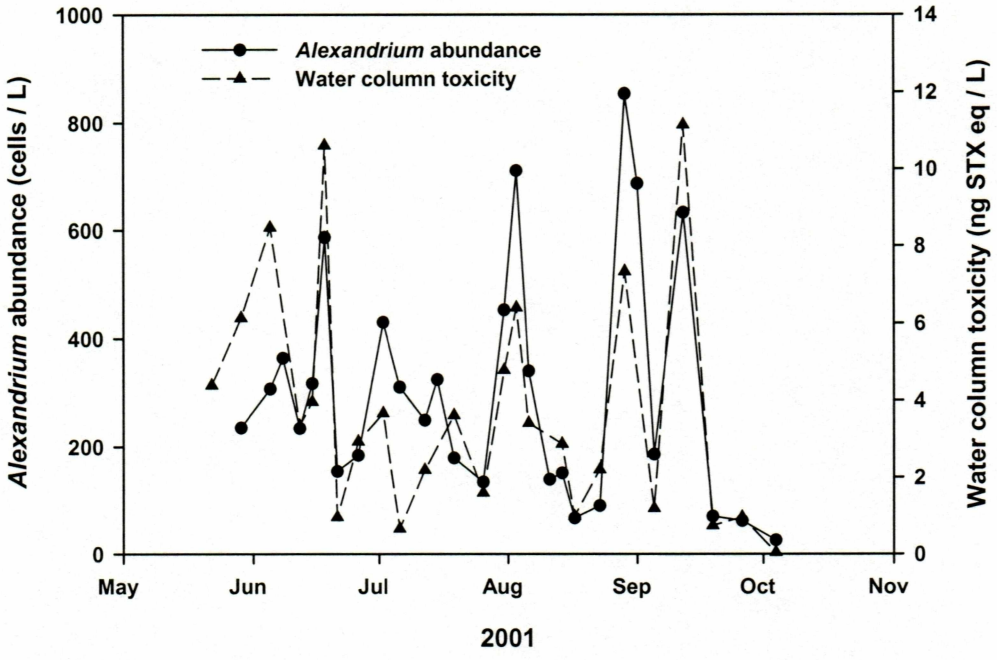

Figure 4.6. *Alexandrium* abundance and water column toxicity at Trident Basin 2001.

4.6 References

Anderson, D.M., 1995. Toxic red tides and harmful algal blooms: A practical challenge in coastal oceanography. Reviews of Geophysics. U.S. National Report to International Union of Geodesy and Geophysics 1991-1994. American Geophysical Union, 95RG00440, pp.1189-1200.

Anderson, D.M., 1997. Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. Limol. Oceanogr. 42, 1009-1022.

Anderson, D.M., 2002. ECOHAB-GOM: the ecology and oceanography of toxic *Alexandrium* blooms in the Gulf of Maine. In: Xth International Conference on Harmful Algae Book of Abstracts, pp. 13.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104, 511-524.

AOAC., 1995. Official Methods of Analysis, 16th ed., P. Cunniff, (Ed.), AOAC International, Arlington, pp. 46-49.

Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of paralytic shellfish toxins from the dinoflagellate *Alexandrium fundyense* in the mussel *Mytilus edulis* Mar. Ecol. Prog. Ser. 63, 177-188.

Burbank, D.C., 1977. Circulation studies of Kachemak Bay and Lower Cook Inlet. Environmental Studies of Kachemak Bay and Lower Cook Inlet. 3. Alaska Dep. of Fish and Game, Anchorage, 207 pp.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 381-403.

Cho, E.S., Lee, H.J., 2001. Thecal plates, toxin content and growth of five clones of *Alexandrium tamarense* (Dinophyceae) isolated from Jinhae Bay, Korea. Phycologia 40, 435-439.

Elbrächter, M., 2002. Sexual reproduction of dinoflagellates-an overview. In: Xth International Conference on Harmful Algae Book of Abstracts, pp. 82.

Fraga, S., Anderson, D.M., Bravo, I., Reguera, B., Steidinger, K., Yentsch, C.M., 1988. Influence of upwelling relaxation on dinoflagellates and shellfish toxicity in Ria de Vigo, Spain. Estuar. Coast. Shelf Sci. 27, 349-361.

Franks, P.J.S., Anderson, D.M., 1992a. Alongshore transport of a toxic phytoplankton bloom in a buoyancy current: *Alexandrium tamarense* in the Gulf of Maine. Mar. Biol. 112, 153-164.

Franks, P.J.S., Anderson, D.M., 1992b. Toxic phytoplankton blooms in the southwestern Gulf of Maine: testing hypothesis of physical control using historical data. Mar. Biol. 112, 165-174.

Hall, S., 1982. Toxins and toxicity of *Protogonyaulax* from the northeast Pacific. Ph.D. Thesis, Univ. Alaska.

Hamasaki, K., Horie, M., Tokimutsu, S., Toda, T., Taguchi, S., 2001. Variability in toxicity of the dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions. J. Plank. Res. 23, 271-278

Horner, R.A., Garrison, D.L., Plumley, F.G., 1997. Harmful algal blooms and red tide problems on the U.S. west coast. Limnol. Oceanogr. 42, 1076-1088.

McGillicuddy, D.J., Signell, R.P., Stock, C.A., Keafer, B.A., Keller, M.D., Hetland, R.D, Anderson, D.M., 2002. A mechanism for offshore initiation of harmful algal blooms in the coastal Gulf of Maine. In: Xth International Conference on Harmful Algae Book of Abstracts, pp. 191.

Neal, R.A., 1967. Fluctuations in the Levels of Paralytic Shellfish Toxin in Four Species of Lamellibranch Mollusks near Ketchikan, Alaska, 1963-1965. Ph.D. Dissertation, Univ. Washington.

Price, D.W., Kizer, K.W., Hansgen, K.H., 1991. California's paralytic shellfish poisoning prevention program, 1927-89. J. Shellfish Res. 10, 119-145.

Reed, R.K., Schumacher, J.D., 1986. Physical Oceanography. In: Hood, D.W., Zimmerman, S.T. (Eds.), The Gulf of Alaska Physical Environment and Biological Resources. Minerals Management Service publication number: OCS study, MMS 86-0095. pp. 57-75.

Royer, T.C., 1979. On the effect of precipitation and runoff on coastal circulation in the Gulf of Alaska. J. Phys. Oceanogr. 9, 555-563.

Schantz, E.J., Magnusson, H.W., 1964. Observations on the origin of the paralytic poison in Alaska butter clams. J. Protozool. 11, 239-242.

Scholin, C., Marin III, R., Massion, E., Jensen, S., Cline, D., Roman, B., Doucette, G., 2002. Remote detection of HAB species using the environmental sample processor (ESP): progress and future directions. In: Xth International Conference on Harmful Algae Book of Abstracts, pp. 254.

Shumway, S.E., Sherman-Caswell, S., Hurst, J.W., 1988. Paralytic shellfish poisoning in Maine: monitoring a monster. J. Shell. Res. 7, 643-652.

Sobey, E.J.C., 1980. Physical Oceanography. In: Environmental Assessment of the Alaskan Continental Shelf: Kodiak Interim Synthesis Report 1980. Science Applications, Inc. Boulder, CO, pp. 47-86.

Stabeno, P.J., Bond, N.A., Hermann, A.J., Mordy, C.W., Overland, J.E., 2001. Meteorology and Oceanography of the Northern Gulf of Alaska. Submitted to Continental Shelf Research.

Trainer, L.V., Poli, M.A., 2000. Assays for dinoflagellate toxins, specifically brevetoxin, ciguatoxin, and saxitoxin. Rochat, H., Martin-Eauclaire, M.-F. (Eds.), In: Methods and Tools in Biosciences and Medicine Animal Toxins. Birkhauser Verlag Basel/Switzerland, pp. 1-17.

Chapter 5

Observations on *Alexandrium* Bloom Dynamics in NE Kodiak Waters

5.1 Introduction

Spring phytoplankton blooms in temperate coastal waters are related to a combination of increased light, thermal stratification of the water column, and high nutrient levels present in the photic zone due to winter wind-mixing events (Mann and Lazier1996). Increased water temperatures and freshwater input, in the form of rainfall and river runoff, stratify the water column, enabling phytoplankton cells to remain within the photic zone (Sverdrup 1953). Algal cell densities rapidly increase in these conditions. In temperate waters, dinoflagellates comprise a secondary bloom, often occurring after the initial spring diatom bloom (Holligan 1979; Margalef et al. 1979). Possible explanations for this secondary bloom include increased stratification and nutrient dynamics favoring dinoflagellates.

Dinoflagellate blooms typically occur in conditions of low turbulence, often in association with frontal systems and stratification of inshore waters (Margalef et al. 1979; Holligan et al. 1984; Larocque and Cembella 1990). The blooms appear in association with decreased vertical mixing and often disappear as a result of reactivated circulation (Margalef et al.1979). While diatoms predominate in tidally mixed waters (Holligan et al. 1984), and may be biophysically stimulated by motion (Smayda 1997), turbulence inhibits dinoflagellate growth and mechanically damages cells (White 1976; Pollingher and Zemel 1981).

Dinoflagellates are thought to have a high nutrient affinity, i.e., low K_s (the half saturation or affinity constant), compared to diatoms (Margalef 1978). Selection would favor low K_s species in nutrient depleted conditions (e.g., stratified waters after the primary spring bloom) while high K_s species would have a competitive advantage in high nutrient conditions (e.g., mixed waters). However, Smayda (1997) reviewed data that collectively showed that HAB species lack the high nutrient affinity thought to give them a competitive advantage in low nutrient conditions. He postulated that dinoflagellates have evolved adaptations to offset the ecological disadvantages of a high K_s that include: 1) nutrient retrieval migrations, 2) mixotrophic nutritional tendency, 3) allelochemically enhanced interspecific competition, and 4) allelopathic antipredation defense mechanisms (Smayda 1997). Some *Alexandrium* species have been found to exhibit these

adaptations (Blanco and Campos 1988; White et al. 1989; Nygaard and Tobiesen 1993; MacIntyre et al. 1997).

Dinoflagellates account for ~75% of harmful algal bloom species (Smayda 1997). In Alaska the problem of PSP is due to dinoflagellates of the genus *Alexandrium* (Horner et al. 1997). Ecosystem dynamics are complex and the conditions in which *Alexandrium* spp. persist are not well understood. To better understand *Alexandrium* bloom dynamics in the Kodiak Island region, meteorological data for the Kodiak Island area were collected and chlorophyll *a*, macro-nutrients, and water temperature were measured coincident with plankton and mussel sampling in 2000-2001 (Chapters 2-4). The data presented here are discussed in the context of existing bloom hypotheses, specifically regarding stratification and nutrient conditions that are related to increases in the abundance of *Alexandrium*.

5.2 Materials and Methods

5.2.1 Description of the study area

Water samples were collected in Trident Basin located in Chiniak Bay, Kodiak Island, Alaska, USA (57° 47' N 152° 24' W). Trident Basin is approximately 27 m in depth and is relatively well mixed (E. Munk (NOAA), pers. comm.) with three entrances into Chiniak Bay. Although salinity was not measured in Trident Basin, salinity in Chiniak Bay (station location 57° 43' N 152° 18' W; depth 190 m; data provided by Brad Stevens, NOAA) was on average 32.5 ‰. The average monthly summer (May through September) air temperatures and rainfall during 2000 and 2001 were 10.7° C and 9.6 cm, respectively.

5.2.2 Water collection

Water samples were collected every 3-14 days from May 9 to Nov 11 in 2000 (Net collection) and from May 22 to Oct 5 in 2001 in Trident Basin (Niskin collection), as described in Chapters 2 and 3. In 2000, surface nutrient concentrations and surface water temperatures were measured at each collection event. In 2001, nutrient, temperature, and chlorophyll samples were collected from depths of 1, 3, 5, 10 and 17 m and depth-averaged.

5.2.3 *Alexandrium abundance*

Alexandrium cell densities were determined using the sandwich hybridization assay as described in Chapters 2 and 3. Net (2000) or Niskin bottle depth-averaged values (2001) were plotted over time.

5.2.4 *Chlorophyll a*

Chlorophyll *a* samples were collected and analyzed as described in Childers (2001). Seawater (250 mL to 1L) was filtered onto 47 mm Whatman GF/F (0.7 μm retention) filters and stored at –70° C until analyzed. Filters were extracted in 20 mL scintillation vials in 10 mL acetone/DMSO solution (six parts 90% aqueous acetone and four parts 40% DMSO; Shoaf and Lium 1976) at –20° C in the dark. After 24 hrs the vials were removed from the freezer, brought to room temperature in the dark, mixed, decanted into culture tubes, and centrifuged. Chlorophyll fluorescence was read, before and after addition of 10% HCl, on a Turner model 10 series fluorometer, calibrated with chlorophyll *a* from Turner Designs (Sunnyvale, CA). Chlorophyll (μg/L) values were depth-averaged, and plotted over time. The ratio between chlorophyll *a* and phaeopigments (the degradation product after addition of acid) was calculated.

5.2.5 *Nutrient analysis*

Samples were collected in polyethylene scintillation vials and stored at –70° C until analysis. Nutrients were analyzed with a Technicon AutoAnalyzer II or an Alpkem model 300 continuous nutrient analyzer. The yearly averaged 95% confidence intervals for the nutrient standards produced ranges of ±0.09 μmol L^{-1} NO_2^-, ±0.10 μmol L^{-1} NH_4^+, ±0.25 μmol L^{-1} PO_4^{-3}, ±0.78 μmol L^{-1} $NO_2^- + NO_3^-$ and ±1.35 μmol L^{-1} $Si(OH)_4$ (Childers 2001).

5.2.6 *Water temperature*

Water temperatures were measured with a mercury thermometer (± 0.5° C). Surface temperatures were collected during the 2000 field season. Temperature data were collected from each collection depth during the 2001 season and depth-averaged.

5.2.7 *Meteorological data*

Average daily temperature and precipitation data were collected by the NOAA National Weather Service, Kodiak, AK (57° 45' N 152°, 29' W) at a ground elevation of 111 ft.

5.2.8 Data analysis

Simple linear correlation (Pearson's correlation; Zar 1999) was run for *Alexandrium* abundance versus chlorophyll *a*, and *Alexandrium* abundance versus each macronutrient (NO_3^-, PO_4^{-3}, $Si(OH)_4$, NH_4^+, NO_2^-). The relationship between *Alexandrium* abundance and water temperature was analyzed with simple linear regression. Data were also plotted over time to identify potential patterns.

5.3 Results

5.3.1 Alexandrium abundance

Alexandrium densities started to increase during the second week of May 2000 (Figure 5.1) and the third week of May 2001 (Figure 5.2). Cell densities varied between undetectable levels and 1200 cell/L (2000) and 800 cells/L (2001). In both years cell numbers decreased to undetectable levels by October. Two clear *Alexandrium* peaks were apparent in 2000; five small peaks were detected in 2001.

5.3.2 Chlorophyll a

Chlorophyll *a* levels varied throughout the summer of 2001 with five relatively small but distinct peaks (Figure 5.2). The largest peak (5.9 µg Chl *a*/L) occurred in mid August. Chlorophyll *a* levels were less than 1µg/L by late September. The ratio of chlorophyll *a*:Phaeopigments in early May was 1.0 with a marked increase in late July (data not shown).

5.3.3 Nutrients

Major nutrient concentrations were high in the early spring of both 2000 and 2001. Nutrient concentrations decreased rapidly from late April to May 9, 2000, when phytoplankton sampling was initiated (Figure 5.1). The decrease of nutrient concentrations in 2001 occurred slightly later, between May 7 and May 22, when regular sampling was initiated (Figure 5.2). There were variations throughout the spring and summer season in the concentrations of all nutrients, with a general rise in concentrations starting around the beginning of September in both years.

5.3.4 *Alexandrium abundance, chlorophyll a, and phosphate*

Alexandrium abundance was not clearly correlated with any of the measured parameters (Table 5.1). However, visual inspection of the plots suggested potential patterns in both 2000 (Figure 5.3a) and 2001 (Figure 5.3b) that may point toward *Alexandrium* bloom triggering events. Increased *Alexandrium* cell densities were related to increases in phosphate levels during both the 2000 and 2001 field seasons, although not all increases in phosphate led to increased *Alexandrium* abundance. Also in 2001 the five *Alexandrium* blooms of greater than 400 cells/L trailed the chlorophyll *a* peaks by 3-14 days.

Table 5.1. Pearson's correlation coefficient (r) for *Alexandrium* abundance versus chlorophyll *a* and macro nutrient concentrations. Chlorophyll *a* was not measured in 2000.

Parameter	Correlation coefficient (r) for surface values (2000)	Correlation coefficient (r) for depth-averaged values (2001)
Chlorophyll a	N/A	0.09
NO^{3-}	-0.31	-0.27
PO_4^{3-}	0.14	0.24
$Si(OH)_4$	-0.39	-0.06
NH_4^+	-0.26	0.05
NO_2^-	-0.26	0.14

N/A; not available (freezer malfunction; lost samples)

5.3.5 *Depth contour plot: Alexandrium abundance*

Depth contour plots of *Alexandrium* abundance (Figure 5.4) show that cells were most abundant in the upper 10 m for most (4 of 5) bloom periods, but were also present at depth during one bloom.

5.3.6 *Water temperature*

Temperatures were typically 7° C at the onset of routine spring sampling in both 2000 and 2001. Surface waters reached their maximum temperature in late August of 2000 and mid July in 2001 (Figure 5.5b; note that depth averaged values were plotted for 2001; surface values followed the same pattern; data not shown). No correlation was found between *Alexandrium* abundance and water temperature (r^2=0.136 for surface temperatures in 2000; r^2=0.009 for depth averaged temperature in 2001). Graphically, no relationship was apparent between *Alexandrium*

abundance and water temperature (Figure 5.5a and b) either. However, *Alexandrium* cell densities began to increase in May of both years when water temperatures reached 7° C and began to decline in late September when water temperatures dropped to approximately 10° C.

5.3.7 Precipitation and air temperature

Based on visual inspection of Figures 5.6b and 5.7b, *Alexandrium* abundance was weakly associated with periods of low precipitation in both 2000 and 2001. No relationship with air temperature was apparent.

5.4 Discussion

Overall, no clearly discernable chemical or meteorological patterns unambiguously signaled blooms of *Alexandrium*. However, phosphate and chlorophyll *a* levels (Figure 5.3) as well as low rainfall (Figures 5.6 and 5.7) were weakly associated with increases of *Alexandrium* abundance. These putative signals are described within the context of overall nutrient dynamics for *Alexandrium*.

Nutrient concentrations were high in late April/early May in both 2000 (Figure 5.1) and 2001 (Figure 5.2) and decreased rapidly by mid to late May. These declines in nutrient concentrations are most likely explained by spring bloom events (Mann and Lazier1996). Although diatom abundance data were not collected in this study, the initial spring bloom in coastal Alaska waters is typically dominated by diatoms (Sambrotto and Lorenzen 1986), which are generally able to out-compete other phytoplankton species when nutrient concentrations are high. Although both phosphate and nitrate concentrations were reduced during these early spring blooms, the sharp decrease in silicate, a necessary component of diatom frustules (Lalli and Parsons 1993), most clearly signaled a diatom bloom. The chlorophyll *a* to phaeopigment ratio, at the onset of the 2001 sampling season, was close to 1.0 (data not shown), indicative of heavy grazing pressure that would maintain phytoplankton biomass at moderate to low levels. The initial *Alexandrium* blooms in 2000 and 2001 followed the spring diatom blooms suggested by the substantial nutrient (especially SiO_4) draw down seen both years. The five periods of peak *Alexandrium* cell densities throughout the summer of 2001 appeared to follow chlorophyll *a* peaks (Figure 5.3 b). This pattern supports a previously reported HAB model (Holligan 1979; Margalef et al. 1979) except that in this study, five peaks (not one) were observed during the summer for both

chlorophyll *a* and *Alexandrium* abundance. It would be interesting to have the 2000 chlorophyll *a* data, as there were two more readily discernable peaks in *Alexandrium* abundance (Figure 5.1). Based on the 2001 data, it is reasonable to postulate a spring diatom bloom that ended in early May of 2000 followed by a second diatom bloom in mid August.

In pairwise comparisons, no correlations were found between *Alexandrium* abundance and nitrate, nitrite, silicate or ammonium (Table 5.1; Figures 5.1 and 5.2). Nutrient depletion in the marine environment is usually associated with nitrogen depletion (Tyrell 1999). In this study, *Alexandrium* blooms often coincided with low nitrate concentrations but moderate to high ammonium concentrations. However, as nitrate, nitrite, and ammonium are all suitable nitrogen sources for *Alexandrium* (Cembella 1998) it is unlikely that nitrogen was a limiting factor for growth. In some instances increases in cell densities were associated with increases in phosphate (Figure 5.3a and b). This may be associated with freshwater runoff delivering phosphate from terrestrial organic debris (Tyrell 1999).

Increases in *Alexandrium* cell densities in 2000 and 2001 were weakly associated with periods of low precipitation that were preceded by rainfall (Figures 5.6b and 5.7b). Three possible explanations for this observation include (1) freshwater input, preceding the dry periods, may have favored *Alexandrium* growth and/or increased water column stability, (2) periods of low precipitation were related to higher solar radiation and (3) the rainfall that preceded the period of reduced precipitation brought added terrestrial nutrients to the coastal waters. These possibilities are not mutually exclusive.

Previous studies have related increased *Alexandrium* densities to low salinities (Iwaski 1979; Cembella et al. 1988; Weise et al. 2002), supporting explanation (1). However, Trident Basin is tidally mixed, and unlike some areas in Chiniak Bay, does not directly receive large amounts of freshwater runoff. Further study is needed to determine the importance of salinity and stratification to *Alexandrium* growth in this area. In support of explanation (2), periods of high solar radiation have been positively related to high *Alexandrium* abundance (Gayoso 2001, Carreto et al. 1993). Like many dinoflagellates, *Alexandrium* spp. have high concentrations of photo-protective carotenoids and mycosporine-like amino acids giving them a competitive advantage at high light intensities (Carreto et al. 1989; Carreto et al. 1990; Carreto et al. 1993)

and allowing them to remain active in surface waters. With the ability to vertically migrate, some red tide dinoflagellate species have been found to accumulate at relatively high light intensities (Cullen and Horrigan 1981; Anderson and Stolzenbach 1985). In this study, high *Alexandrium* cell densities were found predominantly in the upper 10 m of the water column (Figure 5.4), a distribution that may be related to their photo-protective carotenoids. In support of explanation (3), growth of 14 species of red tide flagellates in culture was enhanced with the addition of soil extracts (Iwasaki 1979). Terrigenous organic matter is high in humic substances that are natural trace metal chelators (Anderson 1978). Chelators may be beneficial either because they bind toxic metal ions and/or increase the availability of metal ions needed as micronutrients. Additionally, *Alexandrium* spp. require vitamins (Iwasaki 1979) and some *Alexandrium* spp. are able to use organic nitrogen sources (Ogata et al.1996), either of which could have been supplied from terrigenous sources. It would be interesting to determine the role of organic nutrients in *Alexandrium* growth in the Kodiak Island region.

Because no correlation was found with any one chemical or meteorological parameter, it is likely that a number of factors play a role in *Alexandrium* bloom dynamics in the Kodiak region. More research is needed to understand the role of stratification, nutrients, and current dynamics in relation to *Alexandrium* abundance in the area. Further, because the data suggest that *Alexandrium* increases lag behind environmental events by days to a week or two, very frequent sampling would likely be needed to identify the causes of blooms. The sandwich hybridization assay tools used here may prove to be an effective aid in understanding *Alexandrium* blooms and PSP events.

5.5 Acknowledgements

I thank the students and technicians in Dr. Terry Whitledge's lab, specifically Amy R. Childers, at the University of Alaska Fairbanks for analyzing the nutrients for this study. I thank Dr. Dean Stockwell at the University of Alaska for his assistance with chlorophyll *a* analysis. I also thank Brad Stevens from the NMFS RACE shellfish division in Kodiak for providing salinity data from Chiniak Bay.

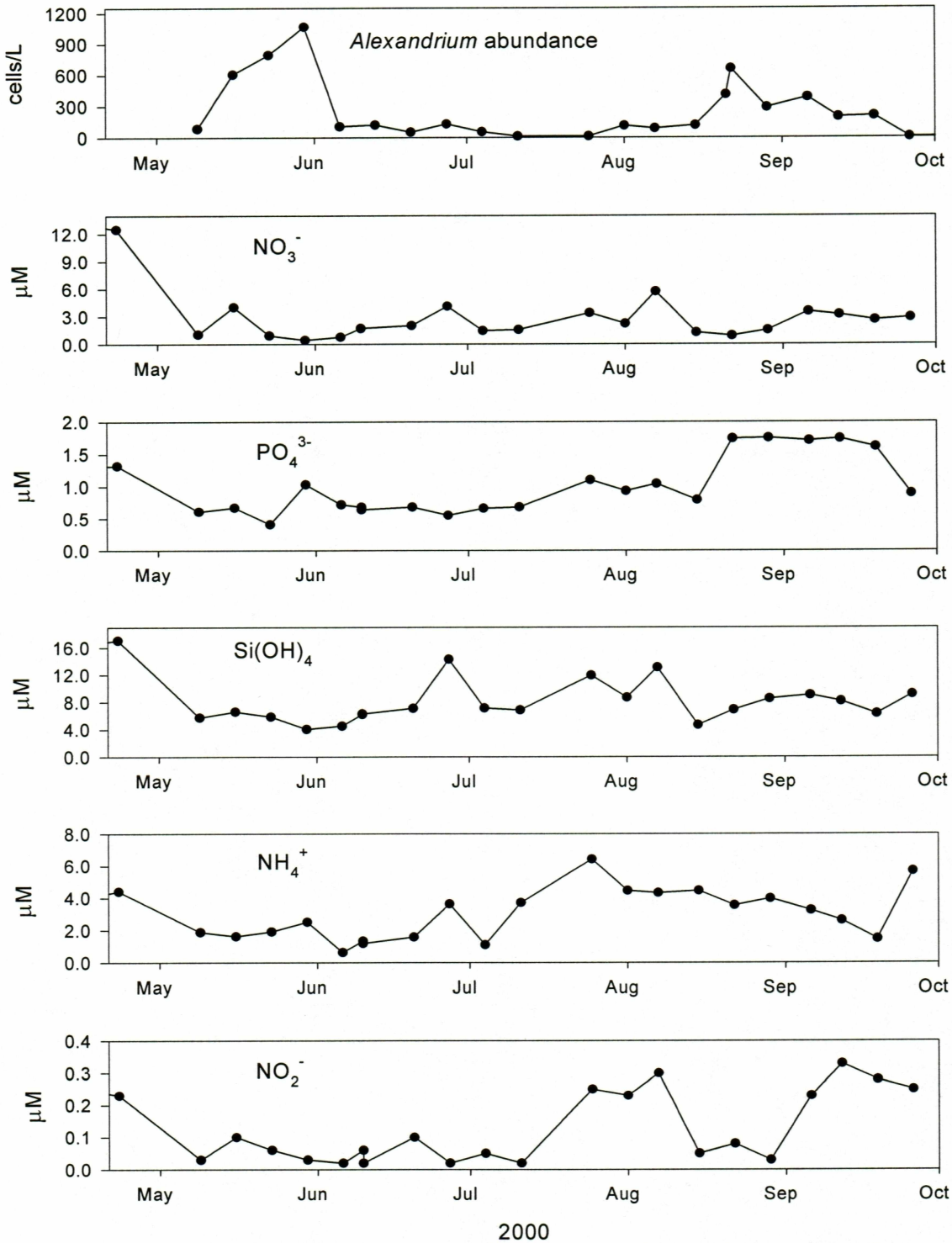

Figure 5.1. *Alexandrium* abundance from net collection and surface nutrient concentrations at Trident Basin, 2000.

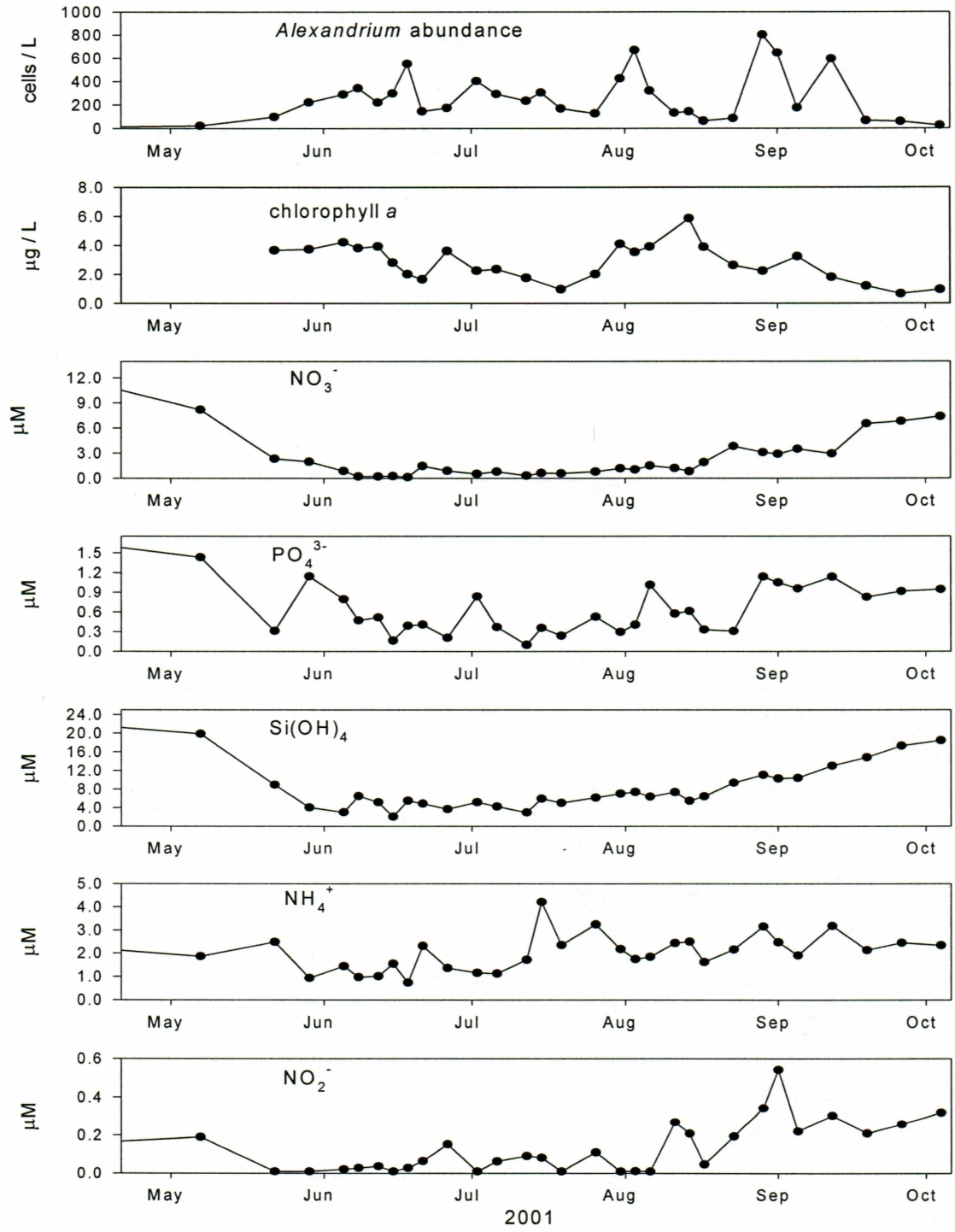

Figure 5.2. Depth-averaged *Alexandrium* abundance, chlorophyll *a* and nutrient concentrations at Trident Basin, 2001. *Alexandrium* and nutrient data prior to May 22 reflect net and surface samples respectively.

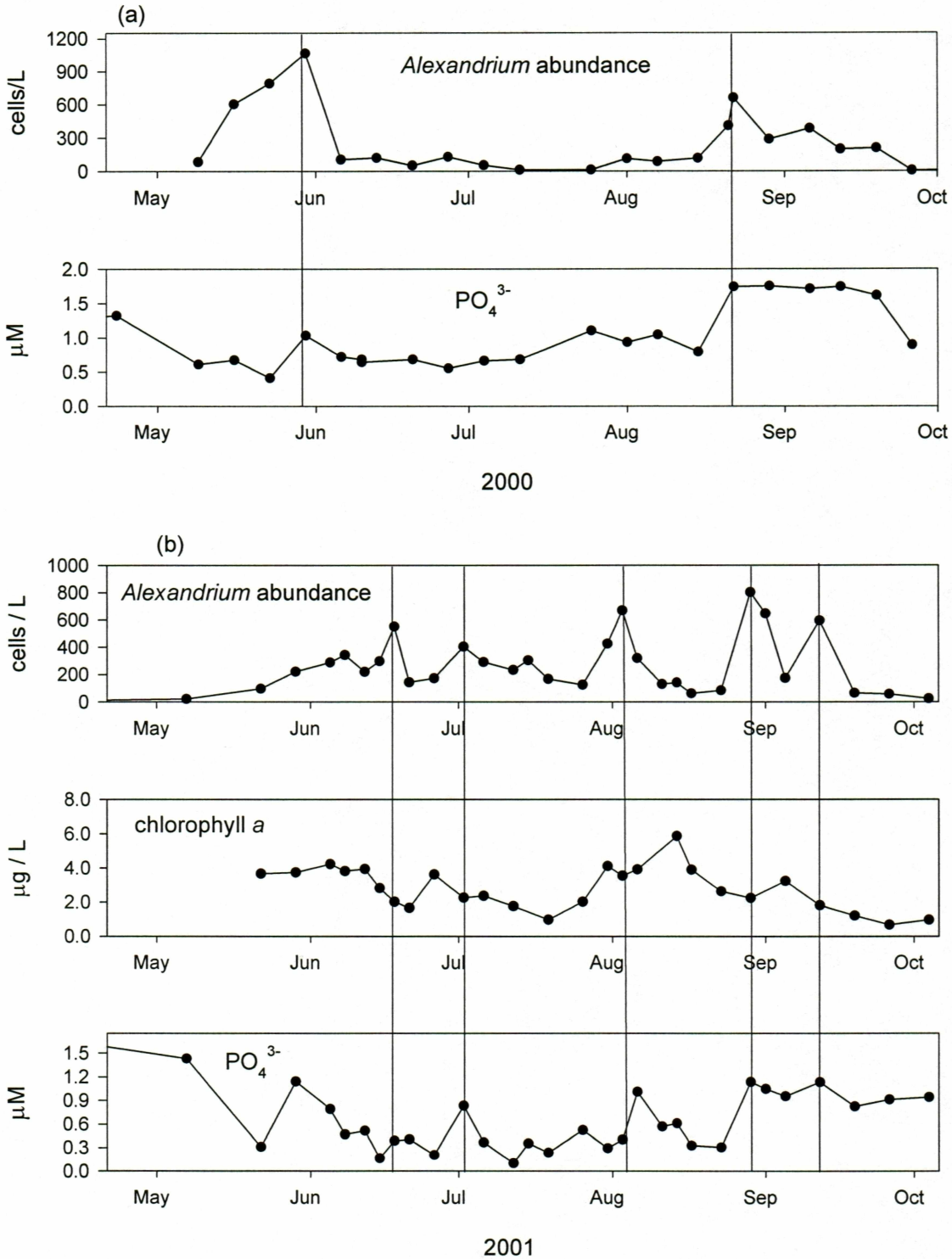

Figure 5.3. Potential triggering events associated with *Alexandrium* blooms and phosphate (2000) chlorophyll *a* and phosphate (2001). Vertical lines mark peaks in *Alexandrium* abundance.

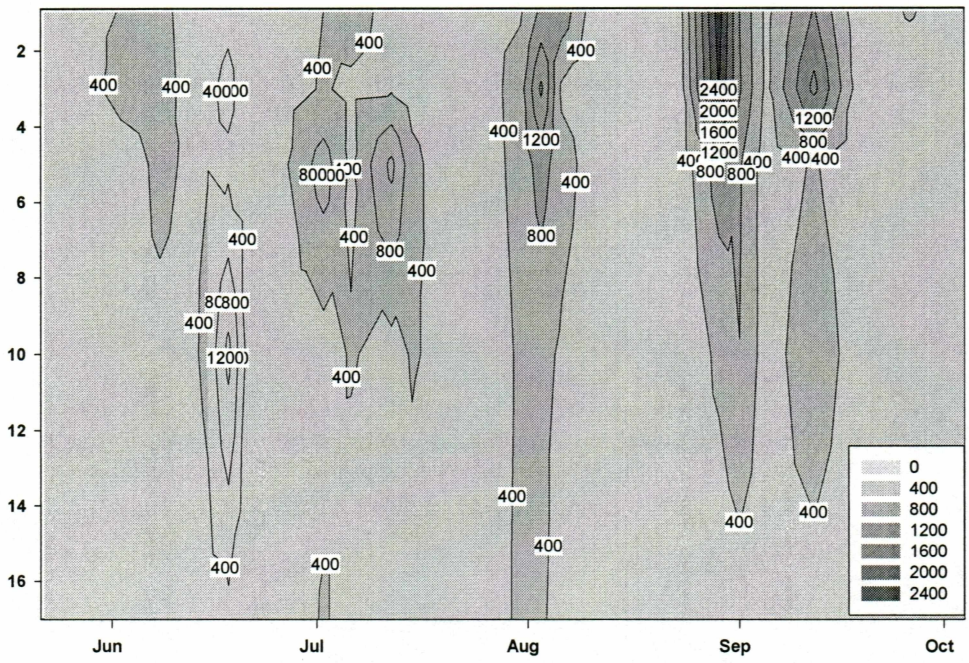

Figure 5.4. Depth contour of *Alexandrium* cell densities (cells/L) collected from 1, 3, 5, 10 and 17 m at Trident Basin, 2001.

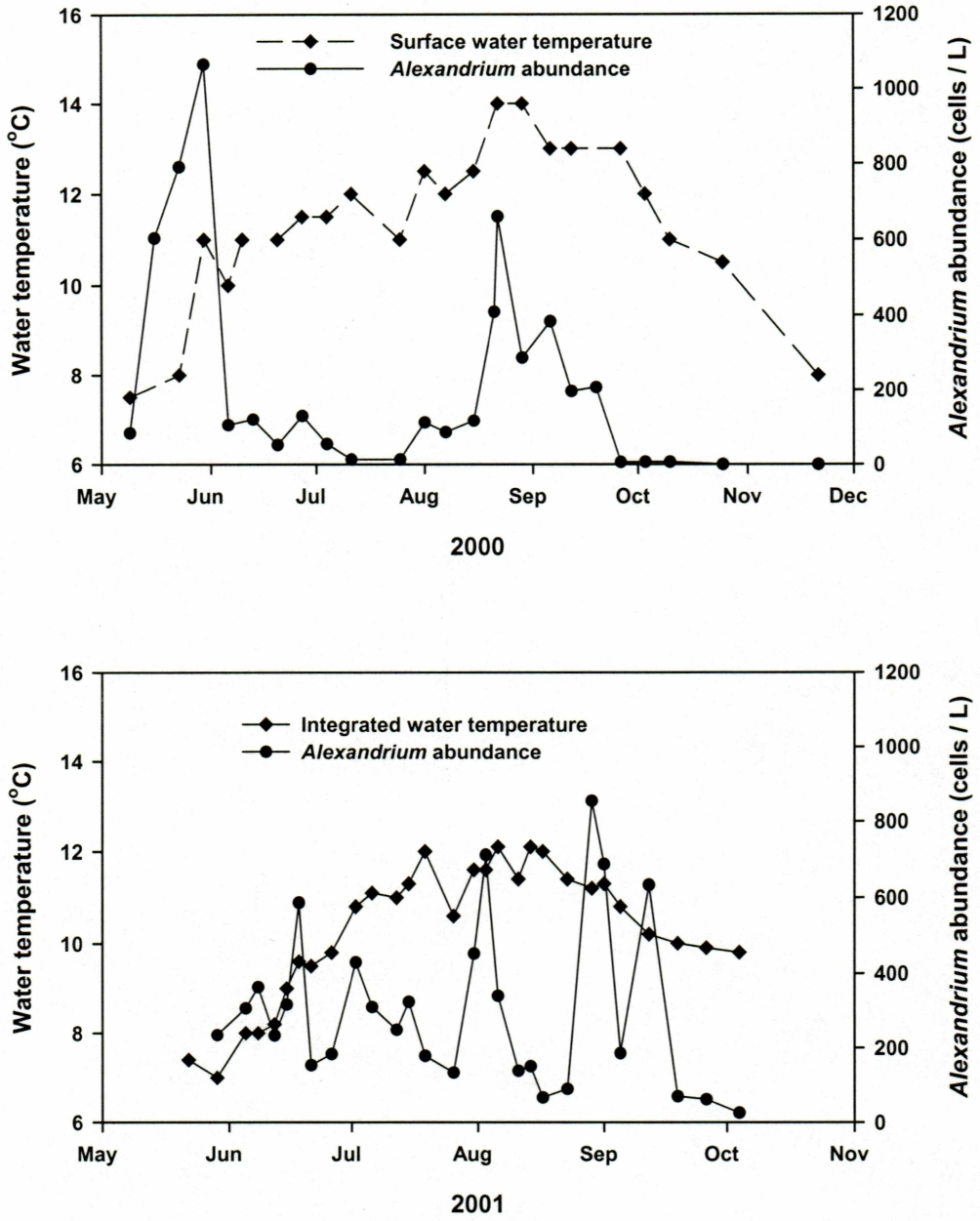

Figure 5.5. Water temperatures at Trident Basin, 2000 and 2001, overlaid with *Alexandrium* abundance.

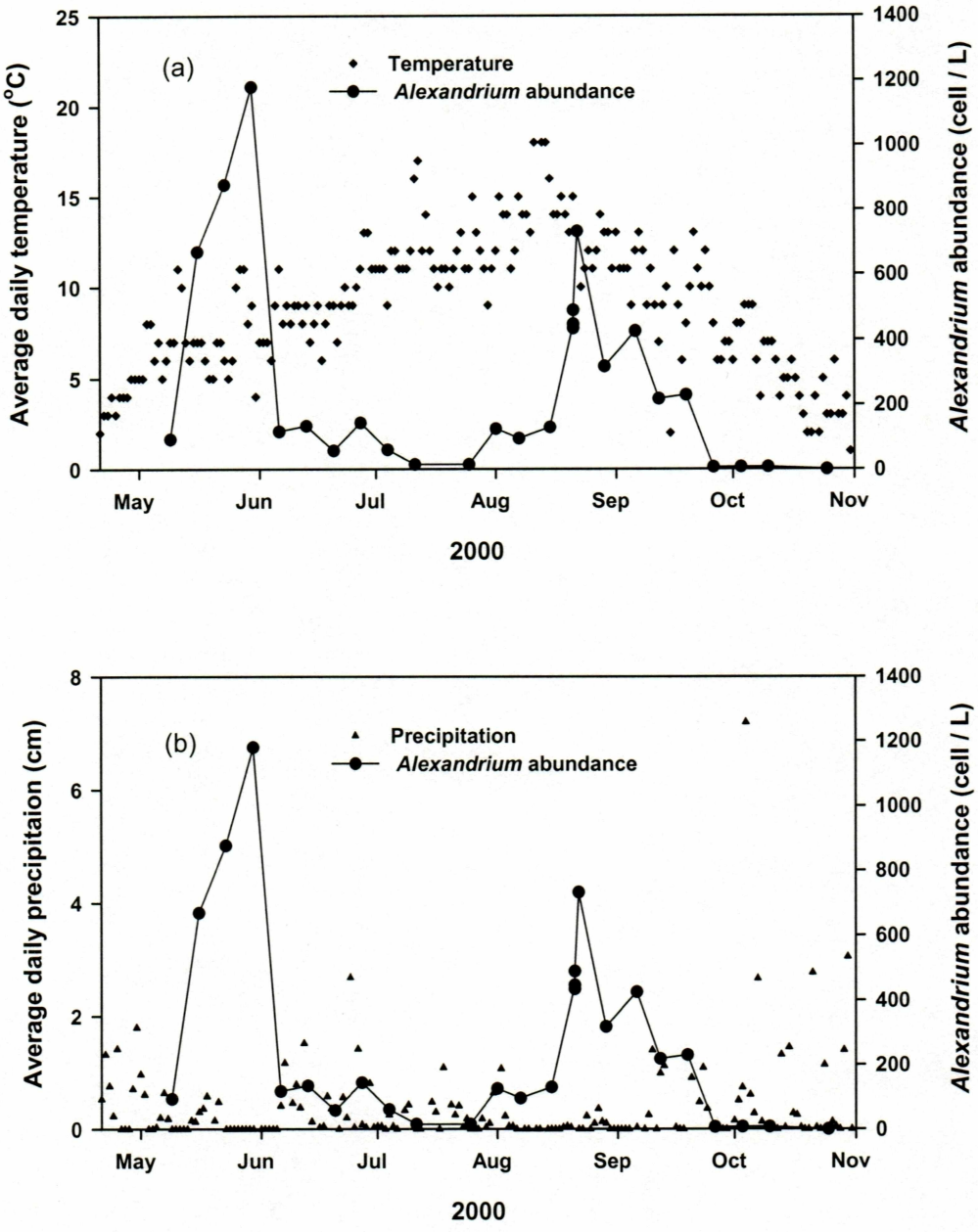

Figure 5.6. Average daily air temperatures (a) and average daily precipitation (b) from Kodiak Island, 2000, overlaid with *Alexandrium* abundance. Temperature and precipitation data were provided by the NOAA National Weather Service, Kodiak AK.

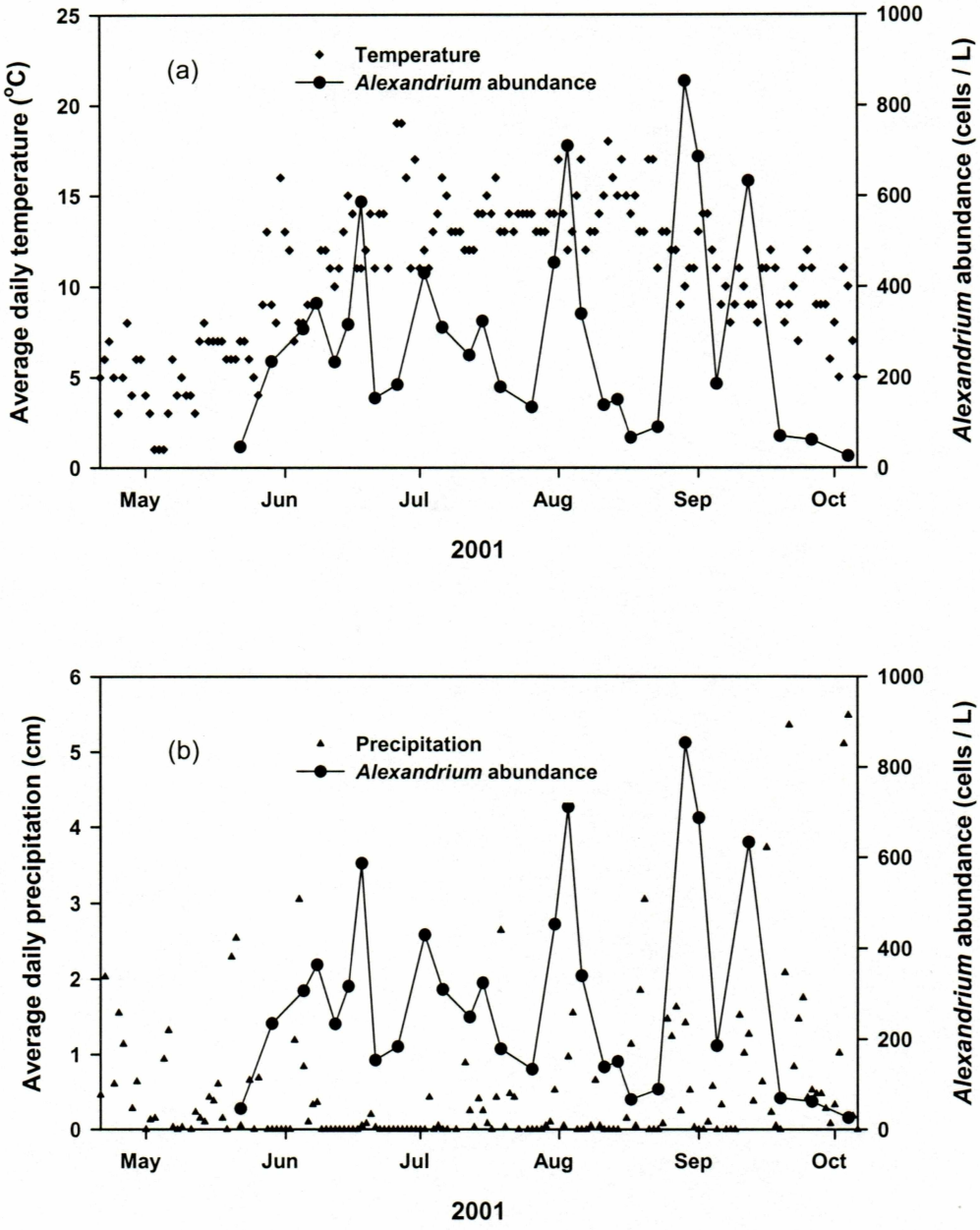

Figure 5.7. Average daily air temperatures (a) and average daily precipitation (b) from Kodiak Island, 2001, overlaid with *Alexandrium* abundance. Temperature and precipitation data were provided by the NOAA National Weather Service, Kodiak AK.

5.6 References

Anderson, D.M., 1978. The role of chelators and trace metals in toxic algal blooms. In: Taylor, D.L., Seliger, H.H. (Eds.), Toxic Dinoflagellate Blooms. Elsevier/North-Holland, New York, pp. 463-467.

Anderson, D.M., Stolzenbach, K.D., 1985. Selective retention of two dinoflagellates in a well mixed estuarine embayment: the importance of diel vertical migration and surface avoidance. Mar. Ecol. Prog. Ser. 25, 39-50.

Blanco, J., Campos, M.J., 1988. The effect of water conditioned by a PSP-producing dinoflagellate on the growth of four algal species used as food for invertebrates. Aquaculture 68, 289-298.

Carreto, J.I., De Marco, S.G., Lutz, V.A., 1989. UV-absorbing pigments in the dinoflagellates *Alexandrium excavatum* and *Prorocentrum micans*- effects of light intensity. In: Okaichi, Anderson, Nemoto (Eds.), Red Tides: Biology, Environmental Science, and Toxicology. Elsevier, New York, pp. 333-336.

Carreto, J.I., Carignan, M.O., Daleo, G., De Marco, S.G., 1990. Occurrence of mycosporine-like amino acids in the red-tide dinoflagellate *Alexandrium excavatum*: UV-photoprotective compounds? J. Plank. Res. 12, 909-921.

Carreto, J.I., Elbusto, C., Sancho, H., Carignan, M., Colleoni, D.C., De Marco, S., Fernandez, A., 1993. An exploratory analysis of the Mar del Plata shellfish toxicity area (1980-1990). In: Smayda, T.J. ,Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, Amsterdam, pp. 377-382.

Cembella, A.D., Turgeon, J., Therriault, J.C., Beland, P., 1988. Spatial distribution of *Protogonyaulax tamarensis* resting cysts in nearshore sediments along the north coast of the lower St. Lawrence estuary. J. Shell. Res. 7, 597-609.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D. Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 381-403.

Childers, A.R., 2001. Major Nutrient Distributions in Relation to the Physical Structure of the Gulf of Alaska Shelf. M.S. Thesis. Univ. Alaska.

Cullen, J.J., Horrigan, S.G., 1981. Effects of nitrate on the diurnal vertical migration, carbon to nitrogen ratio, and the photosynthetic capacity of the dinoflagellate *Gymnodinium splendens*. Mar. Biol. 62, 81-89.

Gayoso, A.M., 2001. Observations on *Alexandrium tamarense* (Lebour) Balech and other dinoflagellate populations in Golfo Nuevo, Patagonia (Argentina). J. Plank. Res. 23, 463-468.

Holligan, P.M., 1979. Dinoflagellate blooms associated with tidal fronts around the British Isles. In: Taylor, D.L., Seliger, H.H. (Eds.), Toxic Dinoflagellate Blooms. Elsevier/North-Holland, New York, pp. 249-256.

Holligan, P.M., Williams, P.J., Purdie, D., Harris, R.P., 1984. Photosynthesis, respiration, and nitrogen supply of plankton populations in stratified, frontal and tidally mixed shelf waters. Mar. Ecol. Prog. Ser. 17, 201-213.

Horner, R.A., Garrison, D.L., Plumley, F.G., 1997. Harmful algal blooms and red tide problems on the U.S. west coast. Limnol. Oceanogr. 42, 1076-1088.

Iwasaki, H., 1979. The physiological characteristics of neritic red-tide flagellates. In: Taylor, D.L., Seliger, H.H. (Eds.), Toxic Dinoflagellate Blooms. Elsevier/North-Holland, New York. pp. 95-100.

Lalli, C.M., Parsons, T.R., 1993. Biological Oceanography: An Introduction. Butterworth Heinemann Ltd. Oxford, pp. 45-73.

Larocque, R., Cembella, A.D., 1990. Ecological parameters associated with the seasonal occurrence of *Alexandrium* spp. and consequent shellfish toxicity in the lower St. Lawrence Estuary (eastern Canada). In: Graneli, E., Sundström, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 368-373

MacIntyre, J.G., Cullen, J.J., Cembella, C.D., 1997. Vertical migration, nutrition and toxicity in the dinoflagellate *Alexandrium tamarense*. Mar. Ecol. Prog. Ser. 148, 201-216.

Mann. K.H., Lazier, J.R.N., 1996. Dynamics of marine ecosystems. Blackwell Science, Inc. Cambridge Massachusetts, 394 pp.

Margalef, R., 1978. Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanol. Acta. 1, 493-509.

Margalef, R., Estrada, M., Blasco, D., 1979. Functional morphology of organisms involved in red tides, as adapted to decaying turbulence. In: Taylor, D.L., Seliger, H.H. (Eds.), Toxic Dinoflagellate Blooms. Elsevier/North-Holland, New York, pp. 89-94.

Nygaard, K., Tobiesen, A., 1993. Bacterivory in algae: a survival strategy during nutrient limitation. Limnol. Oceanogr. 38, 273-279.

Ogata, T., Koike, K., Nomura, S., Kodama, M., 1996. Utilization of organic substances for growth and toxin production by *Alexandrium tamarense*. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO 1996, pp 343-346.

Pollingher, U., Zemel, E., 1981. In situ and experimental evidence of the influence of turbulence on cell division processes of *Peridinum cinctum* forma *WestII* (Lemm.) Lefevre. Br. Phycol. J. 16, 281-287.

Sambrotto, R.N., Lorenzen, C.J., 1986. Plankton and Primary Production. In: Hood, D.W., Zimmerman, S.T. (Eds.), The Gulf of Alaska: Physical Environment and Biological Resources. Minerals Management Service publication number: OCS study, MMS 86-0095. pp. 249-282.

Shoaf, W.T , Lium, B.T., 1976. Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide. Limnol. Oceanogr. 21, 926-928.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42, 1137-1153.

Sverdrup, H.U., 1953. On conditions for the vernal blooming of phytoplankton. J. Cons. Perm. Int. Exp. Mer. 18, 287-295.

Tyrell, T., 1999. The relative influences of nitrogen and phosphorous on oceanic primary production. Nature 400, 525-531.

Weise, A.M., Levasseur, M., Saucier, F.J., Senneville, S., Bonneau, E., Roy, S., Sauve, G., Michaud, S., Fauchot, J., 2002. The link between precipitation, river runoff, and blooms of the toxic dinoflagellate *Alexandrium tamarense* in the St. Lawrence. Can. J. Fish. Aquat. Sci. 59, 464-473.

White, A.W., 1976. Growth inhibition caused by turbulence in the toxic marine dinoflagellate *Gonyaulax excavata*. J. Fish. Res. Board. Can. 33, 2598-2602.

White, A.W., Fukuhara, O., Anraku, M., 1989. Mortality of fish larvae from eating toxic dinoflagellates or zooplankton containing dinoflagellate toxins. In: Okaichi, Anderson, Nemoto (Eds.), Red Tides: Biology, Environmental Science, and Toxicology. Elsevier, New York, pp. 395-398.

Zar, J.H., 1999. Biostatistical Analysis. 4th Ed. Prentice Hall, Upper Saddle River, N.Y.

Chapter 6
General Conclusions

This study was funded by the Alaska Science and Technology Foundation to examine *Alexandrium* abundance in relation to shellfish toxicity in the NE Kodiak Island vicinity. A primary focus of the study was to evaluate the sandwich hybridization (SH) probe assay, automated by Saigene Corporation, as a means to detect and quantify *Alexandrium.* Specifically, the goal was to determine if the SH assay was a useful tool in detecting and quantifying *Alexandrium* abundance in the water column and if the information from this assay could be used to predict PSP toxin events in mussels. A minor objective was to evaluate environmental parameters in relation to *Alexandrium* abundance to determine if bloom-triggering mechanisms could be identified.

Dinoflagellates have been linked to PSP events in marine environments since the 1930s. In the North Pacific, PSP is caused by dinoflagellates of the genus *Alexandrium.* However shellfish toxicity in Alaska has been found to be geographically patchy and often difficult to explain based on the presumed distribution of *Alexandrium.* Because PSP toxins have also been associated with bacteria and cyanobacteria, there was considerable speculation at the onset of this study that PSP toxins could be associated with organisms other than *Alexandrium.* Results from this study clearly demonstrate that *Alexandrium* is the causative organism for the presence of PSP toxins in this area. *Alexandrium* abundance patterns tracked water column toxicity and average toxicity per cell was well within the expected range recorded from other studies. Mussel toxicity was positively related to *Alexandrium* abundance. In most cases, shellfish toxicity lagged slightly behind *Alexandrium* abundance, due to mussel feeding and toxin uptake kinetics.

PSP toxin production and growth of *Alexandrium* in Alaska, and worldwide, are erratic and patchy, with considerable variation on both temporal and spatial scales. We observed large variations in mussel toxicity among sites during each field season as well as substantial year-to-year variation in mussel toxicity at the same sites. However, similarities in the timing of toxicity events were seen among sites within each sampling season. The fact that bloom timing in this study was relatively consistent among sites within a year, suggests that the ecosystem under study (the northeastern end of Kodiak Island) is affected by large-scale advective processes. However,

much work is needed to identify the location and initiation of bloom inoculation and to determine the physical oceanographic dynamics that dictate bloom dispersal.

Screening and monitoring of PSP toxins is difficult. Shellfish monitoring cannot be used as a predictive tool and researchers have turned to plankton monitoring as a "first line" strategy for estimating shellfish toxicity. Because of the difficulties associated with studying *Alexandrium* and other HAB species, molecular probes are now facilitating identification and enumeration of these causative organisms. This study focused on a newly developed nucleotide probe assay (SH assay) as a tool to detect *Alexandrium* and to monitor the State's coastline.

We found that the SH assay can be used in a semi-quantitative manner to estimate *Alexandrium* abundance in water samples from Kodiak Island, Alaska. The SH assay results generally tracked results from whole cell counts throughout the 2001 season. Data from the SH assay were strongly associated with water column toxicity and, with a lag incorporated, with shellfish toxicity. Despite the overall positive results reported here, several factors must be evaluated further before the SH assay is fully deployed. There were several sources of error associated with the SH assay, which were sometimes substantial, due to a combination of inconsistent plate manufacture and operator error. Another problem was that the probe signal varied from week to week, presumably due to fluctuations in the rRNA content of the target cells. This complicated the generation of a calibration curve. However, the variations in rRNA content appeared to be sufficiently small that the SH assay can be used to rapidly detect *Alexandrium*, with the caveat that the cell density estimates may vary by a factor of approximately three.

Despite the problems in obtaining absolute cell numbers, the SH assay was sufficiently sensitive to detect changes in *Alexandrium* abundance that led to shellfish toxicity levels above quarantine. It appears from these data that *Alexandrium* cell densities of about 600 cells/ L or greater are reason for concern for mussel toxicity within the study site. However more work is needed before the SH assay can be used for predictive purposes.

Overall, the SH assay is rapid, fairly easy to use and has potential to be an excellent tool in gathering real-time *Alexandrium* abundance data. The assay significantly reduces misidentification and microscope time. Because the SH assay is a good proxy for *Alexandrium*

abundance, it would be a useful tool for PSP monitoring studies where high-density sampling and routine analysis are necessary. In locations such as Alaska, where the coastline is extensive and blooms episodic, the SH assay would be useful as a first line strategy in monitoring PSP events. In a commercial setting the SH assay may be used to recognize increases in *Alexandrium* that lead to shellfish toxicity before shellfish are harvested, helping to eliminate product waste due to contamination.

No clearly discernable chemical or meteorological events unambiguously signaled blooms of *Alexandrium*. However, phosphate, chlorophyll *a*, and rainfall provided weak signals. Increased *Alexandrium* abundance trailed chlorophyll *a* peaks, and appeared to be related to increases in phosphate and to periods of low precipitation. It is likely that many factors play a role in *Alexandrium* bloom dynamics and can be better understood with additional physical and chemical oceanographic data. More information is needed on the physiological ecology of *Alexandrium* strains from this area in order to identify environmental and/or meteorological triggers that stimulate blooms. The SH assay used in this study may prove to be an effective aid in understanding *Alexandrium* blooms and PSP events.

Appendix 1

This appendix includes supplementary data that aid in understanding the calibration of the SH assay and the problems, limitations, and sources of error associated with the SH assay as presented in Chapter 2.

Table A.1a. Whole cell counts used for construction of the field calibration curve in 2000. The table shows the mean number of cells counted per filter with standard deviation around these counts; n is the number of filters counted per sample.

Date	Sample #	Net vertical tow (m)	Mean (cells/filter)	Std dev	n
08/21/00	251	5	238	10	3
08/29/00	257	5	52	14	2
08/29/00	258	5	57	8	2
08/29/00	259	5	33	3	2
08/29/00	260	5	17	1	2
09/06/00	268	5	44	15	3
09/06/00	269	5	45	15	3
09/06/00	270	5	47	3	2
Average			67	9	

Table A.1b. Whole cell counts used for construction of the field calibration curve in 2001. The table shows the mean number of cells counted per filter with standard deviation around these counts; n is the number of filters counted per sample.

Date	Sample #	Niskin depth cast (m)	Mean (cells/filter)	Std dev	n
6/12/01	371 c	5	23	4	5
6/21/01	385 c	5	29	4	4
6/26/01	387 b	3	91	11	5
7/05/01	398 a	1	103	11	5
7/11/01	403	3	126	7	5
7/19/01	413 b	3	19	3	5
7/31/01	421 a	1	126	7	5
8/06/01	429 b	3	65	11	5
8/13/01	436 a	1	48	8	5
9/05/01	460 b	3	37	9	5
9/12/01	463 c	5	172	22	5
9/19/01	469 c	5	10	4	5
9/26/01	472 b	3	11	3	4
10/4/01	477 b	3	4	2	3
Average			62	8	

Table A.2. Coefficient of variation among triplicate well OD readings for each replicate analyzed via the sandwich hybridization assay during the 2001 field season. Data shown include a representative subset of samples collected in 2001 and processed by the SH assay (as described in Chapter 2), starting with sample #349 collected in late May and ending with sample 462 collected in early September. Samples were taken with either a plankton net or by Niskin bottle, in which case the sample depth is listed as a, b, c, d, or e (1, 3, 5, 10, 17 m, respectively). All samples were analyzed twice (replicate a and b) and the same volume of concentrated seawater was used for each replicate. RNA from each replicate was run in triplicate on the assay plates. Reported values are the mean OD reading, standard deviation (std dev), standard error (std error), and coefficient of variation (CV) of these triplicate readings. The mean CV and range are shown at the bottom of the table.

Sample #	Replicate	Mean OD	Std dev	Std error	CV %
349	a	0.185	0.019	0.011	**10.14**
349	b	0.152	0.001	0.001	**0.66**
350 a	a	0.062	0.004	0.002	**6.14**
350 a	b	0.054	0.005	0.003	**8.49**
350 b	a	0.068	0.002	0.001	**3.41**
350 b	b	0.054	0.001	0.001	**1.85**
350 c	a	0.060	0.001	0.000	**0.97**
350 c	b	0.055	0.001	0.001	**1.82**
350 d	a	0.058	0.003	0.002	**5.17**
350 d	b	0.055	0.002	0.001	**2.79**
356 a	a	0.108	0.031	0.018	**29.14**
356 a	b	0.129	0.015	0.009	**11.58**
356 b	a	0.119	0.008	0.005	**7.07**
356 b	b	0.117	0.014	0.008	**11.87**
356 c	a	0.109	0.006	0.004	**5.59**
356 c	b	0.086	0.039	0.023	**45.35**
356 d	a	0.116	0.006	0.004	**5.38**
356 d	b	0.119	0.011	0.006	**9.09**
356 e	a	0.067	0.003	0.002	**4.54**
356 e	b	0.069	0.011	0.006	**16.08**
361	a	0.193	0.070	0.041	**36.45**
361	b	0.234	0.037	0.022	**15.98**
362	a	0.428	0.132	0.076	**30.80**
362	b	0.316	0.034	0.019	**10.64**
363 a	a	0.209	0.119	0.069	**56.81**
363 a	b	0.155	0.011	0.007	**7.27**
363 b	a	0.238	0.004	0.002	**1.75**
363 b	b	0.150	0.004	0.002	**2.34**
363 c	a	0.149	0.070	0.040	**46.67**
363 c	b	0.130	0.013	0.008	**10.35**
363 d	a	0.210	0.023	0.013	**10.85**
363 d	b	0.126	0.009	0.005	**7.14**
369 a	a	0.292	0.004	0.002	**1.38**
369 a	b	0.295	0.111	0.064	**37.82**
369 b	a	0.321	0.029	0.017	**8.98**
369 b	b	0.266	0.009	0.005	**3.38**

369 c	a	0.142	0.011	0.006	**7.38**
369 c	b	0.157	0.045	0.026	**28.71**
369 d	a	0.102	0.008	0.005	**7.66**
369 d	b	0.100	0.030	0.017	**29.67**
369 e	a	0.094	0.000	0.000	**0.00**
369 e	b	0.082	0.004	0.002	**4.95**
370	a	0.206	0.032	0.019	**15.62**
370	b	0.152	0.009	0.005	**5.61**
371 a	a	0.096	0.002	0.001	**1.60**
371 a	b	0.089	0.015	0.009	**17.23**
371 b	a	0.106	0.013	0.007	**11.82**
371 b	b	0.114	0.004	0.002	**3.56**
371 d	a	0.205	0.005	0.003	**2.44**
371 d	b	0.100	0.019	0.011	**19.34**
376	a	0.198	0.129	0.075	**65.47**
376	b	0.182	0.013	0.007	**7.05**
376	c	0.114	0.008	0.004	**6.72**
376	d	0.116	0.003	0.002	**2.63**
377 a	a	0.088	0.006	0.003	**6.24**
377 a	b	0.105	0.012	0.007	**11.59**
377 b	a	0.100	0.031	0.018	**31.19**
377 b	b	0.083	0.020	0.012	**24.28**
377 c	a	0.128	0.010	0.006	**8.00**
377 c	b	0.135	0.036	0.021	**26.34**
377 d	a	0.156	0.015	0.009	**9.66**
377 d	b	0.145	0.012	0.007	**8.07**
377 d	c	0.184	0.019	0.011	**10.31**
377 d	d	0.194	0.004	0.002	**2.08**
377 e	a	0.132	0.004	0.002	**3.05**
377 e	b	0.117	0.001	0.000	**0.49**
378	a	0.292	0.002	0.001	**0.52**
378	b	0.204	0.103	0.059	**50.25**
379 a	a	0.148	0.005	0.003	**3.51**
379 a	b	0.146	0.017	0.010	**11.68**
379 b	a	0.181	0.004	0.002	**1.99**
379 b	b	0.167	0.022	0.013	**12.97**
379 c	a	0.162	0.037	0.021	**22.86**
379 c	b	0.113	0.060	0.035	**53.28**
379 d	a	0.529	0.059	0.034	**11.12**
379 d	b	0.347	0.022	0.013	**6.35**
379 e	a	0.110	0.005	0.003	**4.47**
379 e	b	0.110	0.017	0.01	**15.16**
384	a	0.275	0.004	0.002	**1.47**
384	b	0.375	0.218	0.126	**58.07**
385 a	a	0.091	0.012	0.007	**12.67**
385 a	b	0.108	0.005	0.003	**4.24**
385 b	a	0.103	0.018	0.01	**17.56**
385 b	b	0.081	0.019	0.011	**23.27**
385 d	a	0.128	0.004	0.002	**3.24**

RASMUSON LIBRARY
UNIVERSITY OF ALASKA-FAIRBANKS

385 d	b	0.141	0.025	0.015	**17.98**
385 e	a	0.076	0.002	0.001	**2.02**
385 e	b	0.080	0.008	0.005	**9.76**
386	a	0.178	0.043	0.025	**24.11**
386	b	0.246	0.049	0.028	**20.02**
387 a	a	0.142	0.012	0.007	**8.54**
387 a	b	0.169	0.022	0.013	**12.91**
387 c	a	0.128	0.005	0.003	**3.91**
387 c	b	0.136	0.008	0.005	**5.96**
387 d	a	0.099	0.006	0.003	**5.58**
387 d	b	0.108	0.030	0.017	**27.39**
387 e	a	0.082	0.003	0.002	**3.54**
387 e	b	0.089	0.012	0.007	**13.55**
392	a	0.288	0.127	0.073	**44.17**
392	b	0.212	0.026	0.015	**12.20**
393 a	a	0.161	0.081	0.047	**50.20**
393 a	b	0.181	0.036	0.021	**19.64**
393 c	a	0.225	0.003	0.002	**1.29**
393 c	b	0.258	0.088	0.051	**34.12**
393 d	a	0.087	0.029	0.017	**33.07**
393 d	b	0.119	0.024	0.014	**20.12**
393 e	a	0.195	0.017	0.010	**8.57**
393 e	b	0.157	0.017	0.001	**10.80**
398 b	a	0.121	0.039	0.022	**31.88**
398 b	b	0.073	0.012	0.007	**16.65**
399	a	0.167	0.074	0.043	**44.47**
399	b	0.146	0.011	0.006	**7.18**
400 b	a	0.106	0.029	0.017	**27.20**
400 b	b	0.121	0.023	0.013	**19.01**
400 d	a	0.145	0.005	0.003	**3.16**
400 d	b	0.193	0.062	0.036	**32.11**
400 e	a	0.125	0.006	0.003	**4.68**
400 e	b	0.101	0.007	0.004	**6.61**
404	a	0.209	0.017	0.010	**8.11**
404	b	0.175	0.011	0.006	**6.10**
405 a	a	0.103	0.006	0.003	**5.41**
405 a	b	0.106	0.003	0.002	**3.27**
405 b	a	0.110	0.005	0.003	**4.50**
405 b	b	0.096	0.013	0.007	**13.24**
405 d	a	0.098	0.014	0.008	**14.53**
405 d	b	0.099	0.016	0.009	**15.78**
405 e	a	0.073	0.004	0.002	**5.48**
405 e	b	0.076	0.018	0.010	**23.98**
406	a	0.170	0.012	0.007	**6.78**
406	b	0.171	0.014	0.008	**8.12**
407 a	a	0.106	0.004	0.002	**3.83**
407 a	b	0.099	0.003	0.002	**3.03**
407 b	a	0.118	0.012	0.007	**10.41**
407 b	b	0.118	0.031	0.018	**26.64**

407 c	a	0.166	0.029	0.017	**17.74**
407 c	b	0.169	0.005	0.003	**3.04**
407 e	a	0.078	0.008	0.005	**10.01**
407 e	b	0.116	0.068	0.039	**58.29**
412	a	0.207	0.013	0.008	**6.28**
412	b	0.145	0.005	0.003	**3.19**
413 a	a	0.105	0.037	0.022	**35.63**
413 a	b	0.120	0.006	0.003	**4.75**
413 c	a	0.099	0.038	0.022	**38.82**
413 c	b	0.138	0.020	0.011	**14.27**
413 d	a	0.138	0.018	0.010	**12.88**
413 d	b	0.144	0.009	0.005	**5.93**
413 e	a	0.064	0.006	0.003	**8.56**
413 e	b	0.071	0.014	0.008	**19.72**
414	a	0.132	0.023	0.013	**17.61**
414	b	0.145	0.023	0.013	**15.55**
415 a	a	0.174	0.008	0.005	**4.49**
415 a	b	0.191	0.024	0.014	**12.39**
415 b	a	0.133	0.012	0.007	**9.26**
415 b	b	0.114	0.042	0.024	**37.08**
415 c	a	0.120	0.007	0.004	**5.69**
415 c	b	0.125	0.007	0.004	**5.34**
415 d	a	0.092	0.004	0.002	**4.51**
415 d	b	0.100	0.040	0.023	**39.40**
415 e	a	0.074	0.006	0.003	**8.18**
415 e	b	0.089	0.002	0.001	**1.72**
421 b	a	0.141	0.000	0.000	**0.00**
421 b	b	0.143	0.01	0.006	**6.74**
421 c	a	0.176	0.019	0.011	**10.82**
421 c	b	0.130	0.049	0.028	**37.29**
421 d	a	0.148	0.019	0.011	**13.08**
421 d	b	0.131	0.009	0.005	**6.90**
421 e	a	0.148	0.006	0.003	**3.83**
421 e	b	0.146	0.009	0.005	**6.13**
427 a	a	0.178	0.007	0.004	**3.93**
427 a	b	0.106	0.025	0.015	**23.92**
427 b	a	0.321	0.018	0.011	**5.69**
427 b	b	0.247	0.033	0.019	**13.38**
427 c	a	0.162	0.002	0.001	**1.24**
427 c	b	0.144	0.000	0.000	**0.00**
462	a	0.579	0.073	0.042	**12.55**
462	b	0.529	0.114	0.066	**21.59**
				mean CV	**14.08**
				range	**0-65**

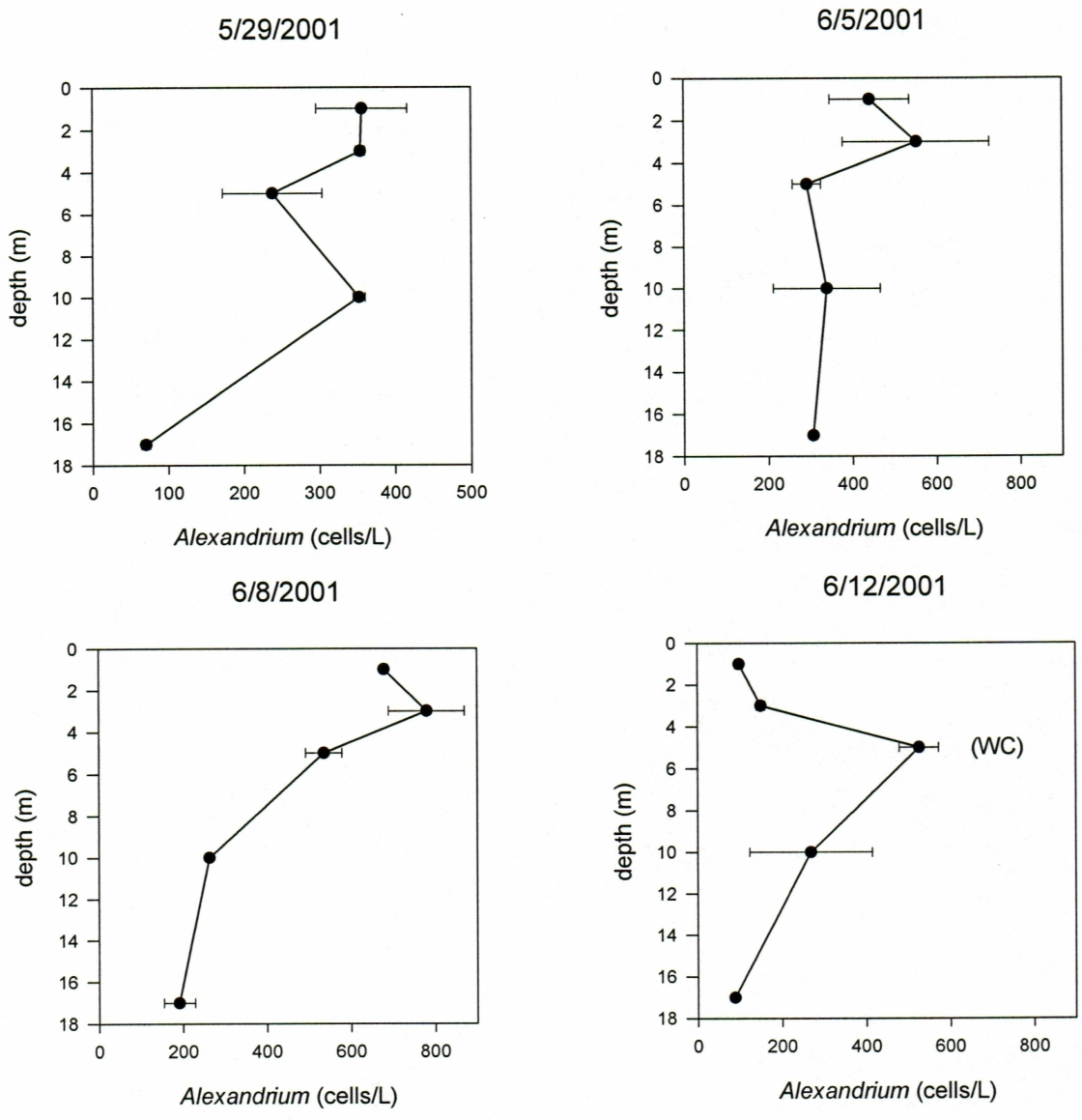

Figure A.1. Depth profiles of *Alexandrium* spp. (± 1 SE) during the summer of 2001 at Trident Basin.

Data shown include all Niskin bottle collections from depths of 1, 3, 5, 10, and 17 m. *Alexandrium* abundance was determined via the SH assay as described in Chapter 2. Two aliquots from each depth sample were filtered (replicates), each of which were analyzed on the SH plates in triplicate. The mean of the triplicate ODs from each replicate were averaged and used to calculate *Alexandrium* abundance. The data presented are the mean abundance data from the two replicates with standard error bars (± 1 SE), except when a sample was used in construction of the standard curve, in which case 4-10 replicates were averaged (data points shown as "WC").

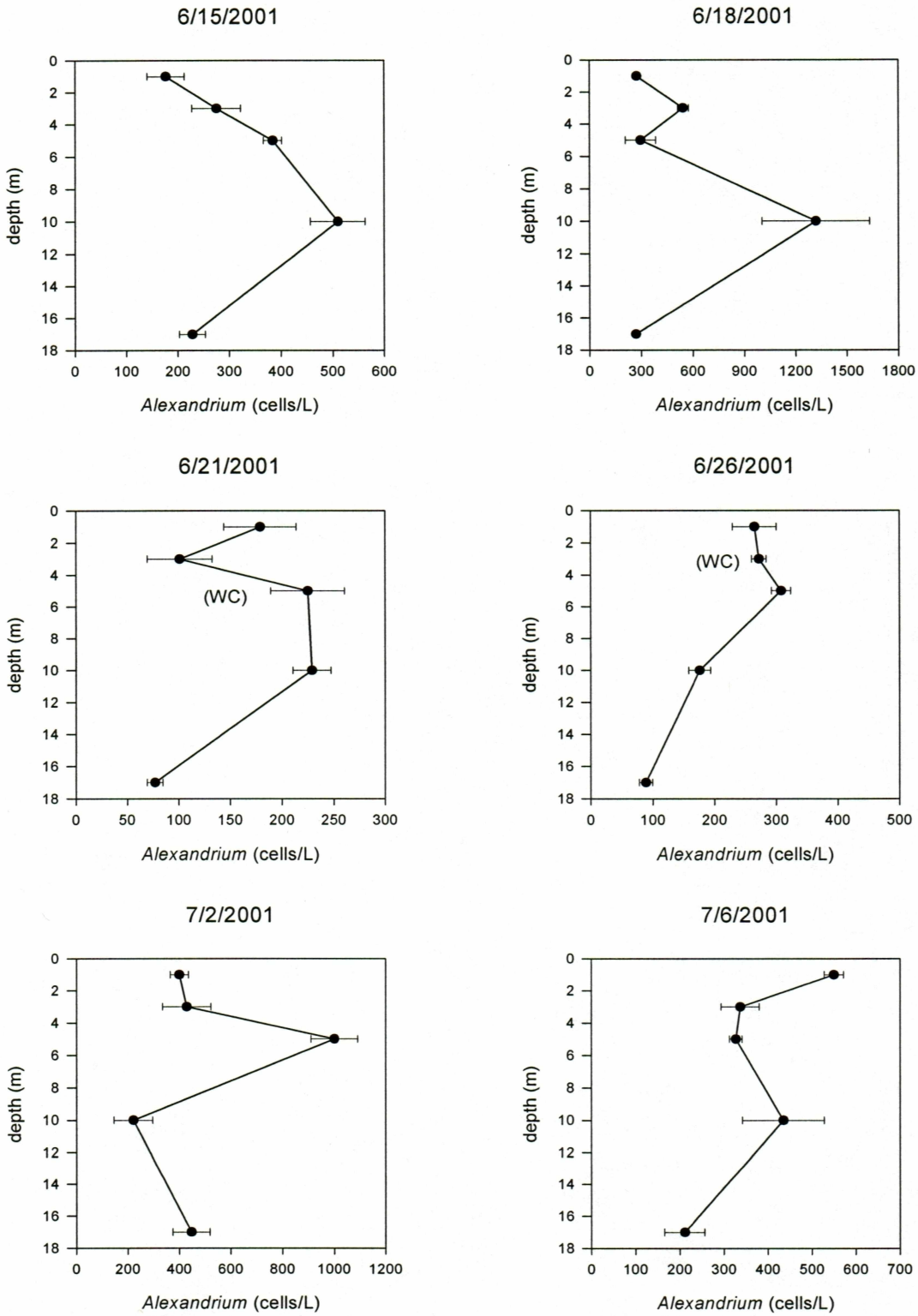

Figure A.1 continued

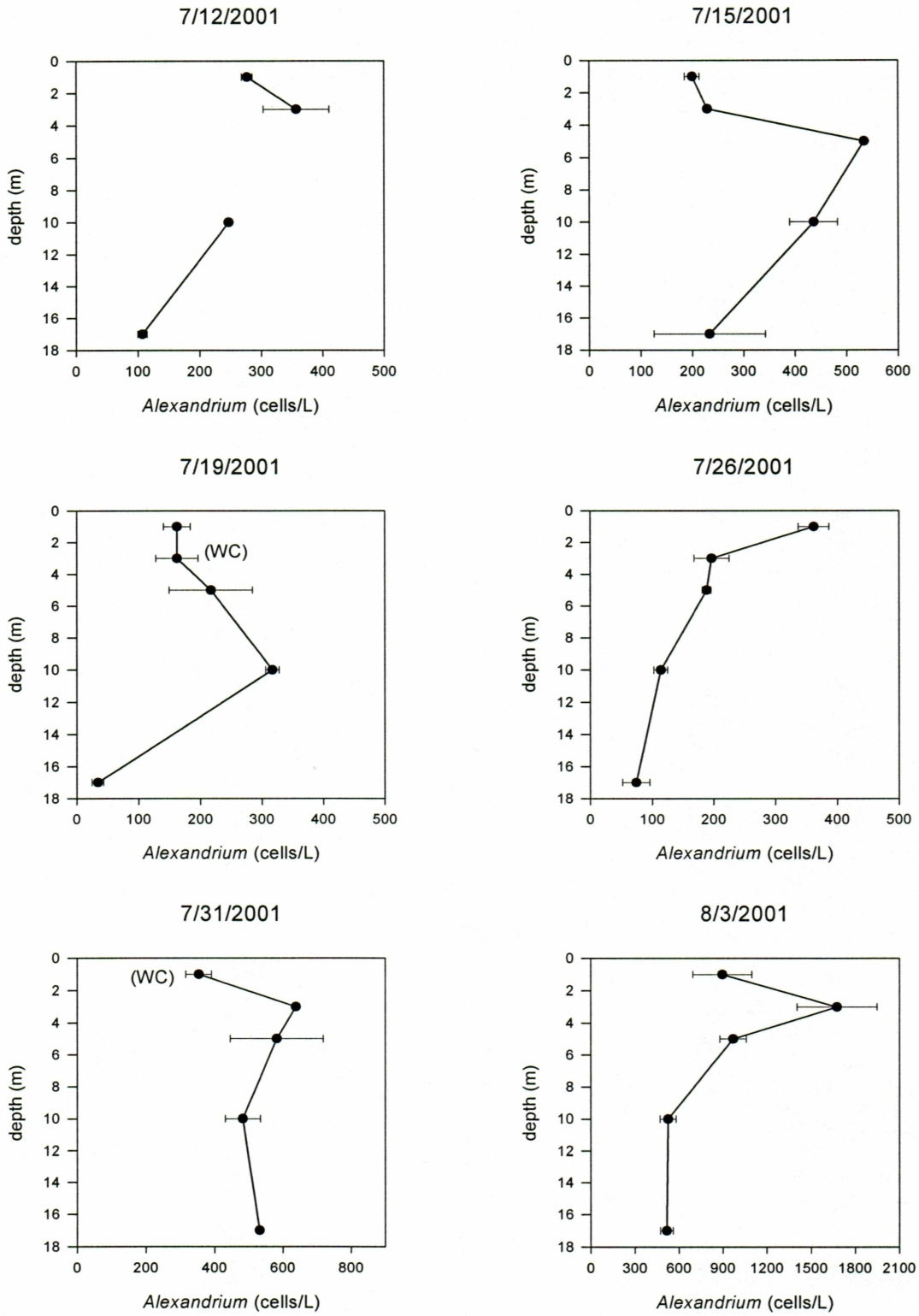

Figure A.1 continued

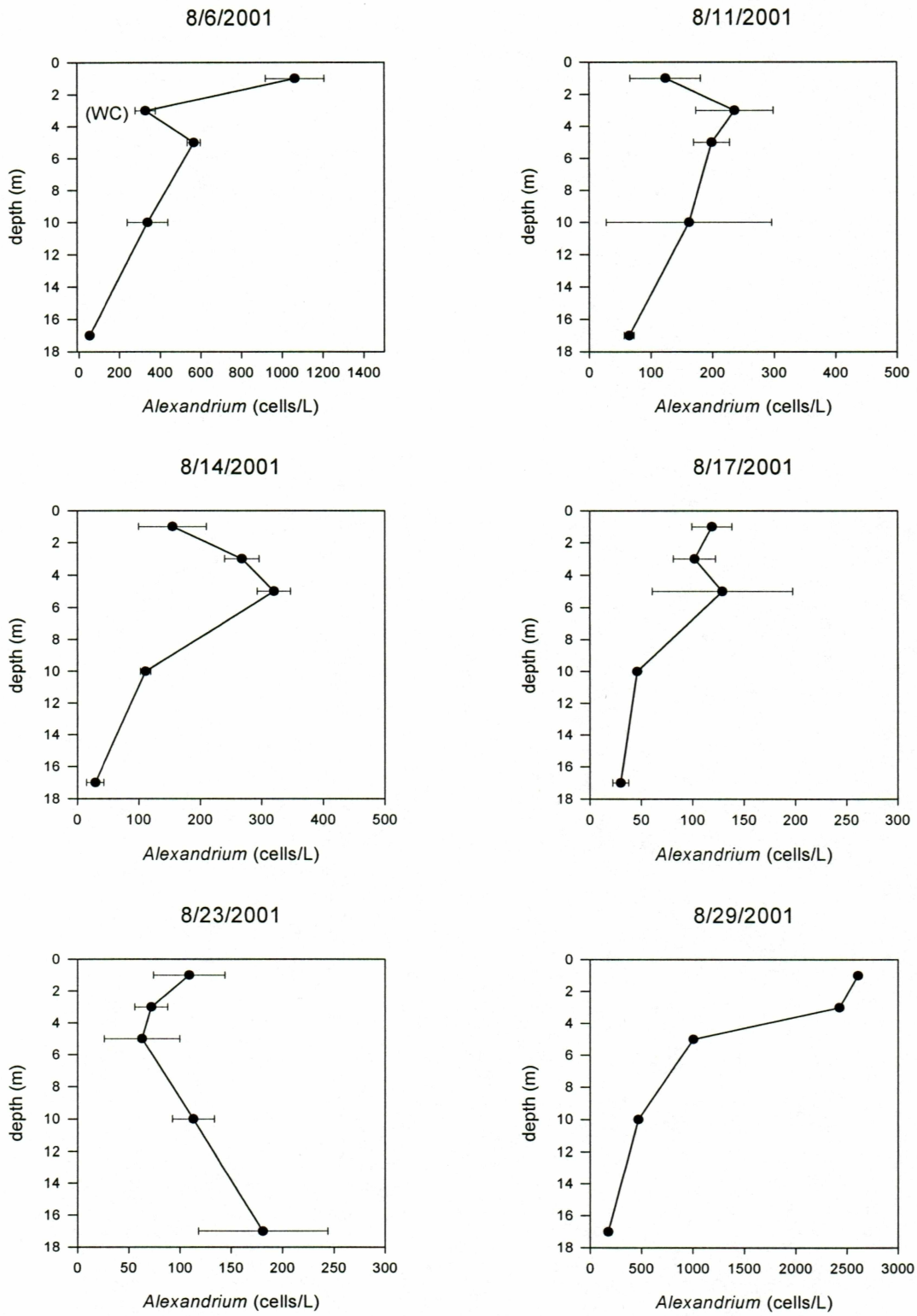

Figure A.1 continued

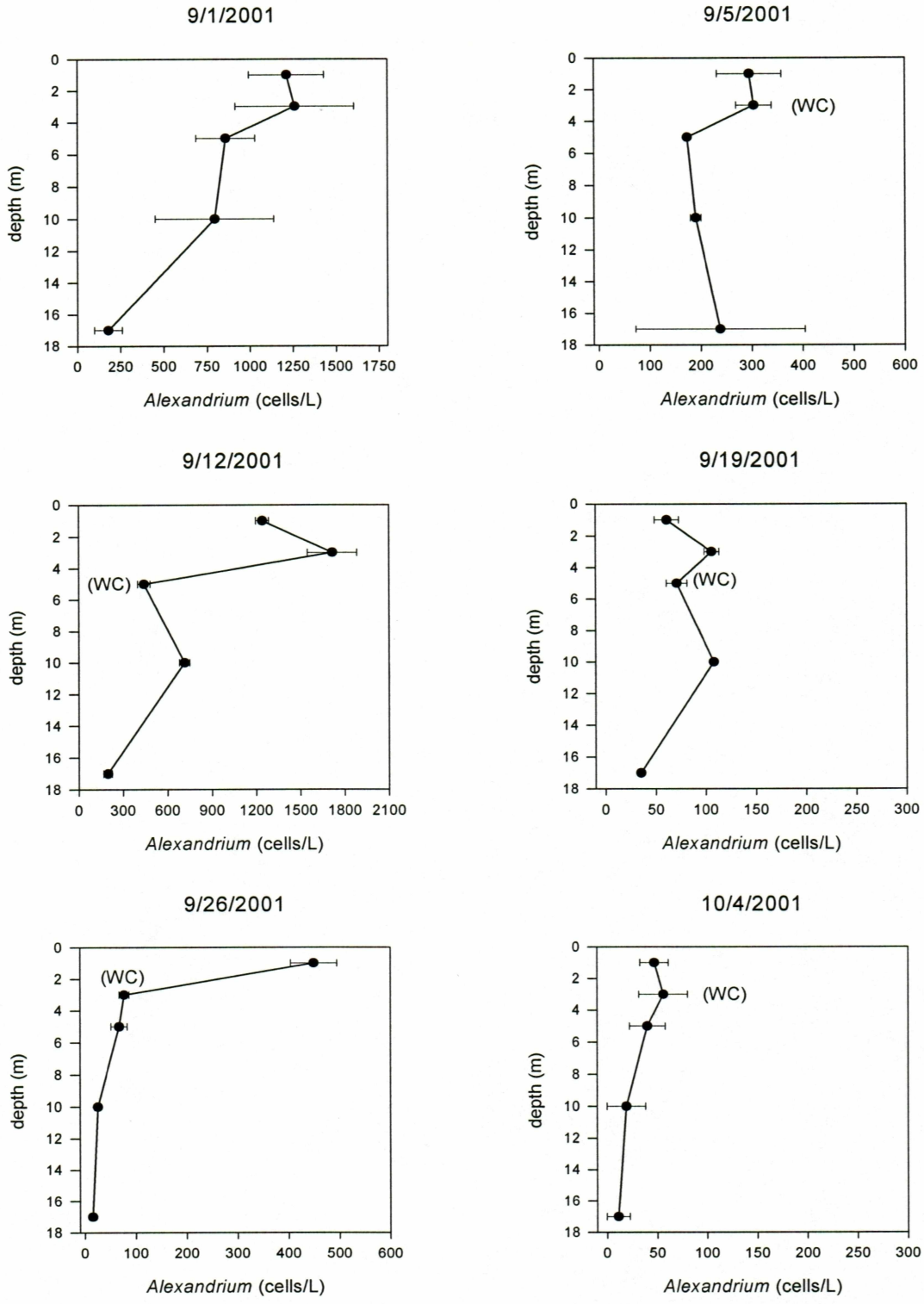

Figure A.1 continued