A study of the survival of *Alexandrium* cysts in natural sediments and their utility to reconstruct harmful algal blooms in the Pacific Northwest

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Abstract

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Harmful algal blooms (HABs) produced by the potentially toxic dinoflagellate genus *Alexandrium* are a natural phenomenon that have been present in the Pacific Northwest since at least the late 1700s. *Alexandrium* can produce a suite of toxins that if concentrated in shellfish, can be lethal within hours of consumption. Detections of *Alexandrium* toxins in shellfish have increased in the Pacific Northwest since the Washington Department of Health began monitoring for paralytic shellfish toxins (PST) in the 1950s but, it is unclear whether the increase is an artifact of increased monitoring efforts or if blooms are expanding geographically and becoming more frequent. It has been hypothesized that *Alexandrium* blooms have become more common in the Pacific Northwest as sea surface temperatures warm. However, the existing PST records in Washington are difficult to standardize or do not have the temporal resolution needed to

statistically assess historical trends of *Alexandrium* detections relative to environmental drivers like sea surface temperature. The focus of this research was to develop a novel method to reconstruct *Alexandrium* bloom patterns using sediment cores and the benthic stage of its life cycle to better understand historical *Alexandrium* variability in relation to the environment.

For most of the year, *Alexandrium* can be found on the sediment floor in a dormant stage known as a cyst. The long-term preservation potential, survival and/or degradation of *Alexandrium* cysts in the sediments are not well-understood. Cysts of the non-toxic dinoflagellate genus *Scrippsiella* commonly co-occur in the sediments with *Alexandrium* in the Pacific Northwest. The primary objectives of this body of work were to (1) better understand the survival and visual appearance of *Alexandrium* and *Scrippsiella* cysts over time in a sediment core (Chapter 3); (2) investigate the relationship between *Alexandrium* cyst densities in sediment cores and the available, nearby historical PST records (Chapters 2 & 4); (3) statistically assess correlations between the cyst record and potential environmental drivers (Chapters 2 & 4); and (4) develop a rapid method to estimate the percentage of *Alexandrium* and *Scrippsiella* cysts able to germinate from a sediment sample (Chapter 5).

Results from this research suggest that *Alexandrium* cysts are able to survive in the benthic environment for >50-80 years and show little evidence of physical degradation as measured by visual appearances and ability to germinate. Cyst records developed from sediment cores are correlated to PST records and can help to illuminate past trends and long-term drivers of HABs. Thus, *Alexandrium* cysts in sediment cores may be a useful proxy of historical HABs and can be used to reconstruct historical bloom patterns at various locations. A better understanding of the historical *Alexandrium* cyst record could help inform predictions of HABs in light of climate change.

- In dedication to my family -

For helping me to find my dreams and

letting them come true.

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1 Introduction

Many planktonic protists produce benthic resting stages in their life histories but little is known about their long term survival while in the sediments. Resting cells, cysts and spores, serve as a seed bank to help a species overwinter and repopulate the water column once conditions suitable for vegetative growth arise. These seed banks can further aid in genetic diversity and enhance species' propagation and dispersion (Bravo and Figueroa, 2014; Wall, 1971). It has been postulated that resting stages evolved to aid in long term survival of plankton during prolonged periods of reduced solar transmission that may have occurred during ice ages and/or after catastrophic meteor strikes (McMinn and Martin, 2013; Ribeiro et al., 2011). The estimated survival of dinoflagellate cysts (Binder and Anderson, 1990; Feifel et al., submitted; Miyazono et al., 2012) and diatom spores (Härnström et al., 2011; Lewis et al., 1999) have ranged from several months to 100+ years.

More than 10% of known dinoflagellate species produce a cyst during their life history (Bravo and Figueroa, 2014). Dinoflagellate cysts can be a product of sexual or asexual reproduction but the sexual cyst, the hypnozygote, is the most resilient type of cyst formed (Figueroa et al., 2008). The cyst wall of the hypnozygote is generally composed of three distinct layers of varying thickness and molecular compositions (Kokinos et al., 1998). The type of cyst (sexual or asexual, long-term or short-term) formed, number of cysts, cyst cell composition and structural integrity of cyst walls can be influenced by many factors, including species, the cyst's age, the nitrogen and phosphorus nutrient levels present in the environment during encystment or other physical environmental variables such as salinity or temperature (Bogus et al., 2014; Figueroa et al., 2005, 2011). Germination of dinoflagellate cysts may be influenced by many external and internal factors including the availability of oxygen (Andersen and Keafer, 1987), parental strains

(Figueroa et al., 2005), light availability (Vahtera et al., 2014) and/or an internal circadian clock (Matrai et al., 2005). Despite a growing body of literature investigating the benthic protist life stages- from encystment, *in situ* survival, to germination- fundamental questions regarding *in situ* long term survival remain unanswered for the majority of protists.

1.1 The harmful genus *Alexandrium* in the Pacific Northwest

Alexandrium is a common coastal temperate-water harmful algal bloom (HAB) forming genus in the Pacific Northwest that produces a suite of paralytic shellfish toxins (PSTs). It is thought that *Alexandrium* has been present in the waters off of the northwest coast of North America for centuries (Horner et al., 1997). During *Alexandrium* blooms, PSTs can accumulate in filter-feeding bivalves to concentrations unsafe for human consumption. State health authorities close shellfish harvesting if PST levels exceed 80 µg STX equiv/100 g shellfish tissue. Periodic shellfish closures cause economic losses to local businesses, shellfish growers and challenge monitoring and resource management authorities.

Many have hypothesized that *Alexandrium* blooms in the Pacific Northwest have increased in spatial extent, duration, and frequency as a direct response to large and small-scale climate drivers (Ebbesmeyer et al., 1995; Erickson and Nishitani, 1985; Moore et al., 2008; Trainer et al., 2003) but there are few long term data sets with the temporal and spatial resolutions needed to statistically assess these relationships. In the Puget Sound, scientists have looked to historical toxicity records from the Washington Department of Health (WDOH) to assess long term sources of regional change in *Alexandrium* blooms. While the WDOH paralytic shellfish toxicity (PST) data set is one of the longest PST records in the world, spanning from 1950s to today in some places, it is complicated by multiple shellfish species with varying toxin

depuration rates, irregular sampling schedules, and large data holes. This has made it difficult for researchers to standardize observations and compare the PST record to potential environmental drivers. Long-term records need to be developed to understand the response of *Alexandrium* to changes in the environment. If there is an ecologically significant, positive relationship between *Alexandrium* populations and sea surface temperature, a warmer future could present more frequent and larger blooms of *Alexandrium* in Washington State.

1.2 *Alexandrium* cysts

The cyst stage of *Alexandrium* has an important role in seeding annual blooms. It serves as a quiescent stage for the dinoflagellate to overwinter and when "ideal" conditions arise, the cyst can germinate, releasing a vegetative, motile cell able to divide and form large, potentially toxic blooms along coastlines. In Puget Sound, Washington, USA, *Alexandrium* cyst abundances in surface sediments vary widely (0 to >12,000 cysts cm^{-3}) and distributions are spatially heterogeneous (Horner et al., 2011). Patchiness in the environment could be caused by hydrographic conditions (i.e. currents, wind and waves), sediment type, or from variability in phytoplankton bloom locations and sizes.

Despite their significant role in initiating harmful algal blooms, the *Alexandrium* cyst stage is generally poorly understood. It is unknown what triggers excystment or how long term cyst viability relates to cyst age or the amount of internal contents in the cysts. The preservation potential of various dinoflagellate cysts, including *Alexandrium*, is an area of active research. Some researchers have claimed to have identified *Alexandrium* cysts in sediments estimated to be 1,000's of years old (Mudie et al., 2002) but further research indicated that the alleged *Alexandrium* cysts were misidentified and were actually cysts of another dinoflagellate,

Scrippsiella (Head et al., 2006). Keafer et al. (1992) concluded that *Alexandrium* cysts in a Massachusetts salt pond had a half-life of 5 years and suggested that all cysts degrade in the environment within 25 years from their initial deposition. In 2012, researchers from Japan successfully germinated *Alexandrium* cysts they estimated to be 100+ years old (Miyazono et al., 2012) which suggested that the long term viability of cysts in the environment may have been underestimated and warranted further investigation.

1.3 Using sediment cores to reconstruct historical *Alexandrium* bloom patterns

This dissertation set out to develop long-term records of *Alexandrium* using sediment cores. The goal of the research was to assess how long *Alexandrium* cysts can survive in the sediments and test the hypothesis that the cyst record can be used to reconstruct historical, century-long bloom patterns of *Alexandrium*. If *Alexandrium* cyst records reflect historical bloom sizes, sediment cores could serve as a valuable source of information from which to infer longer term sources of change. The cyst record could be a natural and direct record of historical blooms, would circumnavigate many of the problems inherent in the PST data, and would allow researchers to develop historical records able to be statistically compared to environmental drivers.

The primary questions motivating this research are:

(1) How long do *Alexandrium* cysts survive in the natural environment and how do you assess long-term survival and viability?
(2) Does the *Alexandrium* cyst record in sediment cores reflect trends in nearby PST records and if so, can the cyst record be used as a proxy of historical blooms?
(3) Are cyst records correlated with any environmental parameters or large-scale climate drivers?

The following is a brief summary of each chapter within the dissertation. Chapter 2 was an investigative study to test the hypothesis that a historical *Alexandrium* cyst record could be developed and reflects nearby PST records. A sediment core from Sequim Bay, WA, was used to develop a 100+ year record of *Alexandrium* cysts and compared to environmental and climate parameters. The goal of Chapter 3 was to better understand the degradation potential of cysts produced by *Alexandrium* and the non-toxic dinoflagellate *Scrippsiella.* A novel, computer-aided image analysis program was developed to help quantify the amount of internal contents (presumed energy reserves) that were present in cysts as they age. Percent germination was then compared to the estimated cyst ages and the amount of internal contents available to energetically support germination. In Chapter 4, a high-resolution *Alexandrium* cyst record was developed from a sediment core extracted from the hypoxic fjord Effingham Inlet, British Columbia. The cyst record is compared to available PST records and nearby sea surface temperature ecords to investigate the complex relationships between the physical environment, *Alexandrium* blooms, PST toxicity, and cyst counts. Lastly, Chapter 5 presents a rapid, quantitative laboratory method that can be used to quickly estimate percent germination and long term viability of dinoflagellate cysts in natural sediments.

2 An *Alexandrium catenella* cyst record from Sequim Bay, Washington State, and its relation to past climate variability

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2.1 Abstract

Since the 1970s, Puget Sound, Washington State, has experienced an increase in detections of paralytic shellfish toxins (PSTs) in shellfish due to blooms of the harmful alga *Alexandrium catenella* (Whedon and Kofoid) Balech. Natural patterns of climate variability, such as the Pacific Decadal Oscillation (PDO) and the El Niño-Southern Oscillation (ENSO), and changes in local environmental factors such as sea surface temperature, air temperature and stream flow have been linked to the observed increase in PSTs. However, the lack of observations of PSTs in shellfish prior to the 1950s has inhibited statistical assessments of longer term trends in climate and environmental conditions on *A. catenella* blooms. After a bloom, *A. catenella* can enter a dormant cyst stage which settles on the seafloor and then becomes entrained into the sedimentary record. In this study, we created a record of *A. catenella* cysts from a sediment core obtained from Sequim Bay, Puget Sound. Cyst abundances ranged from 0-400 cysts per cm^3 and were detected down-core to a depth of 100 cm, indicating that *A. catenella* has been present in Sequim Bay since at least the late 1800s. The cyst record allowed us to statistically examine relationships with environmental parameters over the past century. Local air temperature and sea

surface temperature were positively and significantly correlated with cyst abundances from the late 1800s to 2005; no significant relationship was found between PDO and cyst abundances. This highlights the importance of local environmental variability in controlling *A. catenella* population dynamics.

2.2 Introduction

On a global scale, harmful algal blooms (HABs) appear to be increasing in frequency, duration, and geographically (Sournia and Birrien 1995, Van Dolah 2000). It has been suggested that changes in climate may be a contributor to the worldwide increase in HAB outbreaks (Hallegraeff 1993, Hallegraeff 2010). However, a lack of long time series of HAB occurrences has inhibited statistical assessments of any potential relationships with climate and most investigations have focused on recent, relatively short-term change (i.e., 1-2 decades). Without historical antecedents, it is difficult to quantitatively establish trends of HABs and to relate changes to natural patterns of climate variability or anthropogenic climate change.

Phytoplankton are good indicators of change in the oceans because (1) their abundances can vary seasonally in space and time, (2) their populations exhibit non-linear responses to subtle perturbations to the environment, (3) their population sizes can fluctuate dramatically over short periods of time, and (4) they are not directly commercially exploited, lessening the likelihood that observed changes are due to changes in fishing effort (Hays et al. 2005). While harmful species of phytoplankton typically comprise a small component of the entire phytoplankton community, changes in HAB populations can serve as bioindicators of large-scale marine ecological disturbances such as changes in climate or eutrophication (Harvell et al. 1999). As such, HABs may be a harbinger of change and therefore serve as the quintessential "canary in

the coalmine" for the oceans as well as having direct impacts on human and ecosystem health. Understanding the effects that large-scale climate variability and local environmental conditions have on HAB populations could indicate impending changes in oceanic ecosystems.

After a bloom, some species of harmful algae (i.e., the dinoflagellates) can produce resting cysts that settle onto bottom sediments. Concentrations of these cysts preserved in sediments are useful for reconstructing historical changes in blooms of some dinoflagellate species (Cox et al. 2008) and have been shown to reflect changes in the environment (Dale 2001, Pospelova et al. 2006). Evaluating cyst concentrations in sediment cores can therefore serve as a window into the past, acting as a natural historical record of dinoflagellate communities (Wall et al. 1977). In this study, we have created a historical cyst record of a HAB dinoflagellate species, *Alexandrium catenella*, from Sequim Bay, Washington State, and compared it to changes in local environmental and large-scale climate parameters.

Alexandrium catenella[1] is a common, free-living, coastal cold-water dinoflagellate known to produce a suite of paralytic shellfish toxins (PSTs). During blooms of *A. catenella*, cells containing PSTs can become concentrated in filter-feeding bivalves and ingestion of these PST-laden bivalves by humans can cause paralytic shellfish poisoning. The most potent PST, saxitoxin, is a neurotoxin that causes disruption in the neuromuscular and respiratory systems and can be lethal within a few hours of ingestion (Quayle 1969, Garcia et al. 2004). State health authorities close shellfish harvesting if PST levels exceed 80 µg STX equiv/100 g shellfish

[1] Several species of *Alexandrium*, including *A. catenella*, *A. acatenella*, *A. tamarense*, and "intermediates," have been reported from Puget Sound and other Pacific Northwest waters (e.g., Taylor, 1984; Taylor and Horner, 1994). However, *A. catenella* has been the species identified historically as the dominant *Alexandrium* species in Puget Sound so we have continued to use that name although we recognize the current taxonomic confusion over the identification of species in the *tamarense/catenella/fundyense* complex.

tissue. Periodic closures cause economic losses to shellfish growers and challenge monitoring and management authorities.

Alexandrium catenella exhibits an alternation between a motile, planktonic, vegetative stage with a non-motile, benthic, cyst stage. During sexual reproduction, the gametes of *A. catenella* fuse to form a motile planozygote, which then develops into a non-motile, 40-50 µm in length (Cox et al. 2008) hypnozygote cyst that settles to the sea floor. In laboratory studies, the formation of cysts has been triggered by reduced nitrogen and phosphorus levels (Meksumpun et al. 1994, Kennaway and Lewis 2004), but it is unknown if low nutrients cause encystment in the natural environment. The cell wall of the *A. catenella* hypnozygote is composed of three discrete sections spanning a width of 5 µm. It is primarily composed of silicon and sulphur and has been shown to be resistant to acetolysis in the lab (Meksumpun et al. 1994, Kennaway and Lewis 2004). The protective and resistant biopolymer wall of many dinoflagellate species, commonly referred to as dinosporin, may account for their prominence in the fossil record of sediment cores (Kokinos et al. 1998). *Alexandrium* spp. cysts have been identified 60 cm down a 63 cm in length sediment core from Japan and researchers were able to successfully germinate cysts that were estimated to be older than 8 years (Mizushima and Matsuoka 2004).

It is believed that *A. catenella* has been present in the waters off the Northwest coast of North America for centuries (Horner et al. 1997). In the 1930s, the Washington State Department of Health (WDOH) began working with researchers in California to test regionally for PSTs. At that time, the PST problem was thought to be restricted to the outer coast, but after three deaths occurred in Clallam County in 1942, the coast was closed to shellfish harvesting from Port Angeles on the Strait of Juan de Fuca to the mouth of the Columbia River from April 1-October 31 annually (Fig. 1). In 1957, the WDOH began more frequent PST testing in Puget Sound in

response to increased shellfish toxicity levels in British Columbia, Canada. Between 1942 and 1978, low levels of PSTs were detected along the Washington coast and the Strait of Juan de Fuca, but no toxins were detected in shellfish from inside Puget Sound. There were no reported illnesses during this period. Then in 1978, a major bloom of *A. catenella* caused widespread toxicity in Puget Sound's Whidbey Basin extending to south of Seattle, and toxins reached record breaking levels of 30,360 µg STX equiv/100 g shellfish tissue. In 1988, the first shellfish harvest closures occurred in the South Sound and the WDOH began a Sentinel Mussel Monitoring Program at a few sites using caged mussels. In 1990, the Sentinel Mussel Monitoring Program was expanded and now more than 70 sites throughout the Puget Sound are sampled for PSTs at roughly two week intervals.

Prior to our study, the WDOH PST record was the longest record available in the Pacific Northwest to assess the influence of the environment on *A. catenella*; however, there are complications associated with the dataset that limit its utility to quantitatively establish regional trends in *A. catenella* blooms. The data are collected to protect the health of shellfish consumers, thus sampling intensity by the WDOH increases during toxic events, introducing an unavoidable detection bias within this dataset. Also, care must be taken to standardize the dataset because a variety of shellfish species have been collected and display different toxin accumulation, retention, and depuration rates (Bricelj and Shumway 1998). Furthermore, the magnitude of a bloom may not have a linear relationship with shellfish toxicity (Smayda 1997) and shellfish toxicity may be strongly influenced by environmental parameters, such as temperature (Navarro et al. 2006).

Prior to 1978, there were no known reports of PSTs south of Whidbey Island; today, blooms of *A. catenella* are frequently reported from South Sound. Based upon an analysis of the available

WDOH PST records, it would appear that blooms of *A. catenella* in Puget Sound have increased in frequency and spatial extent since the 1950s (Trainer et al. 2003). However, it remains unclear if this apparent increase in PST detection is simply an artifact of increased monitoring effort by the WDOH or if it truly reflects a Sound-wide trend of increased *A. catenella* blooms. Before the Sentinel Mussel Monitoring Program for PSTs began in 1990, the available shellfish toxicity data (1957-1990) was patchy in space and time and trends were gleaned from a range of shellfish species that have different rates of toxin uptake and depuration. Thus, it is difficult to quantify long-term trends in *A. catenella* blooms and determine any potential relationships with climate prior to 1990 using the WDOH toxicity records alone. In fact, a recent study using the WDOH toxicity data from only the Sentinel Mussel Monitoring Program concluded that bloom prevalence and magnitude had not increased significantly in the Puget Sound from 1993-2007 (Moore et al. 2009).

Alexandrium catenella may display accelerated growth in response to sea surface temperatures (SST) above 13^{o}C (Nishitani and Chew 1984). In the Pacific Northwest, SST varies with air temperature and large-scale climate variations associated with the El Niño-Southern Oscillation (ENSO) and the Pacific Decadal Oscillation (PDO) (Moore et al. 2008). The 1978 expansion of *A. catenella* into Puget Sound coincided with a shift in the PDO from cool phase to warm phase (Ebbesmeyer et al. 1995, Trainer et al. 2003, Moore et al. 2010). This shift was locally manifested by warmer SST in Puget Sound, particularly in the winter (Moore et al. 2008). Other qualitative investigations have also linked increased PSTs in Pacific Northwest shellfish to ENSO (Nishitani and Chew 1984, Erickson and Nishitani 1985, Ebbesmeyer et al. 1995). However, a recent analysis of PSTs in shellfish from Sequim Bay in Puget Sound found no significant relationship with ENSO from 1991-2007 (Moore et al. 2010). The authors

hypothesize that this is because anomalously warm water temperatures created during El Niño winters do not generally persist into the seasonal time period that shellfish in this region accumulate PSTs, typically in the summer and fall (Moore et al. 2010). Instead, a positive relationship between PSTs and periods of warm air and water temperatures in conjunction with low stream flow was suggested to favor blooms of *A. catenella* (Moore et al. 2009). A longer-term record of *A. catenella* cyst or cell abundances or PSTs in shellfish would allow for more powerful quantitative analysis of the potential effects large-scale climate variability and local conditions have on bloom dynamics.

The purpose of our study was to examine the relationship between historical climate variability and *A. catenella* cyst concentrations in a sediment core extracted from Sequim Bay, Washington State. Sequim Bay is an ideal study site because it has the longest record of PSTs available in Washington State and because it has been identified as a regional "hot spot" for PSTs, meaning that the site most often has some of the highest levels of shellfish toxicity relative to other monitored sites within Puget Sound (Moore et al., 2009). Specifically, we wanted to know (1) if the historical numbers of cysts correlate well to past changes in climatic and other environmental parameters, and (2) how long *A. catenella* has been present in Sequim Bay. To answer these questions, a cyst profile was created from the uppermost 100 cm of a 197-cm sediment core taken from Sequim Bay in March, 2005. The core was dated by applying an average sedimentation rate of 0.6 cm yr^{-1} determined using ^{210}Pb analysis (Cox et al. 2008), and historical records of environmental parameters and large-scale climate indices were correlated with the cyst profile. This study provides the first record of *A. catenella* cysts in the Pacific Northwest with sufficient length to determine relationships with long-term and large-scale patterns of climate variability and change.

2.3 Materials and Methods

2.3.1 Study site

Sequim Bay is located near the northwest entrance to the Puget Sound (Fig. 1). It has a surface area of 19.46 km^2 at mean lower low water (MLLW) with a maximum depth of 38.4 m and a tidal range of 3.5 m above to 1.3 m below MLLW. Small streams feeding into Sequim Bay include Dean Creek, Johnson Creek, Jimmycomelately Creek, and No Name Creek. One major stream, the Dungeness River, flows into the neighboring Dungeness Bay and serves as a relative indicator of freshwater input to the area.

The entrance to Sequim Bay is almost entirely enclosed by a naturally occurring spit, Travis Spit, which has been a permanent feature for at least the past 100 years (Elwha-Dungeness planning unit 2005). The water channel joining Sequim Bay to the Strait of Juan de Fuca is approximately 2.57 km long and 152.4 m wide with a depth ranging from 3.9-9.1 m below MLLW. During an inflowing tide, only about 10% of the water is exchanged (Elwha-Dungeness planning unit 2005). As such, it is likely that immigration and emigration of *A. catenella* cells across the sill is limited and that cysts that settle in Sequim Bay sediments are primarily of local origin rather than arriving by advection (Cox et al. 2008). Therefore, Sequim Bay essentially functions as a mesocosm whereby *A. catenella* bloom dynamics are controlled by local environmental conditions which may be modulated by the large-scale climate.

2.3.2 Sediment core

The sediment core was collected from Sequim Bay on March 16, 2005 using a piston corer in a water depth of 27 m at 48.09°N and 123.03°W (Fig. 1). The 197-cm long core was stored in the dark at 4°C until it was sectioned into 2-cm intervals in July, 2007. The 2-cm interval was necessary to provide enough sediment to count cysts and date sections. Sediment intervals were stored in re-sealable plastic bags in the dark at 4°C until processed further.

2.3.3 ^{210}Pb chronology

Excess ^{210}Pb was measured to determine the depth of the surface mixed layer within the sediment core. Assuming a steady-state of ^{210}Pb flux from the atmosphere to the ocean, the radioactive decay of ^{210}Pb can be used to assess sediment mixing parameters (Nittrouer et al. 1979). Activities of ^{210}Pb were calculated by measuring the alpha decay of its granddaughter, ^{210}Po, in each sediment section. The 2-cm sections of the core were well mixed inside the plastic bags, and 5 g of dry weight sediment from each section was spiked with ^{209}Po as a control. Sediment samples were digested in 10 mL of HNO_3, heated to dryness, digested in 10 mL 6 N HCl, and dried again. Five milliliters of 6 N HCl were added to the samples which were rinsed into a centrifuge tube and brought to 45 mL volume with 0.3 N HCl. The samples were centrifuged for 10 min at 2,000 RPM and the supernatant was poured into glass plating jars. The rinse was repeated two more times to assure complete extraction of all alpha-emitting radionuclides from the sediment samples.

The ^{209}Po and ^{210}Po radionuclides spontaneously deposited onto silver discs suspended in the glass plating jars over a period of 24 hrs. Activities of ^{209}Po and ^{210}Po on the silver discs were measured using a silicon surface barrier detector (Ortec Alpha Spectroscopy Workstation, model

Octete Plus and EG&G Ortec Alpha Spectroscopy Workstation, model Ocetete PC, both with ULTRA series detector model BU-017-450-100). ^{210}Po activity was calculated by the ratio of peak area of ^{210}Po to the control, ^{209}Po.

2.3.4 Cyst counts

Cyst counts were completed on the uppermost 100 cm of the 197-cm long core following the method of Yamaguchi et al. (1995). Briefly, the 2-cm sections of the core were well-mixed inside the plastic bags prior to extracting a 5-cm^3 aliquot. The 5-cm^3 subsample was collected using a metal spatula and measured in a modified graduated test tube. Care was taken to assure that there were no bubbles inside the sediment subsamples. Subsamples were then transferred to a 50-mL test tube and were suspended in 25 mL distilled water, probe sonicated for 60 sec at 40 mHz, and then passed through sequential sieves to obtain the size fraction between 20-90 µm. Material gathered on the 20-µm sieve was washed into a 15-mL centrifuge tube and the volume brought to 9 mL with distilled water. One milliliter of 10% formaldehyde was added to the tube which was then placed in a refrigerator for 1 hr. The solution was centrifuged at 2,000 RPM for 10 min, the supernatant removed, the volume brought to 10 mL using methanol, and the tube placed in a refrigerator. After 2 days, the sample was centrifuged at 2,000 RPM twice, first to remove the methanol and then to wash the sample with 10 mL of distilled water. After the distilled water was removed, 1.5 mL of primulin stain was added for 1 hr. The primulin stain was removed from the sediment sample by centrifugation and w ashing with 10 mL of distilled water. Finally, the sediment sample was resuspended in 10 mL of distilled water.

Cysts were enumerated using a standard microscope (Zeiss, West Germany) equipped with an epifluorescence condenser and a filter set composed of an excitation filter (BP 450-490),

chromatic beam splitter (FT 510) and a barrier filter (LB 520). Two 1-mL subsamples were counted at a 1:3 dilution in a Sedgewick-Rafter counting slide. Cysts were identified following Matsuoka and Fukuyo (2000) and based on their fluorescence, shape, size, cell wall and internal cellular contents. Only those cysts that were positively identified as *A. catenella* were included in counts; partially crushed, totally empty or cysts that were not the characteristic ovoid shape were excluded from the analysis. Cysts of other co-occurring dinoflagellate species and pollen were also counted but the data are not presented in this paper.

2.3.5 PST records

The WDOH has monitored PSTs in multiple shellfish species from Sequim Bay since 1957. The longest record of observation from a single shellfish species is for *Saxidomus giganteus*, the butter clam. However, the butter clam PST data set is limited to the time span 1957-1990 because after 1990 the WDOH focused monitoring efforts on the sentinel species, *Mytilus edulis*, the blue mussel.

Saxidomus giganteus can retain PSTs for up to 6 months even in the absence of toxic *A. catenella* cells in the water column (Bricelj and Shumway 1998). Thus, toxin loads within *S. giganteus* tissues are an integrative indicator of blooms over many months. Monthly maximum toxicity values serve as the best index of blooms because maximum shellfish toxicity should occur with or slightly after peak *A. catenella* cell concentrations. The monthly maximum PST level in *S. giganteus* was averaged over an entire year to produce an annual mean value from 1957-1990. The time series was filtered using a 3-yr centered average to match the temporal resolution of the cyst record from the sediment core.

2.3.6 Environmental parameters and climate indices

A historical record of local SST in Sequim Bay was reconstructed using two independent data sets: observations at the Race Rocks Lighthouse and a geoduck clam proxy from Protection Island (Fig. 1). Monthly SST records from 1921-2005 for Race Rocks Lighthouse in the Strait of Juan de Fuca were obtained from the Department of Fisheries and Oceans, Canada, (http://www.pac.dfo-mpo.gc.ca/SCI/osap/data/SearchTools/Searchlighthouse.htm) and used to approximate SST in Sequim Bay using the regression equation

$$SST_{Sequim} = 2.3 \times SST_{Race\ Rocks} - 11.5$$

where SST_{Sequim} is the SST in Sequim Bay and $SST_{Race\ Rocks}$ is SST at Race Rocks Lighthouse ($R^2 = 0.77$, $p < 0.01$, $N = 36$; Moore et al. 2010).

For years where the Race Rocks Lighthouse data set was incomplete, between 1939-1941 and prior to 1921, a geoduck record was used. The width of the growth rings in the shells of the long-lived geoduck clam can provide annually resolved records of SST, similar to tree rings in dendrochronological climate reconstructions (Noakes and Campbell 1992). SST in Sequim Bay for 1868-1920 was estimated by regressing SST in Sequim Bay (determined from the Race Rocks Lighthouse regression equation above) to the geoduck growth index (Strom et al. 2004) from 1921-2005 ($R^2 = 0.87$, $p < 0.05$, $N = 75$). SST in Sequim Bay from 1868-1920 was then calculated using the regression equation

$$SST_{Sequim} = 2.4 \times GGI + 8.27$$

where SST_{Sequim} is the SST in Sequim Bay and GGI is the geoduck growth index as measured by the width of the shell accretion for the corresponding year.

Because PST levels tend to be highest from July-November, and the geoduck proxy for SST correlates best with March-October SST observations at Race Rocks (Strom et al. 2004), annual averages of SST were limited to the months between March and October. A 3-yr centered average was calculated for the period 1868-2005 by combining the two annual indices of SST for Sequim Bay (Fig. 2).

The Pacific Decadal Oscillation (PDO) is defined as the leading principle component of SST in the North Pacific Ocean poleward of 20^{o}N. In the Pacific Northwest, warm or positive phases of the PDO manifest as warmer than average SST, while cool or negative phases of the PDO display cooler than average SST (Mantua et al. 1997). A monthly index of the PDO from 1900 to present was provided by the University of Washington Joint Institute for the Study of the Atmosphere and Ocean (http://jisao.washington.edu/pdo/). An annual average was calculated using July-June values following Moore et al. (2008) and filtered using a 3-yr centered average (Fig. 2). July through June 12-month averages were chosen because high autocorrelation of the PDO time series rapidly declines from June to July.

Observations of local air temperature at Port Townsend (Fig. 1) were obtained from the United States Historical Climatology Network (http://cdiac.ornl.gov/epubs/ndp/ushcn/newushcn.html) for the time period 1887-2006. An annual average was calculated using March-October values and then filtered using a 3-yr centered average (Fig. 2).

2.3.7 Pearson's correlation

Pearson's correlation was used to assess the relationships between local environmental and large-scale climate parameters with cyst abundances from the Sequim Bay core. Oceanographic time series are invariably autocorrelated meaning that each data point is not independent from the

other. This is an important consideration when calculating the number of relevant degrees of freedom for determining the probability of statistical relationships. To account for autocorrelation within the time series of local environmental and large-scale climate parameters described above, the integral time scale for each data set was calculated by integrating a covariance matrix until the zero endpoint and used to reduce the degrees of freedom within a data set following the method of Emery and Thomson (2001). The adjusted numbers of degrees of freedom are used to determine the significance of correlations with the cyst profile.

2.4 Results

2.4.1 Excess ^{210}Pb Chronology

^{210}Pb radioactive decay was used only to assess the depth of the mixed layer in the Sequim Bay core, which extended down to 19 cm (Fig. 3). The mixed layer was identified by the randomly distributed ^{210}Pb values in the upper portion of the sediments. ^{210}Pb values below the mixed layer were considered to be in the permanent sedimentary record once there was evidence of a linear decay. The sediment chronology began below the mixed layer, with the year 2005 corresponding to a depth of 19 cm (Fig. 3). The presence of a prominent mixed layer indicates that waters above the core site are, in general, oxygenated and able to support biological benthic life.

Dating chronology was assigned using the Cox et al. (2008) Sequim Bay sedimentation rate of 0.6 cm yr^{-1}. That study utilized a subsample from a Soutar box corer collected at the same time and directly adjacent to our piston core. The Soutar corer has been shown to better preserve the near surface sedimentary record for ^{210}Pb analysis. Using this sedimentation rate, every 2-cm

section of the piston core used in this study represented an idealized 3.3-yr integrated value of cyst abundance.

2.4.2 Cyst counts

Cysts were identifiable from the top of the core down to 100 cm, indicating that *A. catenella* has been present in Sequim Bay since at least the late 1800s. Intact cysts were found throughout the core but were less common with depth (Figs. 3, 4). Cyst counts ranged from 0-400 cysts per cm^3, with the highest cyst abundances occurring in more recent years. Cyst counts began to steadily increase beginning in the mid-1950s and remained relatively high in the most recent sediments. To visually amplify variations within the cyst data set, values were plotted on a logarithmic scale (Fig. 5).

2.4.3 PSTs, environmental parameters and climate indices

From 1957-1990, annual average PST levels in the butter clam ranged from 81-947 µg STX equiv/100 g shellfish tissue. To assure temporal congruency amongst data sets and to allow for analysis, the annual PST levels were interpolated using a 3-yr centered average, reducing the range displayed graphically and used in subsequent statistical analysis to 85.3-550.4 µg STX equiv/100 g shellfish tissue (Fig. 2).

Sea surface temperatures and air temperatures displayed similar warming trends since the early 1900s. Annual mean sea surface temperature in Sequim Bay, as calculated from our regression equation, ranged from a low of 8.7°C in 1955 to a high of 13.7°C in 1998; temperatures rose rapidly beginning in 1978, with the mid-1990s to 2005 being the warmest years within our

record (Fig. 2). Annual mean air temperature in Port Townsend ranged from a low of 8.4^{o}C in 1955 to a high of 11.3^{o}C in 2004 (Fig. 2).

Over the past century, PDO events have typically persisted for 20-30 years (Mantua and Hare 2002). The PDO was in cool phase from 1890-1924 and from 1947-1976, and was in warm phase from 1925-1946 and from 1978 to at least 1998 (Figs. 2, 5). Since 1998, fluctuations in the PDO have occurred at a much higher frequency (i.e., cool phase from 1999-2002 and warm phase from 2003-2007).

2.4.4 Pearson's correlation

From the late 1800s to 2005, the cyst record was positively and significantly correlated with Sequim Bay SST ($r = .645$, $p < 0.05$, $N = 39$, Fig. 6) and Port Townsend air temperature ($r = .672$, $p < 0.05$, $N = 33$, Fig. 6). SST in Sequim Bay was most correlated with local air temperature ($r = .793$, $p < 0.05$, $N = 33$, Fig. 6, Moore et al. 2008), and secondarily correlated to shifts in large-scale climate patterns such as the PDO ($r = .629$, $p < 0.05$, $N = 32$, Fig. 6). The cyst record was not significantly correlated with the PDO (Fig. 6).

From 1957-1990, the cyst profile was positively and significantly correlated with PST levels in Sequim Bay butter clams ($r = .762$, $p < 0.05$, $N = 10$, Fig. 6). The butter clam PST record was also positively and significantly correlated with local air temperature ($r = .657$, $p < 0.05$, $N = 10$, Fig. 6) and the PDO ($r = .784$, $p < 0.05$, $N = 10$, Fig. 6). This indicated that high concentrations of PSTs in shellfish correspond with high concentrations of *A. catenella* cysts, warm air temperatures and warm phases of the PDO.

2.5 Discussion

The Sequim Bay sediment core analyzed in this study provides the longest record of *A. catenella* available in the Pacific Northwest from which to assess long-term trends and relationships with the local environment and large-scale climate. Cysts were found down-core to a depth of 100 cm indicating that *A. catenella* has been present in Sequim Bay since at least the late 1800s (Fig. 3). This finding agrees with historical, anecdotal evidence that men in Captain Vancouver's crew fell ill after consuming contaminated shellfish in Central British Columbia while exploring the Pacific Northwest in 1793 (Vancouver 1793).

The cyst record presented in this study spans over 100 yr and allows us to begin to quantitatively assess relationships between *A. catenella* and climate patterns that vary on long time scales, but this required a number of assumptions to be made regarding the ecology and biology of the organism. For example, relationships between the magnitude of a bloom, the toxicity of a bloom, and the number of cysts deposited in the sediment at the termination of a bloom are essentially unknown for most species of *Alexandrium*. In our analysis, we must assume a linear relationship between bloom size and cyst deposition. Future research into these variables will help to illuminate relationships between the motile, free-living stage and the non-motile, benthic cyst stage of *A. catenella*.

It is also important to note that the chronology in this study is a best estimate and there is some amount of error associated with the dating due to variable bioturbation intensities, sedimentation rates, and stochastic physical processes over time. Given the estimated sedimentation rate of 0.6 cm yr^{-1} and a mixed layer depth of 19 cm, it is possible that a single *Alexandrium* cyst could have remained in the mixed layer for roughly 30-yr. We could not account for changes in these

variables and so have instead, necessarily assumed that mixing due to biological and physical processes and sedimentation rates have been constant over time. As such, the temporal resolution of the cyst record presented from Sequim Bay is best suited to detect low frequency signals such as shifts associated with large-scale patterns of climate variability that have a frequency of greater than 30 years. Higher frequency disturbances that occur on a seasonal to inter-annual timescale, such as ENSO, cannot be captured by our cyst record. But, as a result, our cyst record is less likely to capture anomalous blooms and high frequency trends and more likely to reflect general trends over longer periods of time and is able to assess relationships with the PDO and long-term trends in SST and air temperature.

The cyst record, spanning from the late 1800s to 2005, provides the first, long-term and quantitative evidence that *A. catenella* populations are responding directly to regional warming trends that have occurred over the past century. Based on our cyst record, a rapid increase in cyst abundance began in the late 1950s in Sequim Bay; this trend best matches long-term increases in local SST and air temperature (Figs. 5, 6). Nishitani and Chew (1984) proposed that *A. catenella* preferentially blooms during periods when SST is above 13^{o}C; our study supports the hypothesis that temperature is a primary driver of *A. catenella* populations in Sequim Bay.

In 1978, a rapid expansion of PST detections throughout Puget Sound was thought to be related to a shift in the PDO from cool to warm phase (Ebbesmeyer et al. 1995, Trainer et al. 2003, Moore et al. 2010). PST levels in Sequim Bay butter clams from 1957-1990 are significantly correlated to fluctuations in the PDO (Fig. 6, Ebbesmeyer et al. 1995, Moore et al. 2010); however, the cyst record from 1900-2005 is not significantly correlated with the PDO (Fig. 6). Even when data from the 1970s to 2005 are excluded from analysis, thereby eliminating the possibility that the large jump in cyst abundances in the late 1970s masked the detection of any

other possible trends, there is still no significant correlation between the cyst record and PDO. The discrepancy between the WDOH PST record and the cyst record in relation to the PDO highlights the need to have long-term datasets that span more than one shift in a large-scale climate pattern to statistically assess any possible influences. These results also indicate that local environmental variability such as regional SST and air temperature may be more important in controlling *A. catenella* blooms compared to larger-scale climate variations such as PDO.

A. catenella cysts were positively and significantly correlated with the PST record in shellfish from Sequim Bay (Fig. 6). This lends support to the argument that the cyst record provides a good proxy for blooms and that *A. catenella* bloom magnitude and frequency has increased over time (Cox et al 2008). However, it could also be argued that this correlation is an artifact of the monotonic increase in cyst concentrations resulting from the natural degradation of cysts over time. A previous study estimated an *Alexandrium* spp. cyst half-life of roughly 5 yr based upon cyst counts down a 12 cm sediment core (Keafer et al. 1992). In contrast, our study would suggest that *A. catenella* cysts can persist in the environment for multiple decades. Fully intact cysts with internal cellular contents were found throughout the top 100 cm of the sediment core extracted from Sequim Bay and cysts remained identifiable throughout the depth of the core analyzed in this study and in one other study from Sequim Bay (Fig. 4, Cox et al. 2008). Further, *Alexandrium* spp. cysts have been identified at 60 cm depth in a sediment core collected from Kure Bay in the Seto Inland Sea, Japan (Mizushima and Matsuoka 2004) and at depths of 7.5 m from an 11 m long sediment core collected from Smygen Bay, Sweden (Yu and Bergland 2007).

However, we cannot rule out the possibility that some *Alexandrium* cysts may have naturally degraded or were ingested by benthic organisms over the time period covered by our core. Various studies have assessed the percent viability of cysts of *Alexandrium* spp. after passing

through the guts of benthic organisms with estimates ranging from 9% (Tsujino and Uchida 2004) to 50-90% survival (Laabir and Gentien 1999). Thus, we interpret the results of our study cautiously and recognize the need for further research in this field. Our analysis is predicated upon the ideal that *A. catenella* cysts may degrade through time but have a half-life on the order of decades to centuries rather than a few years. More research needs to be done to refine and assess the degradation rates and long-term viability of *Alexandrium* spp. cysts in different benthic environments.

Our cyst record indicates that *A. catenella* has been present in Sequim Bay since at least the late 1800s. Cyst abundances ranged from 0-400 cysts per cm^3 with the highest values occurring in more recent years. Of the climate and environmental parameters examined here, cyst abundances were most strongly correlated with local air temperature and sea surface temperature. Since the 1950s, there has been an average global warming of 0.31^oC in the upper 300 m of the oceans (Levitus et al. 2000). At Race Rocks Lighthouse, the long-term warming trend is 0.9°C since 1921 and 1.0°C since 1950 (Snover et al. 2005) – more than three times the global average. This warming trend is likely to continue into the future (Mote and Salathe 2010). Given the relationship reported here and elsewhere (Norris and Chew 1975, Nishitani and Chew 1984, Moore et al. 2009) between *A. catenella* populations and SST, a warmer future could increase the frequency, duration, and magnitude of *A. catenella* blooms and possibly other HAB species in Puget Sound (Moore et al. in press). Reconstructing historical HAB blooms using cyst records to help understand past changes in abundances may help to foretell the future of HABs in a changing climate.

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Figure 2.1 Map of study site (Sequim Bay) and other local environmental information sources: SST – Race Rocks and Protection Island; Air temperature – Port Townsend.

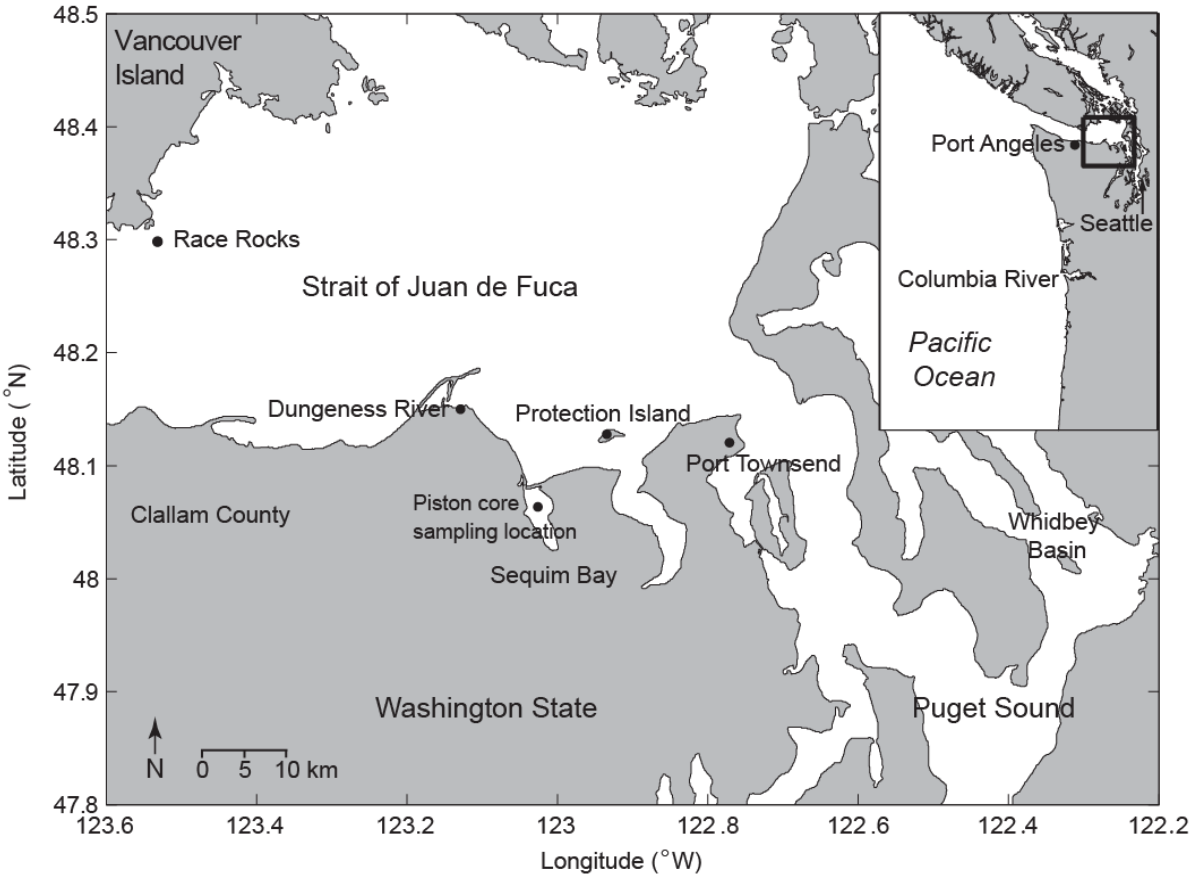

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Figure 2.2 Environmental data sets compiled for comparison with the cyst record. Dots indicate the annual average value of the variable and the line indicates a three-year centered average. The geoduck record is indicated by the x's on the SST graph.

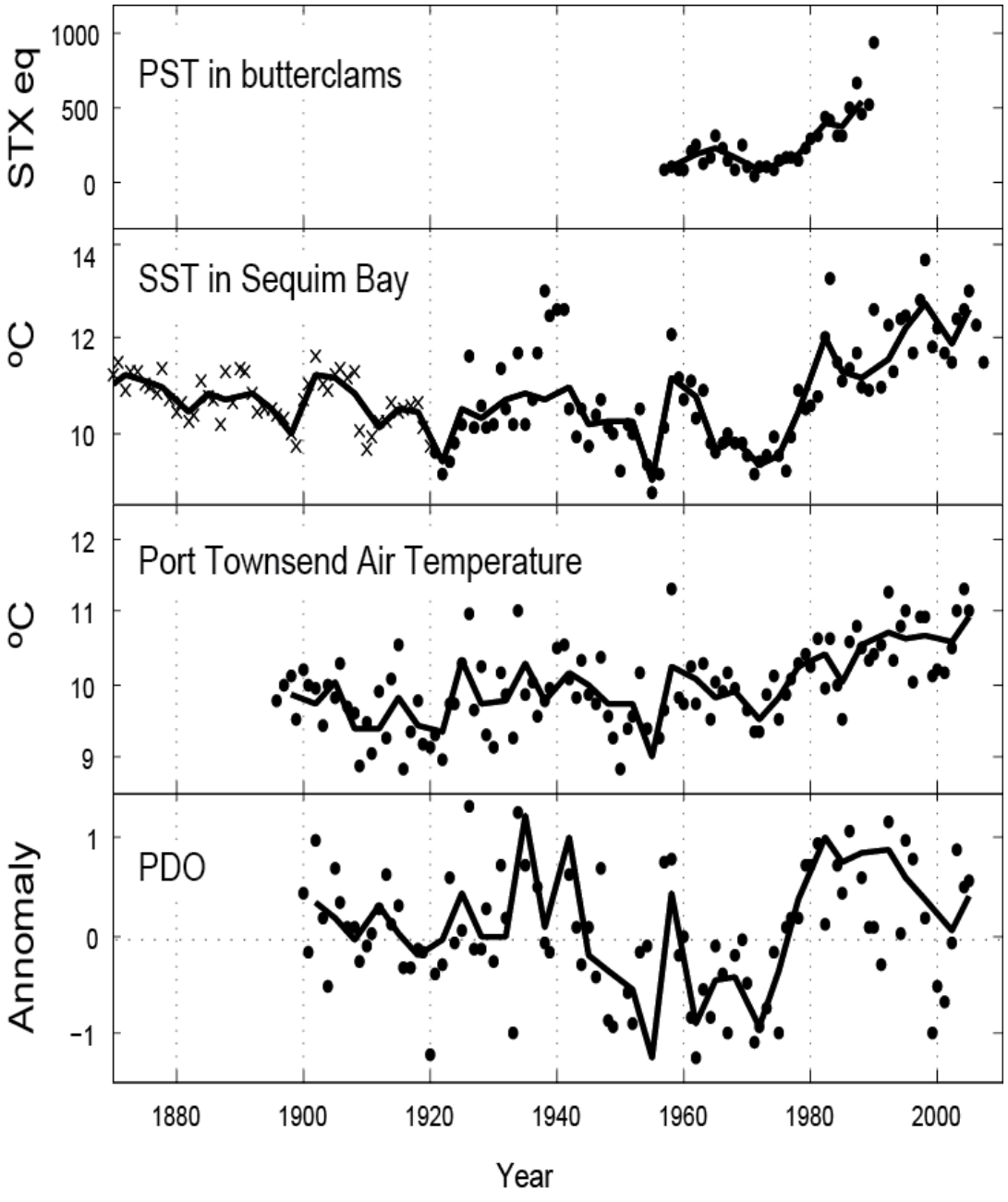


Figure 2.3 ^{210}Pb profile (open circles) and raw cyst counts (stars) of sediment core. The mixed layer is indicated by the non-linear profile of ^{210}Pb in the upper 19 cm. The roughly linear decay below 19 cm indicates the beginning of the permanent record and is where dating chronology begins.

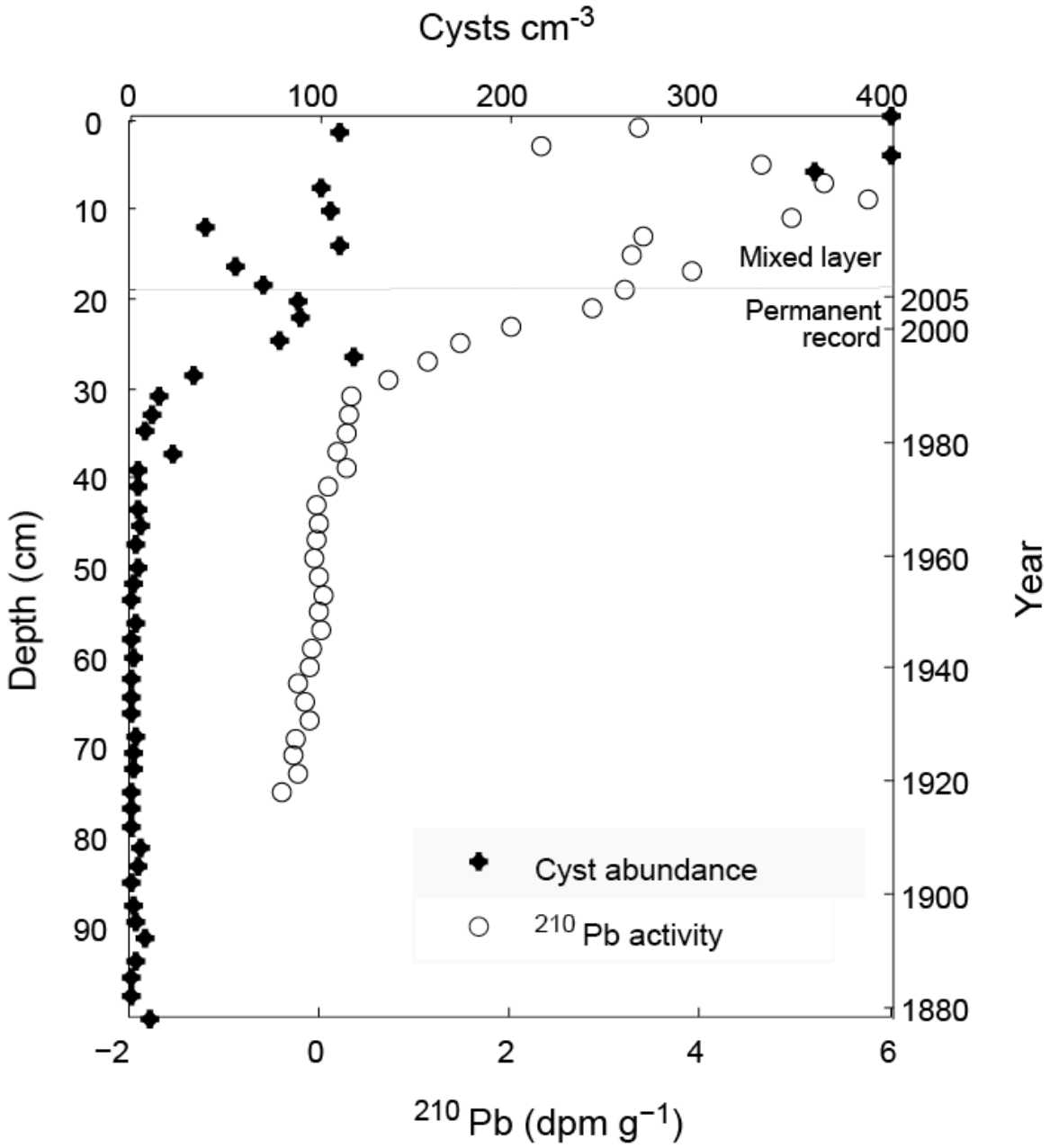


Figure 2.4 Images of *A. catenella* cysts taken from the top (5 cm), middle (33 cm), and bottom (91 cm) of the sediment core at 160x magnification. The left image is captured under transmitted light, the right image under epifluorescence microscopy.

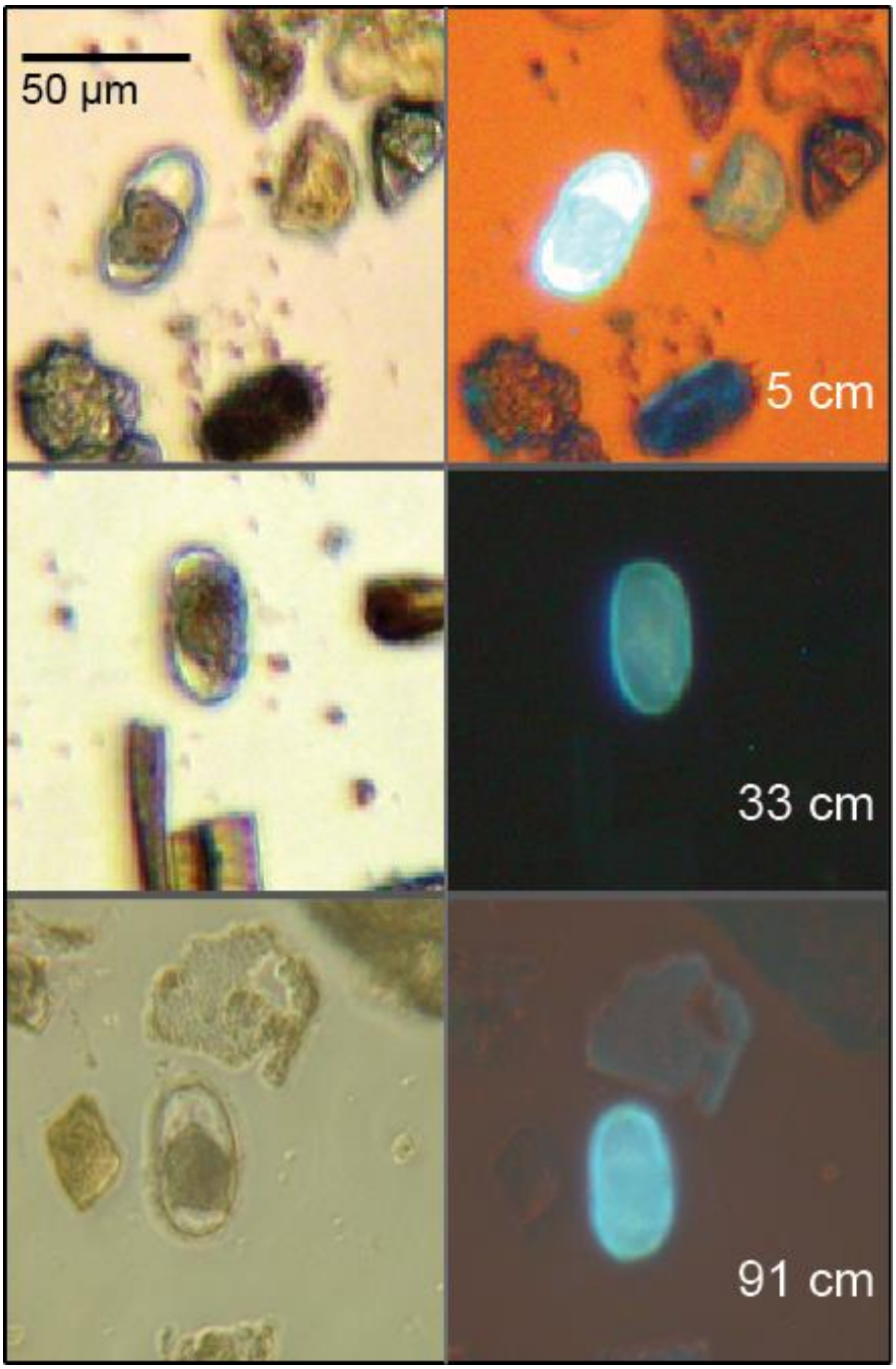


Figure 2.5 Log plot of cyst record (stars) compared to sea surface temperature (SST) (open circles) and the PDO (bar graph).

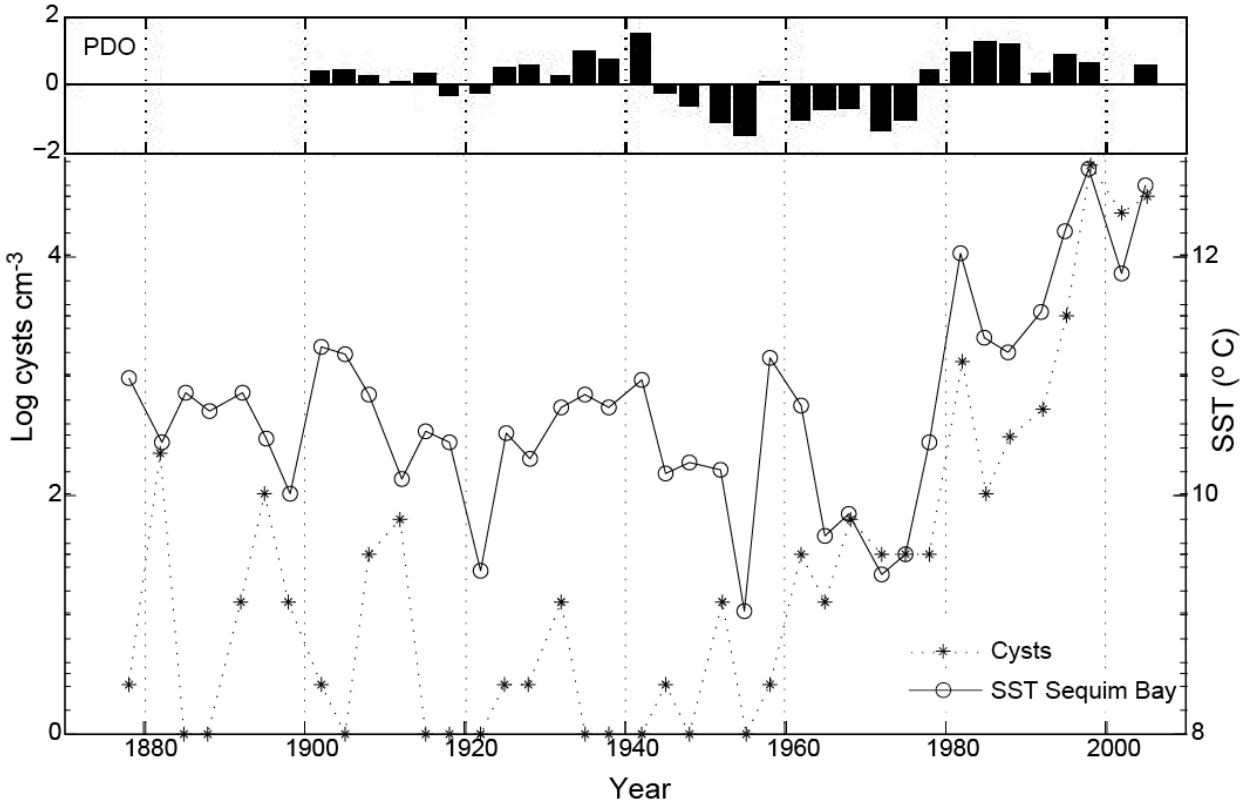
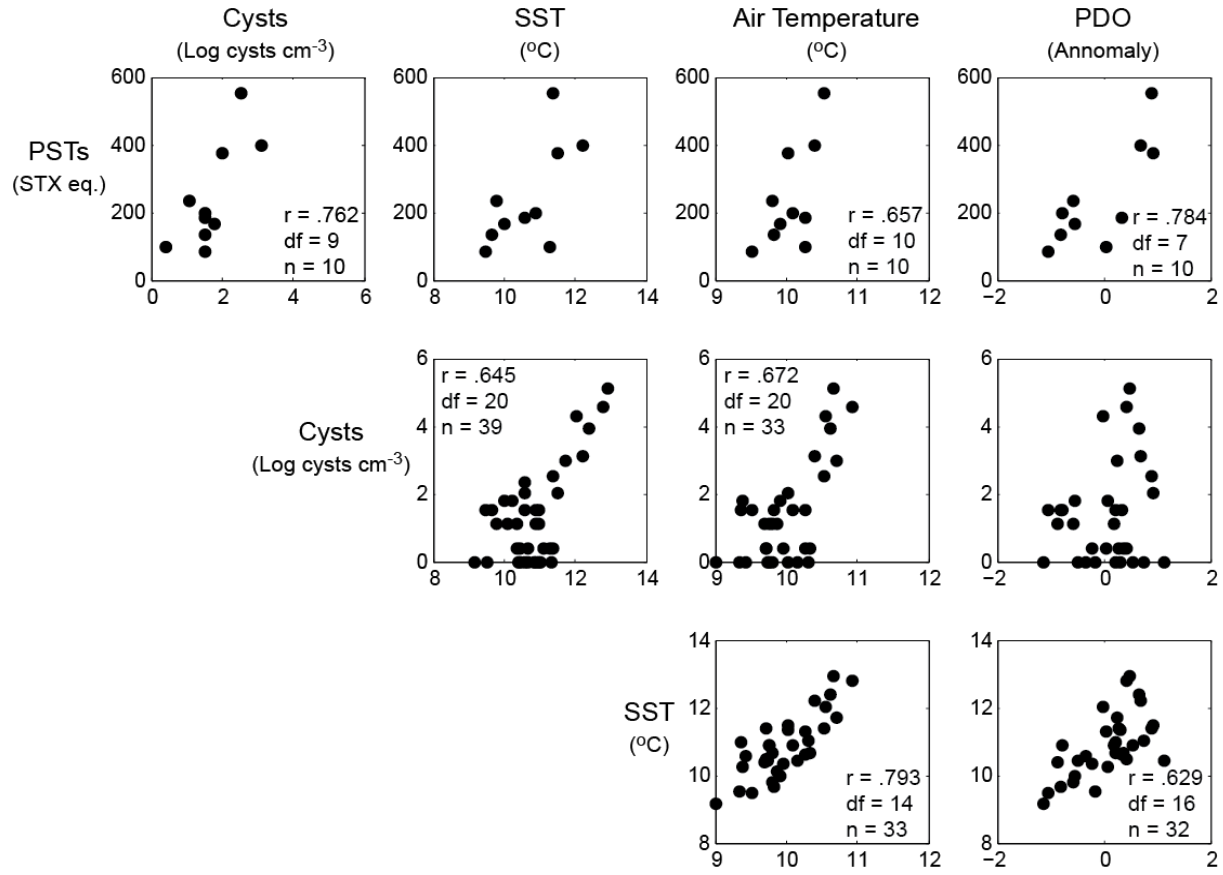


Figure 2.6 Pearson's correlation plots of cyst record and environmental variables. All r-values displayed are significant at the $p < 0.05$ level. If no r-value is displayed, there is no significant relationship.



3 The cytoplasmic fullness of Alexandrium and Scrippsiella cysts is not a primary indicator of cyst age or viability

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3.1 Abstract

Many marine protists produce a benthic resting stage during their life history. This non-motile cyst stage can either germinate near the sediment surface and provide the inoculum for subsequent blooms or be buried by sediment deposits over time and entrained into the sedimentary record. Buried cysts can be re-suspended by deep mixing events (wind and storms) or other disturbance events (e.g., dredging); however, it is not clear how long the living cysts can survive while buried in the sediments and if they are capable of germinating. Here we report on the germination success of the potentially toxic dinoflagellate *Alexandrium* and the non-toxic dinoflagellate *Scrippsiella* from a 60-cm sediment core collected in Sequim Bay, WA, in December 2011. Cysts of *Alexandrium* and *Scrippsiella* were isolated from 2-cm sections of the core, imaged, placed in individual wells of a 96-well plate with growth medium, incubated at favorable conditions, and monitored for germination. An image analysis program, DinoCyst, was used to quantitatively measure the cytoplasmic fullness of the cysts to test the hypothesis that older cysts located deeper in the sediment core will have a smaller amount of granular cytoplasm and will be less likely to germinate. An index of cytoplasmic fullness and age, based on ^{210}Pb

dating of surrounding sediments, were compared with germination success or failure. Our research indicates that cysts of *Alexandrium* and *Scrippsiella* can remain viable in sediments for an estimated 50-80 years, show little visual evidence of cytoplasmic deterioration over this timescale (as measured by cytoplasmic fullness), and that germination success is relatively constant for cysts at depths in the sediment ranging from 0-60 cm. These results suggest that a cyst's cytoplasmic fullness does not indicate their germination potential and that cysts located up to 60 cm deep in the sediments are as likely to germinate as surface cysts under favorable growth conditions.

3.2 Introduction

Alexandrium is a common, cyst-forming dinoflagellate found in temperate coastal waters that can produce a suite of toxins known to cause paralytic shellfish poisoning (PSP). Its presence can cause shellfish harvest closures resulting in economic losses to the aquaculture industry and coastal communities (Hoagland et al., 2002). Because of the human health and economic impacts of *Alexandrium* blooms, there is broad interest in developing tools to predict bloom occurrence (e.g. McGillicuddy et al., 2011).

The cyst stage of *Alexandrium* has an important role in seeding annual blooms; it serves as a quiescent stage for the dinoflagellate to overwinter and can germinate when favorable conditions arise, releasing a motile vegetative cell able to divide and form toxic blooms. However, not all of the cysts germinate; some are buried by sediment deposits and are entrained into the sedimentary record. It is not clear how long the living cysts can survive buried in the sediments or if they deteriorate over time. Understanding *in situ Alexandrium* cyst survival in marine sediments and long-term viability of cysts will help to elucidate the potential for older cysts that are re-

suspended into the surface layer via storms, dredging, bioturbation, or other disturbances to the seafloor to germinate and initiate toxic blooms (Butman et al., 2014). However, it is difficult to quantitatively assess *in situ* cyst survival because sediment cores extracted from oxygenated waters are usually vertically mixed by benthic organisms and other processes making it difficult to develop an exact chronology. While it is generally unknown how the long-term viability of *Alexandrium* cysts buried in the sediments relates to cyst age, some dinoflagellates may survive for decades and even centuries (Lewis et al., 1999; Lundholm et al., 2011). Keafer et al. (1992) suggest a half-life of 2-10 years for buried *A. tamarense* cysts in shallow salt pond in Massachusetts; however, Miyazono et al. (2012) recently germinated an estimated 100-year old *A. tamarense* cyst from Funka Bay, Hokkaido, Japan.

The cysts of *Alexandrium*[2] are readily identified by their uniform size and shape but can show large variability in the amount of visible material in the cytoplasm. Cellular content ranges from almost entirely full of what are presumed to be carbohydrate and lipid reserves to almost empty with only a small bundle of golden brown-colored bodies. Few studies have assessed the viability of *Alexandrium* cysts relative to their internal cell contents but it is generally assumed that emptier cysts are less viable (e.g. Genovesi et al., 2009). Most photosynthetic protist resting stages are thought to lose their capacity to photosynthesize, dramatically reduce their respiration rates and rely on internal carbohydrate and lipid stores in the cytoplasm for survival (Binder and Anderson, 1990). In a study using laboratory-produced *Alexandrium* cysts, Genovesi et al. (2009) suggested that the internal cellular contents of *A. tamarense* cysts systematically evolved

[2] The *Alexandrium* found in western Washington waters historically has been called *A. catenella* but because of the current taxonomic questions in the *A. tamarense/catenella/fundyense* complex, we have chosen to use the genus *Alexandrium* for classification.

from a newly formed resting cyst full of cytoplasmic contents to a final stage cyst that exhibited degradation of cytoplasmic content as the cysts aged over one year. In contrast, Binet and Stauber (2006) found that the viability of laboratory-produced *A. catenella* cysts could not be predicted based upon cyst size, natural fluorescence, or cytoplasmic fullness; no correlation between a cyst's physical appearance and germination success was found. To date, we are not aware of any study that examines the cytoplasmic fullness of naturally-produced cysts of *Alexandrium* in relation to cyst viability or age.

In general, the preservation potential, survival and/or degradation rate of protist cysts are not well understood (Lundholm et al., 2011). A variety of methods have been used in an attempt to better constrain the lifetime of cysts in the natural environment. Previous studies have estimated cyst degradation and survival rates based on mathematical models (Keafer et al., 1992; Shull et al., 2014), physical examination of a cyst's structural integrity (Head et al., 2006), available energy reserves and respiration rates (Binder and Anderson, 1990) and long-term viability in stored and natural sediments (Lewis et al., 1999; Ribeiro et al., 2011). Resting cysts have survived and germinated from sediments that are decades to >100 years old (Lundholm et al., 2011; Miyazono et al., 2012).

In this study, we assessed the long-term viability of dinoflagellate cysts in natural sediments and looked for evidence of systematic deterioration over an estimated 50-80 year period. We set out to test the hypotheses that cysts buried deeper in natural sediments and hence, older relative to surface cysts, will have less cytoplasmic fullness and will be less likely to germinate compared to cysts isolated from the sediment surface. We did this by isolating cysts of *Alexandrium* and the

non-toxic dinoflagellate *Scrippsiella*[3] from a 60-cm long sediment core collected in Sequim Bay, WA, in December 2011. A total of 793 cysts were imaged and placed in individual wells of 96-well plates with growth medium and monitored for signs of germination over three weeks. Image analysis was used to quantitatively measure the surface area of the cysts' cytoplasm. An index of cytoplasmic fullness and age was compared with germination success or failure. This research will help to better understand the survival and long-term viability of *Alexandrium* and *Scrippsiella* resting cysts in natural sediments.

3.3 Methods

3.3.1 Sediments

The sediment core was extracted from Sequim Bay, WA (48.09 ^{o}N, 123.03 ^{o}W) in a water depth of 27 m in December, 2011 using a kasten corer with a 10.2 cm diameter. The core was sectioned into 2-cm intervals and the outer perimeter of sediment was removed as the section of sediments was extracted from the corer on a metal plate. Each interval was transferred to a plastic bag, sealed with no air spaces, and placed in the dark at 2 ^{o}C for 5 months. Core sections were processed to a maximum depth of 60 cm.

Each core section was processed following methods modified from Feifel et al. (2012). Sediments were dated by measuring excess ^{210}Pb (Ortec Alpha Spectroscopy Workstation, model Octete Plus and EG&G Ortec Alpha Spectroscopy Workstation, model Ocetete PC, both with ULTRA series detector model BU-017-450-100), a naturally produced radioactive granddaughter from ^{222}Rn. To obtain the dinoflagellate cysts, a 5 cm^3 subsample was diluted into 30 mL of 2 ^{o}C

[3] The most common *Scrippsiella* found in the waters of coastal western Washington is *S. trochoidea*.

filtered sea water (FSW), sonicated at 40-50 mHz for one minute while sitting in an ice bath and then sequentially sieved through a 20/90 µm Nitex screen filter set and rinsed using 2 ^{o}C FSW. The sediment captured on the 20 µm filter was then concentrated, washed back into a 50 mL centrifuge tube and the volume was raised to 30 mL using FSW.

The 30 mL sediment slurry was vortexed for one minute and one 7 mL subsample was taken and transferred into a sterile 15 mL centrifuge tube. The sample was treated with a modified density centrifugation method using a 1.3 g cm^{-3} sodium polytungstate (SPT) solution following Bolch (1997) to concentrate and separate cysts from their surrounding sediments. This treatment is not meant to be a quantitative process thus we did not account for any cysts that were not captured at the density-gradient interface. Three mL of the SPT solution were carefully layered underneath the 7 mL sediment slurry for a total volume of 10 mL; the sample was centrifuged (International Equipment Co., Needham Hts., MA, USA, International Clinical Centrifuge Model 42283M-6) for 10 minutes at 2,000 rpm (relative centrifugal force of 313 g). Three layers were present after centrifugation: a clear top layer, a cloudy thin layer at the 3 mL mark, and a sediment pellet at the bottom of the centrifuge tube. Cysts that were still adhered to heavier sediment particles after sonication may have passed through the density gradient. The particles caught at the SPT density interface were pipetted into a clean 15 mL test tube and the volume was increased to 3 mL using FSW. The cyst concentrate was stored in the dark at 2 ^{o}C prior to cyst isolation.

3.3.2 Cyst isolation and imaging

Within 24 hours, *Alexandrium* and *Scrippsiella* cysts were isolated from the concentrate. The 3 mL sample was vortexed and 1 mL was pipetted into a Sedgewick-Rafter counting slide (Hausser Scientific, Blue Bell, PA, USA). Individual cysts were isolated using a mouth pipetted

drawn-out capillary tube under a standard microscope (Zeiss, West Germany, 47 30 28) into wells of a 96-well plate filled with 100 µL of f/2 –Si media. The well plate was kept on ice during the isolation process. Isolated cysts were imaged on an inverted microscope (Zeiss, West Germany, Axiovert 35) using a digital SLR camera (Canon, Melville, NY, USA, EOS Rebel T3iDSLR) fitted with a mm-SLR adaptor (Martin Microscope, Easley, SC, USA, mm-SLR 2.5x Universal DSLR to Microscope T-mount Adaptor). The approximate location of each cyst in each well was recorded to ease future monitoring; any well with more than one cyst was excluded from the experiment.

The well plate edges were taped to minimize evaporation and plates were incubated at 13 ^{o}C on a 12 dark:12 light (20 µE m^{-2} s^{-1}) photoperiod. The well plates were visually examined for cyst germination on days 2, 7, 14 and 21 following Lundholm et al. (2010). If the cyst remained intact, no excystment (N) was recorded. If there was a live cell swimming that had divided at least once in the well plate, successful germination (Y) was recorded. If the cyst was empty but no live germling was ever seen swimming in the well plate over the three week monitoring period, empty cyst but no live cell (ECNC) was recorded (Figure 1).

3.3.3 Image analysis

Cyst images were analyzed using a custom-designed image analysis program called DinoCyst (Figure 2). DinoCyst is a plug-in which utilizes the freeware ImageJ software (Schneider et al., 2012) to determine the dimensions and area of the cyst and the area of intracellular contents using a photo image. For each photograph of a cyst, the cyst is computer-located by thresholding the image to identify and outline the cyst (Figure 2b). The threshold value is found by calculating the maximum entropy of a histogram of image values. DinoCyst masks the cyst in the photo and

all further analysis is based upon the masked image. The length and width of the cyst is found by calculating the longest distance between two edge pixels of the cyst and setting this as length and then setting the longest distance between two edge pixels perpendicular to the length as width (Figure 2d). The intracellular content area is calculated by thresholding the inside of the cyst (Figure 2c). The cell wall is then subtracted from the intracellular contents area. The units are converted from pixels and pixels squared to micrometers and micrometers squared by applying a conversion factor that is calculated by measuring the length in pixels of a micrometer ruler under the same magnification as the photo images.

To ensure that DinoCyst was accurately identifying and masking the cysts, we developed a method to estimate image analysis error. Less accuracy might result from blurry images or if cysts have sediment and other particles attached to their mucilaginous outer cell layer (see Figures 1, 2). To estimate DinoCyst's image analysis accuracy, a polygon was created based on the cyst mask by drawing straight lines around the outermost pixels (Figure 2b). The resulting polygon was then compared to the cyst mask image (Figure 2e). If there was perfect overlap between the two polygons, the confidence that DinoCyst is accurately identifying the cyst would be 100%. The percent difference between the confidence polygon (Figure 2e) and the cyst mask (Figure 2b) allowed us to estimate our confidence that the picture was of high-enough quality to be included in our analysis. Images were kept for further analysis if image confidence was estimated to be above 85% for *Alexandrium* and 90% for *Scrippsiella* (see Figure 2).

3.4 Results

3.4.1 Photo image analysis

All of the isolated cysts were immediately imaged for further analysis. Of the 285 *Alexandrium* and 508 *Scrippsiella* cyst isolations and corresponding images, 152 and 361 images respectively were of high-enough quality (i.e. not blurry, no attached particles to mucilaginous layer) to be accurately processed using DinoCyst. The mean (± standard deviation [SD]) dimensions of the imaged cysts of *Alexandrium* and *Scrippsiella* were 44.1 ±0.7 × 27.6 ± 0.5 µm and 32.5 ± 0.6 × 22.6 ± 0.4 µm respectively. The mean (± SD) cytoplasmic fullness of *Alexandrium* cysts that successfully germinated was 72.1 ± 7.4%, with a range of 58.3-82.8%. *Alexandrium* cysts that excysted but did not produce a live germling had a mean cytoplasmic fullness of 71.9 ± 9.4%, and cysts that did not germinate had a mean (± SD) fullness of 72.7 ± 10% (Figure 3). There was no statistically significant difference between the cytoplasmic fullness of cysts that germinated, excysted but had no live cell and did not germinate as determined by one-way ANOVA ($F(2, 149) = .080$, $p = .923$). There was no significant trend in *Alexandrium* cyst cytoplasmic fullness with depth ($r^2 = 0.005$, $p = .393$, $N = 152$, Figure 4).

Scrippsiella cysts that germinated throughout the core had a mean (± SD) cytoplasmic fullness of 74.7 ± 11.0%, with a range of 46.7-92.6%. Cysts that excysted but did not produce a live cell had a mean (± SD) fullness of 73.9 ±- 11.0%, with a range of 38.2-90.0%. Cysts that did not germinate had a mean fullness of 75.3 ± 10.0%, ranging from 49.0-95.0% (Figure 3). There was no statistically significant difference between the cytoplasmic fullness of *Scrippsiella* cysts that germinated, excysted but had no live cell and no germination as determined by one-way ANOVA ($F(2, 358) = .632$, $p = .532$). There was a weak, but statistically significant negative

trend with *Scrippsiella* cytoplasmic fullness and depth (r^2 = .023, p = .004, N = 361, Figure 4); cysts are 0.001% less full for every 1 cm down core. However, this trend should be interpreted cautiously because it is within the SD of estimated cytoplasmic fullness within the same depth.

3.4.2 Chronology

Lead-210 analysis indicated that the mixed layer extended down to 21 cm (Figure 5). The mixed layer is identified by the randomly distributed ^{210}Pb values in the upper portion of the sediments. The linear decay of the ^{210}Pb values below the mixed layer are considered to be indicative of the permanent sedimentary record and are no longer disturbed by bioturbation. The best-fit line to the linear decay of ^{210}Pb (r^2 = 0.73) was used to estimate the sediment accumulation rate of 0.69 cm y^{-1}. Our accumulation rate agrees with published sedimentation rates in Sequim Bay (Cox et al., 2008). Using this sedimentation rate, every 2-cm interval from the core represents roughly 2.89 ± 15 years due to the effects of bioturbation in the mixed layer. The residence time of any particle in the mixed layer was estimated to be 31.1 years.

Cyst age was estimated based on their depth in the core by binning sediment samples into 10 cm intervals below the mixed layer following results from the ^{210}Pb analysis. Cysts isolated from the upper 21 cm, the mixed layer, are assumed to be between 0-30 years old. Below the mixed layer, cyst age can be estimated if we assume that sedimentation rates have remained constant over the timescale of this study. For example, a cyst isolated from depths of 21-30 cm would be estimated to be between 0-45 years old because it would take just under an estimated 45 years to accumulate 30 cm of sediment assuming a constant sedimentation rate (see Figure 5).

3.4.3 Germination and seasonality

A total of 285 *Alexandrium* and 508 *Scrippsiella* cysts were isolated and observed for germination from May, 2012 to January, 2013. For the germination analysis, successful germination was considered to occur if a live cell(s) was observed in the wells or if an opening in the cyst was observed but a long-lived cell could not be found (i.e. ECNC+Y, Binder and Anderson, 1987; Vahtera et al., 2014). Germination success of *Alexandrium* cysts in each 2-cm sediment interval varied from 0-60% with a mean of 22.6%; *Scrippsiella* germination success varied from 9-88% with a mean of 55.8%. Of the 71 *Alexandrium* cysts that germinated, 12-41% produced a germling cell that successfully divided in the wells. Zero to 55% of the 277 *Scrippsiella* cysts that germinated successfully produced viable germling cells (Figure 6). *Alexandrium* and *Scrippsiella* cysts successfully germinated and produced live germling cells when isolated from depths up to 55 and 51 cm respectively (data not shown). Based upon our sediment accumulation rate, these cysts' ages are estimated to be 64 and 58 years old (± 15 years) and were most likely entrained into the sediment sometime in the 1950s and 1960s.

There was a statistically significant difference in germination success of different cyst age groups for both *Alexandrium* ($F(4, 280) = 4.343$, $p = .002$) and *Scrippsiella* ($F(4, 207) = 8.018$, $p = .000$). Post-hoc Games-Howell tests revealed that *Alexandrium* cysts estimated to be 30-75 years old exhibited higher germination success, as determined by the proportion of isolated cysts that germinated, compared to cysts estimated to be 0-30 years old (41% compared to 14% respectively). Similarly, *Scrippsiella* cysts estimated to be 15-60 and 30-75 years old exhibited higher germination success, 68% and 73% respectively, compared to cysts estimated to be 0-30 years old, 44%.

The germination experiments for both *Alexandrium* and *Scrippsiella* cysts from the age groups of 0-30 and 0-45 years occurred over two seasons; spring/summer (May-August) and winter (November-January, Figure 6). We used logistic regression to assess whether the decreased germination rates observed for the 0-30 year age classes could have been an artifact of the time of year when cysts were isolated. Including the season that the cyst was isolated and cyst age as predictors of germination increased the regression model's accuracy for *Alexandrium* by 10% (χ^2 = 20.02, p = .001, df = 5) and for *Scrippsiella* by 8% (χ^2 = 31.165, p < .001, df = 5) based upon Nagelkerke's R^2 (Nagelkerke, 1991). Using the Wald criterion, *Alexandrium* germination success was significantly influenced by the season it was isolated (p = .05) but not cyst age (p = .159); in contrast, *Scrippsiella* germination success was significantly influenced by the estimated cyst age (p = .001) but not season (p = .806). Exp(B) values indicated that an *Alexandrium* cyst isolated from the core and incubated at favorable conditions during the winter season was 33% less likely to germinate than a cyst that was isolated in the spring/summer season. *Scrippsiella* cysts that were estimated to be 15-60 or 30-75 years old were respectively 80% and 84% more likely to germinate than other age classes (Figure 6).

3.5 Discussion

In this study, *Alexandrium* cysts isolated from a sediment core, estimated to be between <1-80 years old, did not show any evidence of deterioration in cytoplasmic fullness or germination success with depth/age (Figure 4). In fact, *Alexandrium* cysts that were isolated from sediments 40-50 cm deep (aged 30-75 years) exhibited statistically significant higher germination success than cysts isolated from sediments in the 0-21 cm sediment layer (aged 0-30 years, Figure 6).

We are aware of only two other studies that have studied germination success or other measures of viability in cysts from different depth layers of a sediment core. In Funka Bay, Japan, Miyazono et al. (2012) reported that *Alexandrium* germination success decreased linearly with depth; however, the small number (7-20) of cysts examined for each depth interval could have introduced large statistical errors (Feifel et al., in prep) that were not fully taken in to consideration. Gracia et al (2013) report reduced viability (6%) in *A. tamarense* cysts located 10 cm below the sediment surface and interpreted this as a sign of aging. The results of Gracia et al. (2013) are counter to the results in this study wherein the highest percent of *Alexandrium* germination was from cysts isolated from depths between 40 and 50 cm; although, our finding could be complicated by seasonality (see discussion below).

Binder and Anderson (1990) measured respiration rates and compared them to carbohydrate energy reserves and estimated that *S. trochoidea* cysts stored at 3 ^{o}C would deplete their energy reserves within 240 days. The authors recognized that this was an underestimate of cyst survival because a previous study observed *S. trochoidea* cyst germination after 350 days (Binder and Anderson, 1987). They hypothesized that either respiration rates decrease with cyst age or that other lipid-based energy reserves became important over time and could be generated via internal anabolism. Our study was not designed to test these hypotheses but results do suggest that *Scrippsiella* cysts can survive in sediments for over 50 years, exceeding conventional expectations based upon thermodynamics alone.

It has been assumed that as cysts age, their internal cell content, an indicator of energy stores, would shrink due to respiration. Thus, a cyst with fewer internal contents in its cytoplasm would be expected to be older and have less energy available to successfully develop a vegetative, swimming cell given favorable conditions for germination. The results from our study suggest

that naturally produced *Alexandrium* and *Scrippsiella* cysts that have a wide range of estimated cytoplasmic fullness, from 42-84% and 38-93% respectively, can successfully germinate (Figure 3). Further, we do not find a statistically significant relationship between a naturally produced cyst's cytoplasmic fullness and its ability to germinate, in agreement with results from laboratory produced cysts studied by Binet and Stauber (2006).

Taken together, these results argue that a naturally produced cyst's cytoplasmic fullness is not a primary indicator of its ability to germinate or age over the timescales examined in this study. Presumably there is some lower threshold where a cyst does not have enough stored energy in lipids and carbohydrates to create a long-lived, dividing germling cell. The relatively small sample size of *Alexandrium* cysts (N=11) that germinated, produced a dividing cell and were suitable for image analysis precludes our ability to discern a lower threshold. For *Scrippsiella* (N=73), data from this study would suggest that a cyst needs to have a cytoplasmic fullness of 46.7% in order to produce a viable, dividing germling (Figure 3).

The mean germination success of *Alexandrium* (22.6%) and *Scrippsiella* (55.8%) cysts throughout the core are comparable to other estimates of viability from naturally produced cysts in estuarine sediments. Gracia et al. (2013) used the live/dead stain SYTOX green to determine that on average, 28% of the *A. tamarense* resting cysts in the St. Lawrence estuary were viable although there was a high degree of site heterogeneity. Kremp and Anderson (2000) estimated *Scrippsiella hangoei* cyst viability to be 58% based upon replicate germination experiments and counting the percent of empty and full cysts present after 2 weeks.

Only a small proportion, 29% and 37% respectively, of the *Alexandrium* and *Scrippsiella* cysts that successfully germinated produced long-lived progeny able to undergo meiotic division.

Previous studies have estimated post-meiotic viability ranging from 50-90% for laboratory-produced *Alexandrium catenella* cysts (Figueroa et al., 2005), 27.4% for naturally-produced, surface sediment estuarine *A. tamarense* cysts (Genovesi et al., 2009), and 47% of naturally-produced *A. fundyense* cysts isolated from sediments located in water 120 m deep (Vahtera et al., 2014). The low rates of germling survival from environmental samples have been suggested to be evidence of a mixed-age assemblage of cysts, inferring that older cysts are less viable because they have fewer energy reserves available to support the metabolic demands of germination (Genovesi et al., 2009; Vahtera et al., 2014). Figueroa et al. (2005) postulated that different compatible parental strains had a significant effect on post-meiotic viability and found that specific genetic crosses exhibited higher percent germination rates relative to others. In our study, germling survival was highest from cysts isolated from the mixed layer in May (age 0-30 years) and from depths of 40-50 cm in July and August (age 30-75 years, Figure 6). The cytoplasmic fullness of cysts from these depths is similar based upon the image analysis results (Figure 4). Provided the image analysis is a relative indicator of the energy reserves available for the germling, we can conclude that the loss of energy reserves as a cyst ages cannot fully account for the low post-meiotic survival. This lends support to the suggestion that there is a genetic basis for variations in germling survival in the natural environment.

We found that germination could occur throughout all of the calendar months in which cysts were isolated but, there was a short, pronounced increase in percent germination during the months of July and August (Figure 6). The five month period of cold storage in our study allows for a maturation period and reduces the likelihood that the reduced germination success of cysts in the surface sediments during May was due to a high concentration of immature, dormant cysts. Our results are similar to those of Perez et al. (1998) who found that naturally-produced

cysts of *A. tamarense* from shallow sediments in the St. Lawrence estuary displayed low germination success (i.e., 20%) when exposed to favorable conditions but had a short, 60-day window of enhanced germination success, nearing 50%, in September and October over the three year study. The authors concluded that the variable germination responses indicated the presence of a mixed population of *Alexandrium*, some of which germinate after a mandatory maturation period and some of which germinate based upon an internal biological clock.

Of the variables examined in this study (depth, cyst fullness and season isolated), the only significant predictor of *Alexandrium* cyst germination success was the season when cysts were isolated; there was enhanced germination success in spring/summer (31%) relative to winter (10%). These results could be evidence of a long-term endogenous annual clock and/or a mandatory dormancy period. *A. tamarense* and *A. fundyense* cysts isolated from deeper waters off the East coast of the USA have been shown to have an endogenous annual clock that triggers excystment during spring/summer and prevents germination during winter regardless of surrounding environmental conditions and water temperatures (Andersen and Keafer, 1987; Matrai et al., 2005). Along the US West coast and elsewhere other studies have found that *Alexandrium* spp. cysts in shallow waters can successfully germinate year round but the timing and total percent of excystment varies (Ní Rathaille and Raine, 2011; Perez et al., 1998; Tobin and Horner, 2011). While further testing is needed, there is a suggestion in our data of an internal biological clock that may be retained for decades.

The sediment samples in this study were not stored in anoxic conditions so cysts could have experienced oxidative stress from the date the core was extracted and sectioned in December 2011 to when we processed the sediments beginning in May 2012, with a maximum of just over one year exposure after core extraction. In laboratory-produced *A. tamarense* cysts, Genovesi et

al. (2009) found that oxidative stress experienced during cold-dark storage did not strongly influence cyst survival; excystment rates were steady from 0-7 months, declined from 7-10 months which was later countered by a surge in excystment at 10-14 months. The authors postulate that these results could be evidence of an endogenous clock rather than cyst deterioration related to cold-dark storage (Genovesi et al., 2009). However, others have found that cold-dark storage does reduce germination success of laboratory produced cysts (Binet and Stauber, 2006; Ní Rathaille and Raine, 2011). We find reduced germination success during the winter after 11 months in storage, but increased germination success from May to July (Figure 6), indicating that cold-dark storage in conditions that were not anoxic did not systematically reduce cyst survival over storage time.

It has been suggested that an *Alexandrium* cyst located deeper than the first few millimeters in the sediments is less likely to germinate and successfully populate a bloom due to low oxygen conditions in the sediments and/or because of requirement for germling cells to swim out of the deeper sediments (Anderson, 1998). Our results suggest that any large disturbance from a natural event or dredging that re-suspends sediments could be a source of *Alexandrium* blooms from long buried cysts. Models have predicted that shear stress caused by waves from storms or tidal currents can re-suspend the top millimeter of sediments and associated cysts depending on sediment type and bottom depth (Butman et al., 2014). Kamiyama et al. (2014) found an up to 10-fold increase in maximum *Alexandrium* spp. cyst densities from 2005-2011 in Sendai Bay, Japan, followed by record breaking PSP toxicity in nearby shellfish. They hypothesized that disturbance to deeper sediments (20-100 cm deep) from the Great East Japan Earthquake and subsequent tsunami re-suspended long buried *Alexandrium* cysts into the water column, the cysts slowly settled out of the water column after denser and larger sediment particles and thus, the

new surface accumulations of *Alexandrium* cysts may be from buried cyst beds and hence, the large PSP toxicity the following year. Of similar concern, towed demersal trawl fishing gear has been shown to disturb and re-suspend dinoflagellate cysts in the sediments (Brown et al., 2013).

Our results have important implications for managing the disturbance of sediments at known cyst bed locations in Puget Sound. Areas in Puget Sound with higher concentrations of cysts deeper in the sediments relative to the surface should not be disturbed because cysts located deeper are still viable, if not more so than surface cysts. Our results also assist efforts to model and forecast blooms of *Alexandrium* in Puget Sound; because the germination success of cysts as deep as 50 cm in the sediments was similar to those found at the surface, predictive HAB models can assume that all cysts that find their way to the surface of the seafloor behave the same, negating the need to account for differing values of germination success for old versus young cysts in biological models.

3.6 Conclusion

Image analysis was used to assess the cytoplasmic fullness of cysts of the potentially toxic alga *Alexandrium* and non-toxic alga *Scrippsiella*. We found no evidence of systematic deterioration of cyst cytoplasmic fullness with age/depth in the sediments. The cytoplasmic fullness of older cysts is statistically the same as that of younger cysts and germination success is relatively constant with age/depth. Based on the cysts we successfully isolated in this study, we conclude that the cytoplasmic fullness of a cyst cannot provide an indication of the cyst's age nor can it accurately predict if it will successfully germinate. Naturally produced cysts of *Alexandrium* and *Scrippsiella* estimated to be 64 and 58 (± 15) years old respectively were able to successfully germinate given favorable conditions. We interpret these results to suggest that *Alexandrium* and

Scrippsiella cysts may be more resilient in the natural environment than previously thought. Older cysts that are re-suspended or brought to the surface via bioturbation or other disturbances may have the same germination potential as newly formed, younger cysts.

3.7 References

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Figure 3.1 Examples of *Alexandrium* cysts classified as excysted but no live cell cysts (ECNC). Top panel (A/B) shows cysts isolated from a depth of 56-58 and 54-56 cm, respectively. Lower panel (C/D) illustrates germling cells that were not moving and whose pigments faded over the three weeks that wells were monitored.

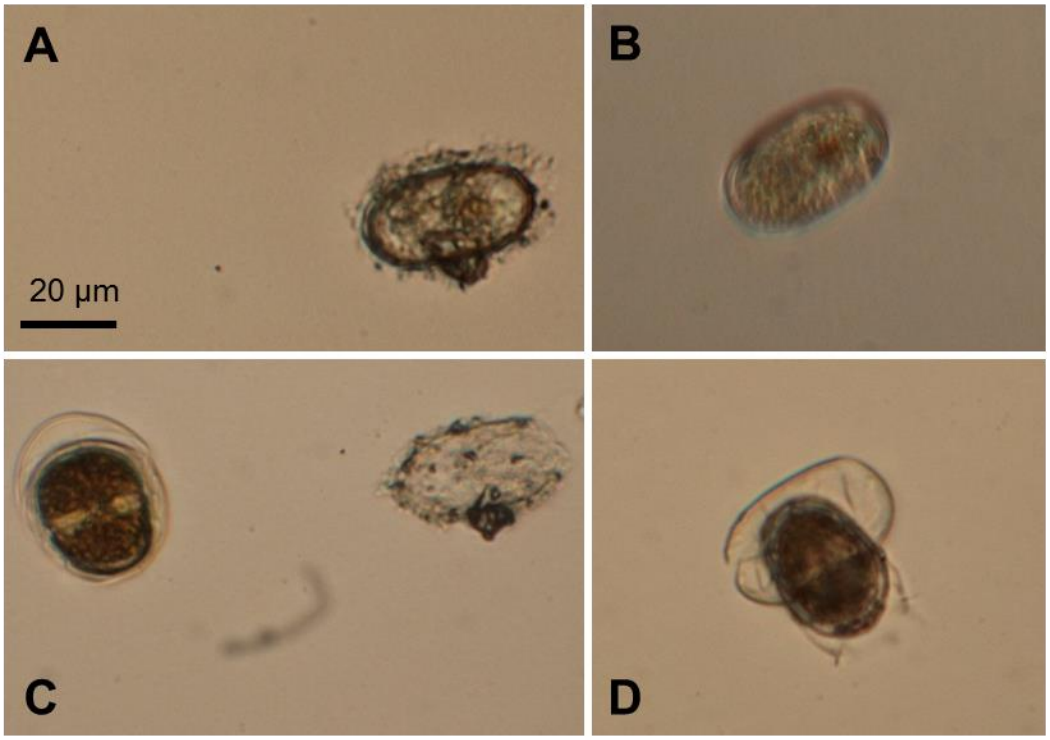


Figure 3.2 Steps in image analysis using DinoCyst. Top row is an *Alexandrium* cyst isolated from a depth of 44-46 cm. Bottom row is a *Scrippsiella* cyst isolated from a depth of 30-32 cm. Panel (A) is the original image, (B) is the image mask created by Dinocyst, (C) is the cellular contents identified by Dinocyst, (D) is the cyst's width and length dimension estimate, (E) is the confidence polygon. The *Alexandrium* cyst has an 80% image analysis confidence based on the percent overlap between image B and E and was thus excluded from image analysis. The *Scrippsiella* cyst has a 98% confidence and was included in the image analysis.

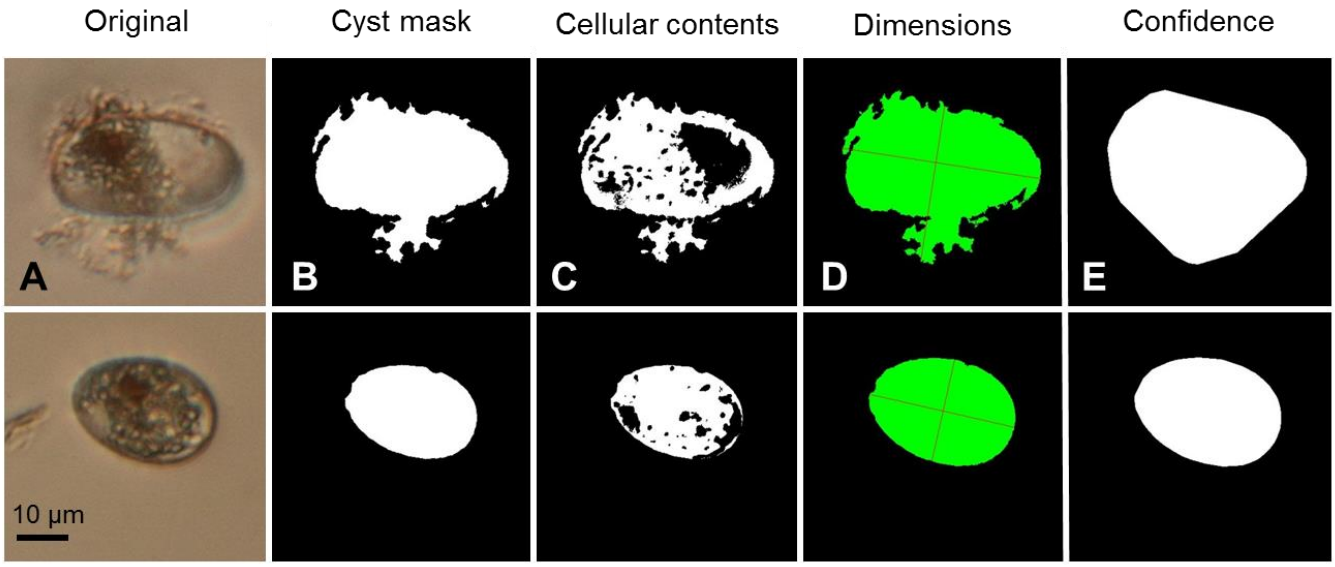


Figure 3.3 Cytoplasmic fullness of *Alexandrium* (solid line) and *Scrippsiella* (dashed line) based on germination capability. The circle is the mean cytoplasmic fullness and the brackets represent the range. The number at the bottom of the bracket is the sample size.

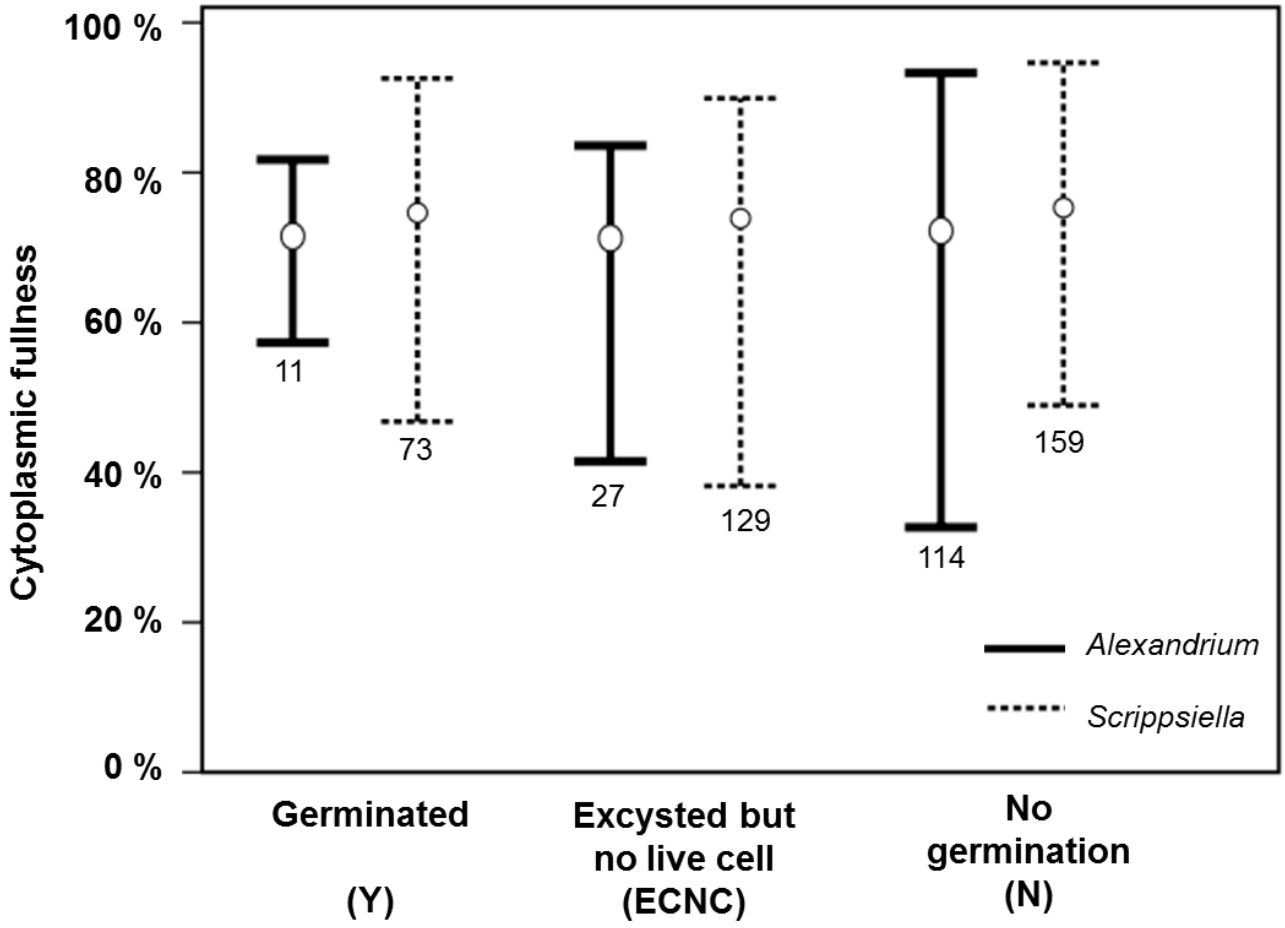
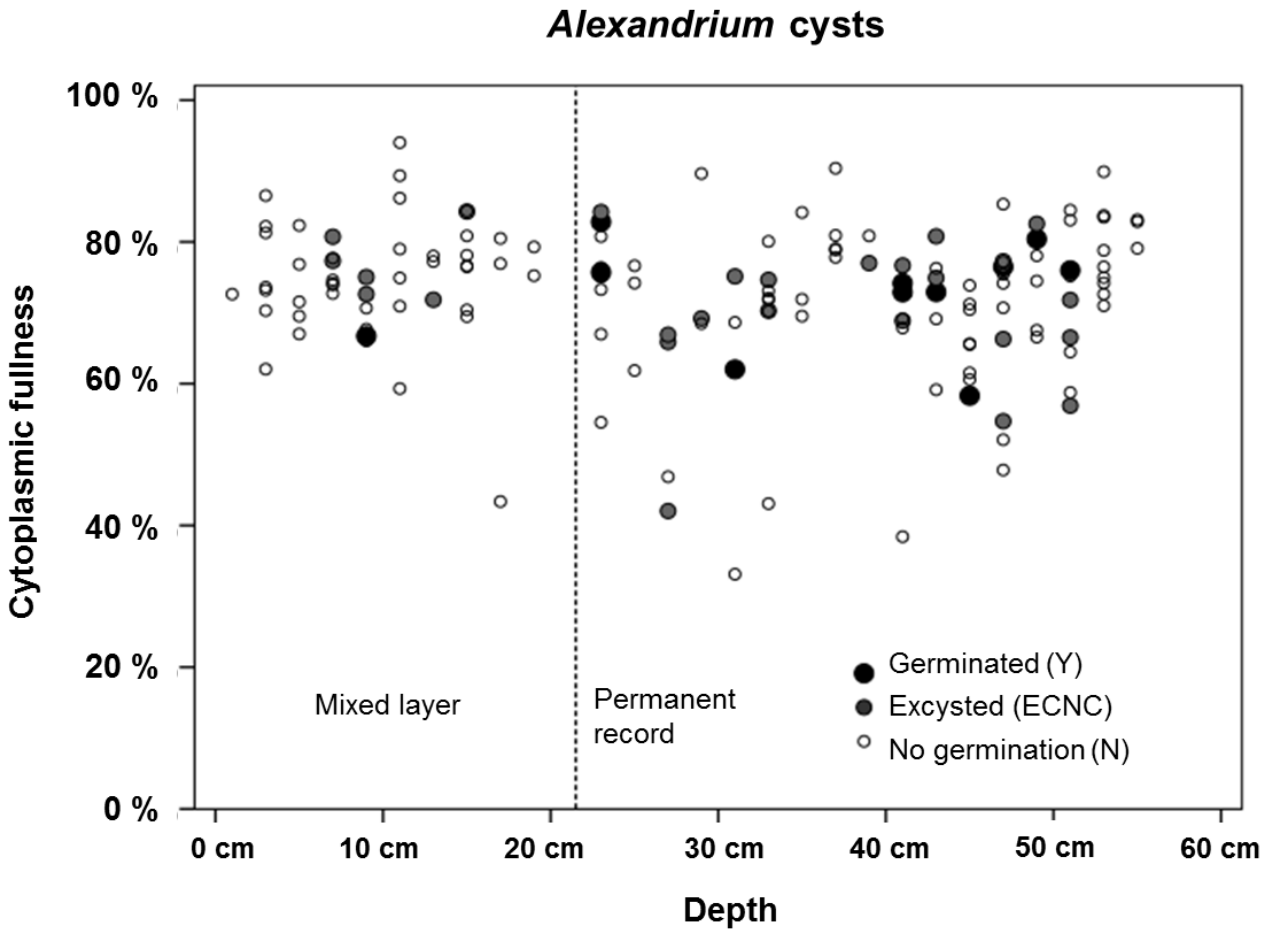


Figure 3.4 Cyst germination success and cyst cytoplasmic fullness as a function of the depth the cyst was isolated from. Black circles represent a successful germination and a viable cell found in the well (Y), gray circles represent a successful germination but no viable cell (ECNC), and the white circles represent no germination (N).



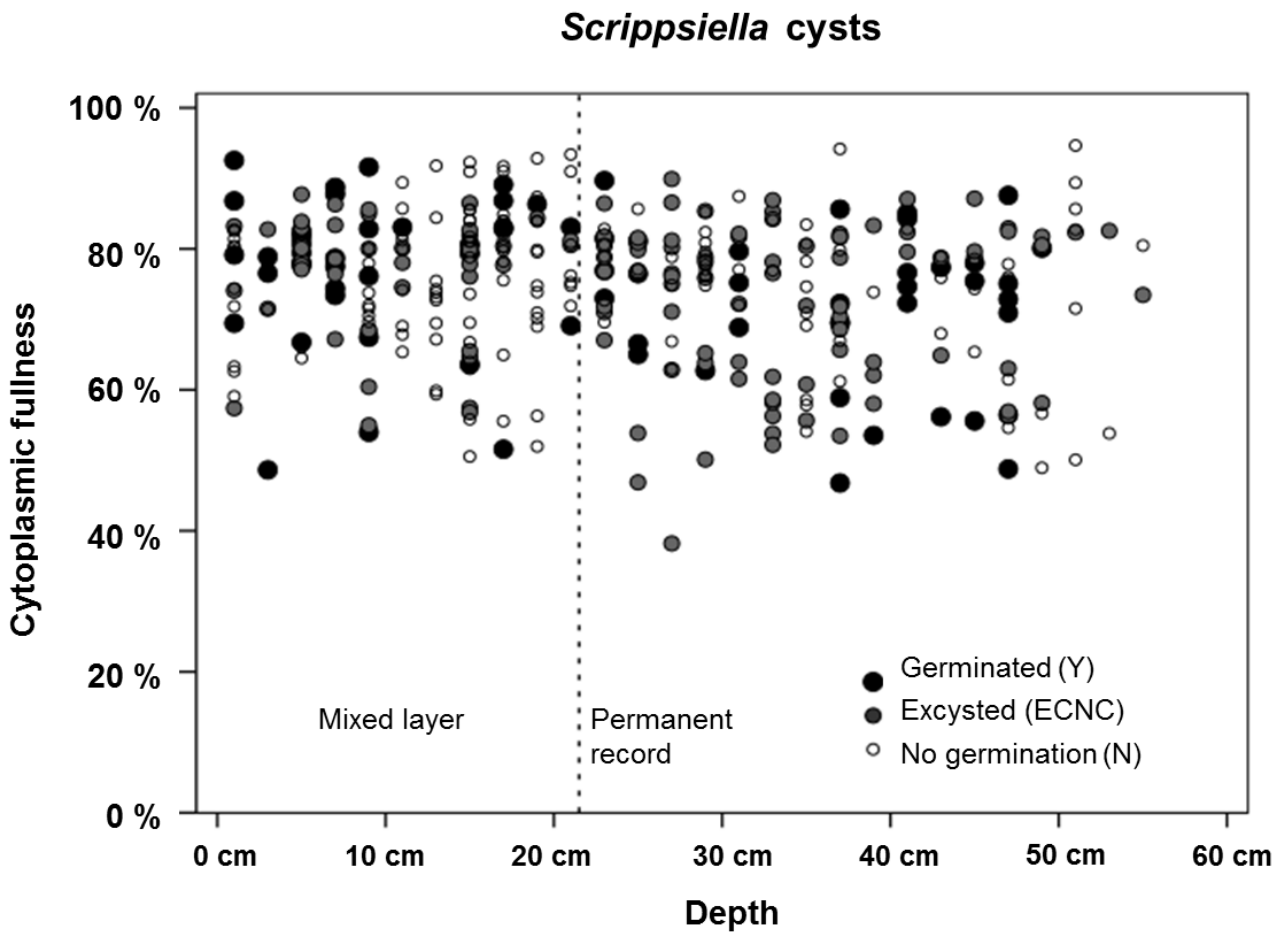

Scrippsiella cysts
Cytoplasmic fullness
100 %
80 %
60 %
40 %
20 %
0 %
0 cm
10 cm
20 cm
30 cm
40 cm
50 cm
60 cm
Depth
Mixed layer
Permanent record
Germinated (Y)
Excysted (ECNC)
No germination (N)

Figure 3.5 Profile of log-transformed total ^{210}Pb activity at the study site. The dashed line at 21.5 cm indicates the beginning of the permanent record, below the bioturbated, mixed layer. The near linear fit of ^{210}Pb decay in the permanent record is used to estimate the site's sedimentation rate to obtain a chronology. The vertical lines show error estimates with dates due to bioturbation.

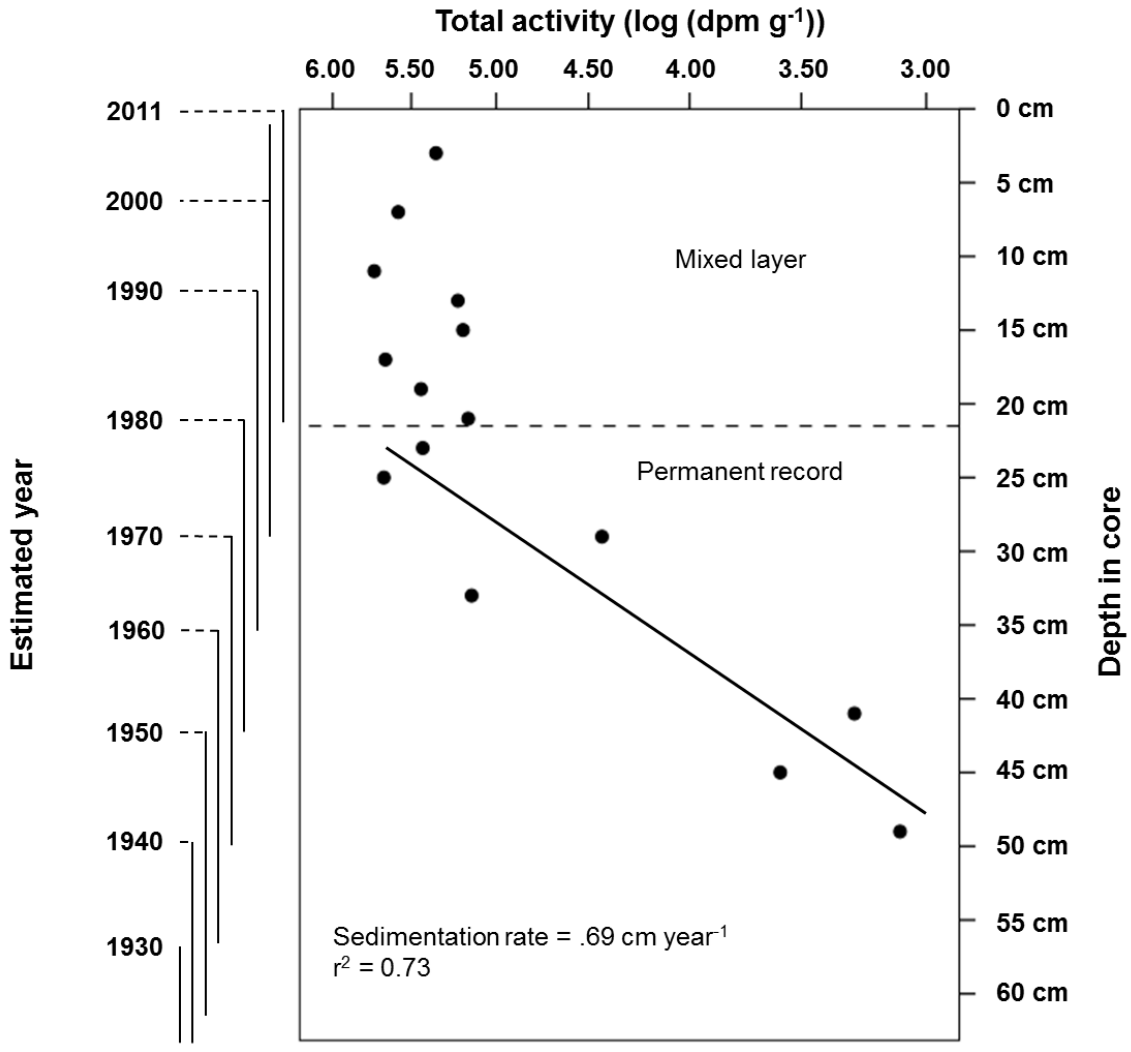
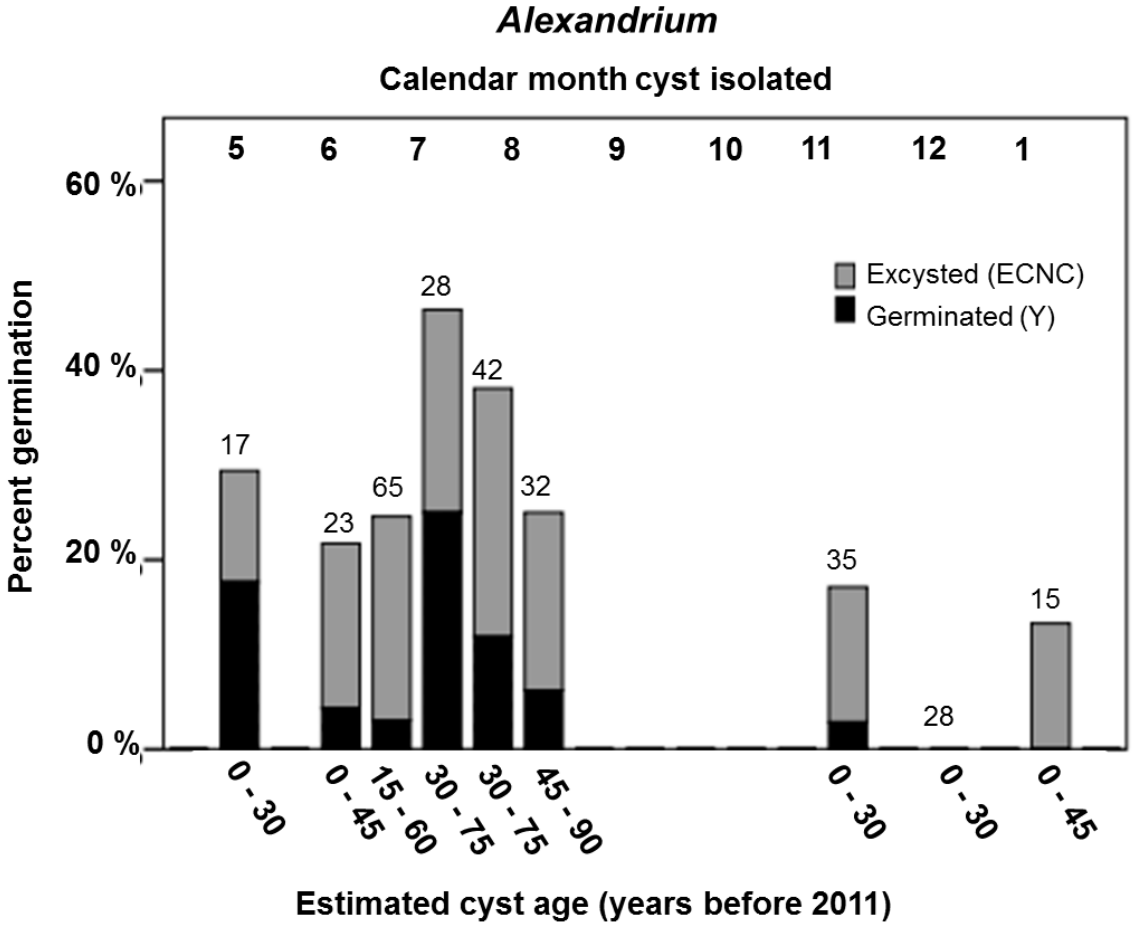
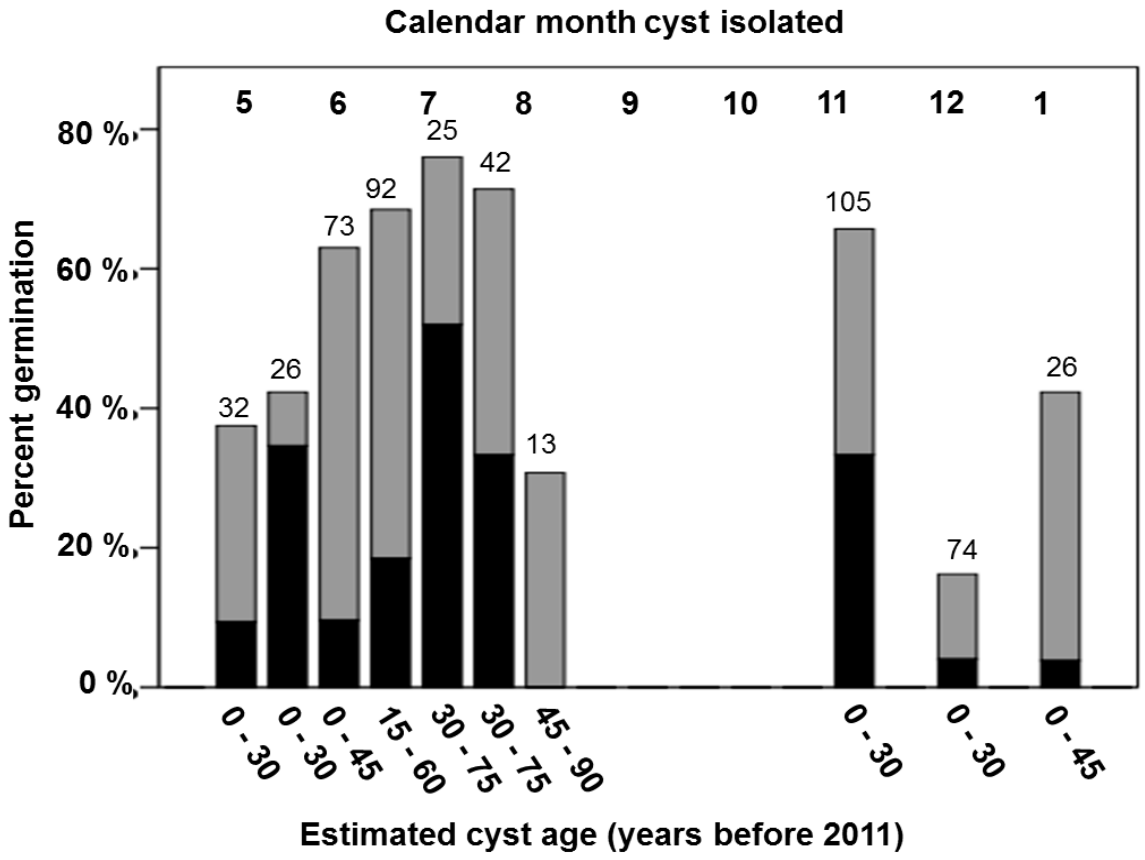


Figure 3.6 Estimated cyst age and germination rates plotted with month the cyst was isolated (top axis). Cyst age was estimated based upon the sedimentation rate and binned every 10 cm. Black bars indicate successful germination (Y), gray bars indicate the cyst germinated but did not produce a viable germling (ECNC). The number above the bar is the total number of cysts isolated in that month at that age range.



Scrippsiella



4 A high resolution *Alexandrium* cyst record from the hypoxic fjord, Effingham Inlet, British Columbia reflects the nearby paralytic shellfish toxin record from 2002-2012

Kirsten M. Feifel

4.1 Introduction

Blooms of the potentially harmful dinoflagellate genus *Alexandrium* can be found worldwide in temperate, coastal environments. They are known to produce a suite of toxins collectively referred to as paralytic shellfish toxins (PST); concentrations of PSTs in filter-feeding shellfish can cause paralytic shellfish poisoning if consumed. The most lethal PST, saxitoxin, can be deadly within hours of ingestion depending on dosage. As such, international health authorities have developed standard protocols to determine if a toxic bloom has contaminated shellfish. If toxin levels are above 80 µg per 100 g shellfish tissue, the area is closed to shellfish harvest in order to protect public health. Over the past few decades, blooms of *Alexandrium* and other harmful algae have increased in geographic scale, frequency and duration but various sampling methodologies make it difficult to standardize global observations (Hallegraeff, 2010).

There are few datasets of *Alexandrium* longer than 60 years sufficient for historical trend analysis but there have been efforts to develop relevant historical records using sediment cores (Feifel et al., 2012). *Alexandrium* exhibits a dual-stage life cycle alternating between a mobile, vegetative cell in the water column and a sessile, benthic cyst stage in the sediments. The cyst stage represents a link between an individual cell and subsequent, potentially dense aggregations

of dividing vegetative cells in the water column. Cysts of *Alexandrium* are able to survive and germinate after being buried in the sediments for 100+ years (Miyazono et al., 2012) and do not display any physical evidence of systematic degradation or decline in percent germination over a 50-80 year timescale (Feifel et al., submitted). Concentrations of cysts on the seafloor are thought to act as the inoculum for annual, regional blooms (Anderson et al., 2005) but in general the relationships between the motile and sessile benthic stages are poorly understood (Estrada et al., 2010).

There is a need to understand how the concentration of *Alexandrium* cysts in the sediments relates to environmental conditions, the following year's bloom and/or if it reflects the previous year's bloom. Some studies have found a strong, positive correlation between cyst densities in the sediments in fall and the subsequent spring bloom (Anderson et al., 2014b). However, Cosgrove et al. (2014) concluded that *Alexandrium* cyst densities in the sediments are not a good predictor of the intensity of the following year's spring bloom but rather, are more representative of the preceding bloom density in the water column. Others have attempted to correlate *Alexandrium* surface cyst (Anderson et al., 2014a) and subsurface cyst (Cox et al., 2008; Feifel et al., 2012) densities with various measures of local toxicity in shellfish. Historical analyses of surface and sub-surface cyst records are often limited by a shortage of nearby shellfish toxicity records, relatively short comparison toxicity time series and imprecise chronologies due to the mixing in the sediments from benthic organisms.

Effingham Inlet, on the west side of Vancouver Island, British Columbia, has been the location of multiple historical dinoflagellate sediment core studies. Earlier studies have used sediment cores from Effingham Inlet to assess the oceanographic and upwelling influence in the inlet (Radi and De Vernal, 2004), to assess water quality and productivity using dinoflagellate cysts

(Radi et al., 2007) and to conclude that the dinoflagellate diversity in the two adjacent sub-basins of Effingham Inlet were distinct from each other (Kumar and Patterson, 2002). However, none of these studies specifically examined *Alexandrium* cysts and compared the cyst record to nearby shellfish toxicity to better understand the relationship between toxicity and subsequent cyst deposition.

Effingham Inlet is seasonally hypoxic which is important to this study for two reasons. First, it reduces the likelihood that benthic organisms could survive in or on the sediments and disturb the sediment floor. Thus, the temporal resolution of the sediment record in Effingham Inlet remains intact allowing for the development of relatively high resolution sediment core record. The chronologies of previous *Alexandrium* sediment core studies have been complicated by bioturbation which reduced their utility to assess short-term, annual to biannual changes (Cox et al., 2008; Feifel et al., 2012). Second, the hypoxic waters present in Effingham Inlet prevent *Alexandrium* cysts from germinating. *Alexandrium* cysts need oxygenated conditions to germinate (Andersen and Keafer, 1987). Therefore the interpretation of the Effingham Inlet cyst record is not complicated by losses due to annual germination of cysts in the sediment.

The objective of this study was to assess the relationship between the concentrations of *Alexandrium* cysts in a hypoxic sediment core and nearby PST concentrations in shellfish collected along the shores of Effingham Inlet. We wanted to develop a high resolution, historical record of *Alexandrium* cysts that was appropriate to compare with the available blue mussel (*Mytilus edilus*) PST record to better ascertain the relationship between cyst densities in the sediments and nearby toxicity. We also compared the PST record to nearby sea surface temperature records to test the hypothesis that more toxic blooms occur during warmer years.

4.2 Methods

4.2.1 Environmental setting

Effingham Inlet is located on the southwestern side of Vancouver Island in British Columbia, Canada. It connects via Barkley Sound to the Pacific Ocean (Figure 1). Effingham Inlet is a 17 km long, glacially carved fjord that is divided into inner and outer basins by two sills remnant from glaciers, 45 m and 65 m deep respectively. The maximum depth of the inner basin is 120 m and 210 m for the outer basin (Kumar and Patterson, 2002). The presence of the sills restricts deep water circulation, limits basin flushing and hence, promotes hypoxic deep water conditions throughout the year and prevents the survival of most benthic macrofauna in the sediments.

Daily sea surface temperature from the nearby Amphitrite Lighthouse (Figure 1) was used as an estimation of historical SST in Effingham Inlet. Amphitrite Lighthouse SST has been taken daily at or near the daytime high tide since the 1930s. The Amphitrite SST record was averaged to weekly or biweekly values to match the sampling temporal resolution of the PST record from 2002-2012. For this analysis we assume that the Amphitrite SST record reflects SST trends at our sampling site.

4.2.2 Sediment processing

A 17 cm in length sediment core was extracted from Effingham Inlet, British Columbia from a depth of 117 m at 49.021944 ^{o}N, -125.1611 ^{o}W on February 2, 2013 using an Ocean Instrument MC200 sediment multi-corer with 1 m long x 10 cm diameter coring cylinders while aboard the R/V Thomas G. Thompson. The sediment core was carefully wrapped in aluminum foil to keep it in the dark and stored upright at 2 ^{o}C until further processing. A concurrent CTD cast indicated

that the coring site was hypoxic with 0.86 mg L^{-1} O^2 at 110 m and a bottom water temperature of 8.2 ^{o}C.

The core was sectioned into 0.5 cm intervals in August, 2013. When the sediment core was unwrapped, a thin nepheloid layer was evident at the sediment-water interface. The excess water was carefully decanted and sediments were extruded from the coring cylinder by pushing a plunger through the tube. There was no visual evidence of sediment lamination. Sediment intervals were measured to be 0.5 cm thick, scraped onto a metal plate and the outer most edge of the sediments was removed and discarded. The remaining sediment sample was transferred into plastic baggies, sealed and stored in 2 ^{o}C dark conditions until further processing. Sediments were processed and cysts enumerated following standard methods (Yamaguchi et al. 1995) and further detailed in Feifel et al. (2012). Triplicate cyst counts were done on the 0.5 cm intervals from 0-4 cm to estimate counting error.

Lead-210 dating was completed by Flett Research LTD (Winnipeg, MB Canada). The surface of the sediment core corresponded to the year the core was extracted, 2013. Below this depth, age was estimated based upon the isotopic decay of ^{210}Pb (Figure 2).

4.2.3 Toxicity data

Historical PST data from 2002-2012 was provided by the Pacific Shellfish Inspection Agency at the Canadian Food Inspection Agency. Data from Effingham Inlet sub-area 23-06 located at 49.023333 ^{o}N, -125.149722 ^{o}W was selected for comparison analysis because the sampling location was closest to the coring location (Figure 1). The Canadian Food Inspection Agency sampled the blue mussel, *Mytilus edilus*, for toxins on a weekly to bi-weekly basis throughout calendar years (red stars, Figure 3).

All the PST data at site 23-6 were plotted from 2002-2012 (Figure 3). Yearly data was integrated below the interpolated PST line, assuming a linear trend between two sampling periods, using MatLab software to provide an estimate of the year's total PST concentrations in blue mussels. If there was not a data point for both the beginning and end of the Julian year (days 1 and 365), a mean of the closest PST data points was input to ensure a uniform timescale across years. Yearly toxicity values were plotted and compared with the cyst record developed from the sediment core (Figure 4).

4.3 Results

4.3.1 Chronology

Lead-210 analysis indicates that the sedimentation rate was on average 0.0991 g cm^{-2} yr^{-1} over the length of the 17.5 cm core, equivalent to 0.33 cm yr^{-1} (Figure 2). Therefore, the temporal resolution of each 0.5 cm cyst sample is estimated to be 1.5 yr. There is little evidence of bioturbation in the core as evidenced by the near linear decay of ^{210}Pb with depth ($r^2 = 0.844$, $p = 0.00$, $N = 11$); hence intense mixing has been limited at this site. Assuming sedimentation rates have remained relatively constant over the timescale of the sediment core, the bottom of the core is estimated to date to approximately 1960.

4.3.2 Toxicity data

PST concentrations exceeded the 80 µg per 100 g shellfish tissue health criteria, causing harvest closures and restrictions, in 7 of the 11 years on record. Effingham Inlet site 23-6 had PST detections above the 80 µg health standard from the beginning of July (Julian day 183), persisting through the winter until the end of February (Julian day 61), but repeatedly reached the

highest toxicity values in the months of July and August (Julian days 190-240) and when temperatures ranged between 12-16 ^{o}C (Figures 3, 5). There were no toxin detections that exceeded the PST health criteria between March and June in the 11 year record.

In years 2005, 2007, 2010, and 2011 there were no PST measurements in exceedance of the PST health criteria. In 2002, 2003, and 2006, PST detections above the health criteria occurred consecutively over a relatively short timeframe (44, 13, and 19 days respectively) suggesting that only one annual bloom of *Alexandrium* occurred in nearby waters those years. Years 2004, 2008, 2009, and 2012 all had PST detections that exceeded health standards multiple times but not over consecutive days suggesting that multiple *Alexandrium* blooms occurred within a year (Figure 3).

4.3.3 Cyst densities

Cyst densities in each of the 0.5 cm intervals ranged from 0-56 cysts mL^{-1}. Counting error generally increased as the cyst density increased; the mean standard deviation in cyst counts of the 8 upper depths (0-4 cm) where triplicate counts were made was ± 4.65 (Figure 4). The highest cyst density occurred at a depth of 1-1.5 cm (est. age 2009). Cysts densities were below 5 cysts mL^{-1} beyond depths of 12 cm (est. age 1975 and older). Cyst densities began to increase from 1975 to 1990, dipped from 1991-1998 and then rapidly increased from 1999 to 2010, coinciding with the available PST record (Figure 4).

4.3.4 SST records

Yearly SST ranged from a minimum of 4.9 ^{o}C to a maximum of 16.2 ^{o}C with a mean of 10.2 ^{o}C. There was no long-term trend in annual mean sea surface temperature at Amphitrite Lighthouse

from 2002-2012. SST temperatures exceeded 13 ^{o}C at some point in all years; waters were above 13 ^{o}C for 37-88 days in the 11 year record. The years 2004 and 2009 had, on average, 85 days where SST was above 13 ^{o}C; the other years had, on average 50 ± 8.5 days above 13 ^{o}C (Figure 3).

4.3.5 Statistics

For statistical analysis, annual PST records were low-pass filtered to match the 1.5 year temporal resolution of the cyst record (blue line, Figure 4). The cyst record and low-pass filtered PST record were significantly correlated from 2002-2012 ($r = 0.8488$, $p = 0.0157$, $N = 7$, $df = 5$).

The daily SST record was resampled to match the sampling resolution of the original weekly to bi-weekly PST record and the 1.5 year resolution of the cyst record. There was no statistically significant relationship between SST and the cyst record ($r = 0.2313$, $p = 0.4938$, $N = 11$, $df = 9$) or the higher resolution SST and PST records ($r = 0.005$, $p = 0.915$, $N = 435$, $df = 433$) although the highest toxicity tended to occur at higher SSTs (Figure 5). The number of days that SST was above 13 ^{o}C was not correlated to the PST toxicity record ($r = 0.3836$, $p = 0.2442$, $N = 11$, $df = 9$) or the cyst record ($r = 0.05$, $p = 0.8704$, $N = 11$, $df = 9$) although 2 of the 4 years with multiple blooms occurred when the number of days above 13 ^{o}C was the highest.

4.4 Discussion

The cyst record developed from Effingham Inlet reflects encystment during and/or after a bloom in overhead waters, as loss of cysts from germination is prevented due to a lack of oxygen in bottom waters. As such, we interpret the relationship between the cyst record in Effingham Inlet and shellfish PST concentrations (Figure 4) to suggest that nearby toxicity records can be used to

predict subsequent cyst densities on the seafloor. This relationship could be complicated by a non-linear relationship between the number of vegetative cells (i.e. the bloom size) and nearby toxicity (Dyhrman et al., 2010; Martin et al., 2014), temperature effects on *Alexandrium* cellular toxicity (Navarro et al., 2006), or variable feeding and depuration rates of mussels in regards to *Alexandrium* cell densities and associated toxins (Velasquez and Navarro, 2014). The relationships between these factors are poorly understood and hard to generalize.

Multiple researchers have used various datasets and analyses to suggest that a bloom size and/or nearby toxicity can be used to predict subsequent cyst densities. Ishikawa et al. (2014) found that *A. catenella* cysts on the seafloor germinated throughout the year but cyst abundance in sediments increased during and directly after a bloom. Cosgrove et al. (2014) were able to predict cyst densities by assuming that 2.5% of the mobile vegetative cell population underwent sexual reproduction and produced a cyst approximately one week after high cell densities were observed in the water column. They found no correlation between the measured cyst densities in previous winters and the following spring/summer *A. minutum* bloom intensity. The relationship between bloom size and the numbers of cyst deposited thereafter is further confirmed by research on *A. catenella* in Chile (Díaz et al., 2014). Because our cyst record from Effingham Inlet only reflects encystment after a bloom, the relationship between the cyst record and toxicity record implies that the size of a bloom influences nearby toxicity and cyst deposition after a bloom rather than the reverse.

The combination of low toxicity and high cyst counts between 2010 and 2011 suggest that either a large cyst deposition occurred during a low toxicity year (i.e. a large bloom of *Alexandrium* occurred but the vegetative cells had low toxin concentrations), there is a temporal resolution mismatch between the annual PST record and 1.5 year cyst record which makes it difficult to do

a direct comparison between the two records or, there is unaccounted for error in our sediment record. Error in the cyst record chronology estimate could arise from physical mixing of the sediments, variable sedimentation rates, sediment compaction or smearing during sediment core processing and extraction. An x-ray analysis of a laminated 15 cm section of a 10 m sediment core extracted from Effingham Inlet, aged to be 4400 ybp, showed that sedimentation rates at that time were not constant over a 60-year period but varied from 0.075 to 0.353 cm yr^{-1} (Chang and Patterson, 2005). This variability was hypothesized to be due to inter-annual changes in diatom productivity and variable input of terrigenous debris from continental runoff. If the sedimentation rate over the ca. 50-year period considered in the current study, it could confound our ability to discern signals with a frequency different than 1.5 years. Sedimentation rates less than 0.33 cm yr^{-1} would act as a low-pass filter to the cyst record, further reducing our ability to correlate the cyst record with any indices that have a temporal resolution >1.5-6 years. In this study, we applied a constant sedimentation rate of 0.33 cm yr^{-1} based upon the ^{210}Pb record and inferred that every 0.5 cm interval represented approximately 1.5 years of integrated environmental data. To counter the potential problem due to variable sedimentation rates, a distinctly laminated core with x-ray-based measurements of varve thickness is needed to properly develop an annual resolution cyst record.

While variable, *Alexandrium* blooms appear to attain higher levels of cellular toxicity when sea surface temperatures range from 12-16 ^{o}C (Figure 5, Nishitani and Chew, 1984). This lends support to the hypothesis that the size and toxicity of a bloom may be moderated by environmental conditions. A study in Japan found that the population size of an *A. catenella* bloom in the water column was not correlated with cyst germination rates or initial cyst concentrations but was rather related to warm water temperatures between 18-23 ^{o}C and other

environmental conditions that promoted vegetative growth (Ishikawa et al., 2014). In this study, we found no statistically significant relationship between SST as measured at Amphitrite Lighthouse and the toxicity or cyst records in Effingham Inlet (Figure 5). The lack of a relationship with SST in our study may have been due to a number of factors. Sea surface temperature at Amphitrite Lighthouse, located on the coast, may not reflect SST in Effingham Inlet. In addition, blooms of *Alexandrium* may be transported into and out of Effingham Inlet or the bloom size could be moderated by multiple environmental factors, temperature being only one of many potential variables. Biological interactions between species (i.e. viruses, grazing pressure) can also potentially have a large influence on phytoplankton populations over relatively small spatial scales (Dale and Murphy, 2014).

Our data suggest that yearly integrated PST concentrations can weakly predict the number of cysts deposited on the nearby seafloor. If the PST record is low-pass filtered to better match the temporal resolution of the cyst record, the two records become significantly correlated. Despite the fact that we cannot adequately account for some potential error in the sediment core chronology, the rapid increase in cyst counts and the toxicity record beginning roughly in 2005 indicates that bloom patterns of *Alexandrium* have changed recently in Effingham Inlet. Provided the relationship between cyst densities and toxicity hold over time, interpretation of the cyst record would suggest that while present, extremely toxic years were not a common feature at this location prior to the 1990's (Figure 4). We could not find any long-term records of historical PST detections or anecdotal evidence to validate this conclusion.

Effingham Inlet is an ideal location to develop long-term records of *Alexandrium* blooms thanks to its hypoxic bottom waters which prevents the loss of cysts to germination and helps to preserve the sediment chronology. One of the most difficult challenges when creating records

from sediment cores is accounting for error due to bioturbation and variable sedimentation rates. Here, we were able to reduce the likelihood of effects from bioturbation but our record could still be complicated by variable sedimentation rates that we could not, ultimately, account for in our analysis. Future studies in Effingham Inlet with sediment cores that have distinct laminations will help develop an accurate, annually resolved record. A dearth of comparable PST records will never be overcome except with time. Effingham Inlet holds the promise to help researchers better understand the dynamics of *Alexandrium* blooms, toxicity, and cyst records.

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Figure 4.1 Site map. The '*' indicates the location where the sediment core was extracted. The 'x' indicates where the PSP record samples were taken. The 'Δ' is the location of Amphitrite Lighthouse where the SST records were taken.

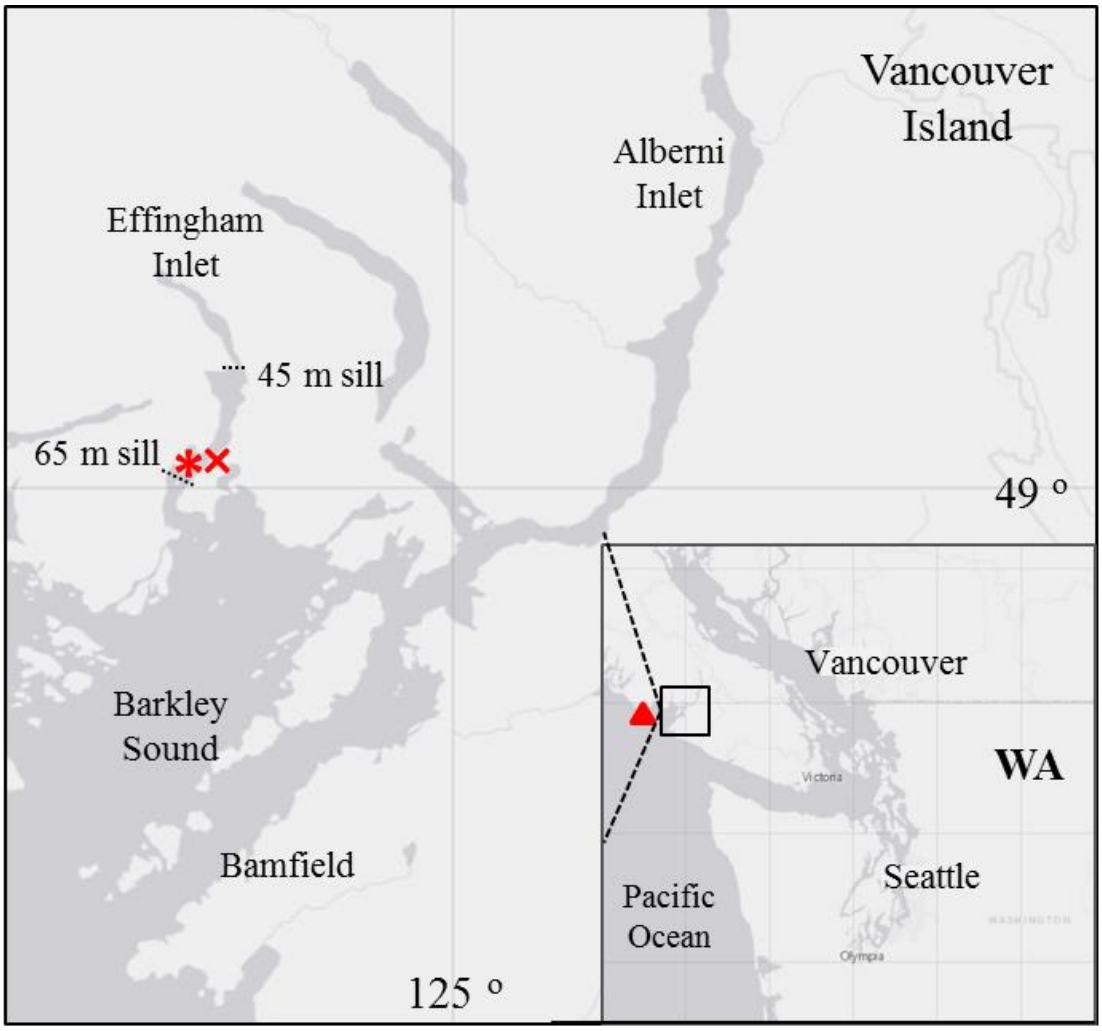


Figure 4.2 Lead-210 record from Effingham Inlet sediment core. The intersecting crosses indicate laboratory measurements of the isotope ^{210}Pb and their associated error bars. The roughly linear decay of the isotope suggests that there has been very little disturbance to the sediments at this location.

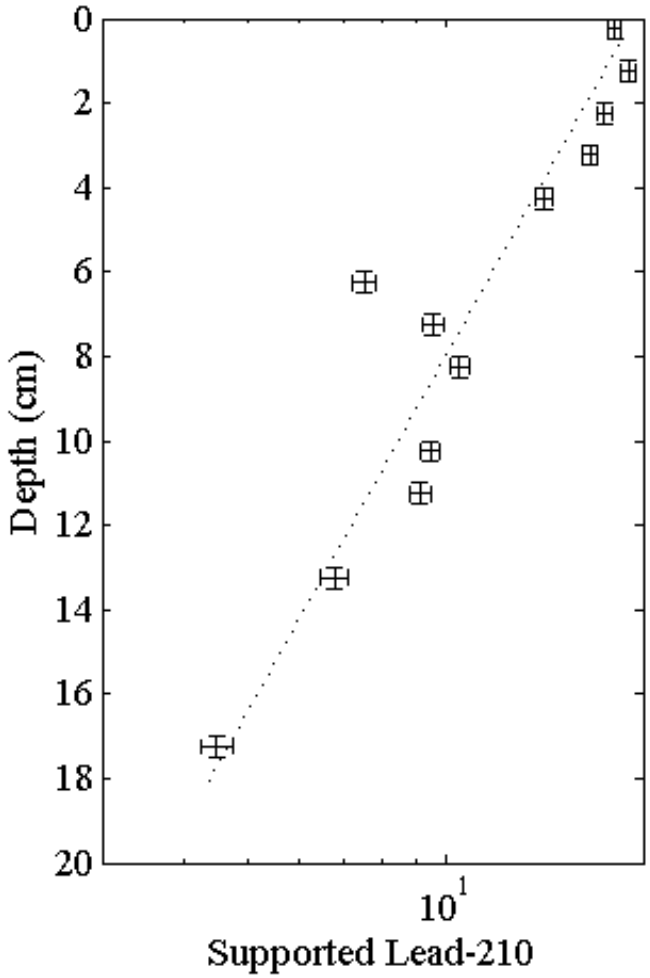


Figure 4.3 PST and SST records. The annual, interpolated PST record was estimated from toxin concentrations in the blue mussel, *Mytilus edilus*. The red stars indicate when a blue mussel sample was taken. SST as recorded at Amphitrite Lighthouse is in blue; the gray line is a 13 ^{o}C threshold.

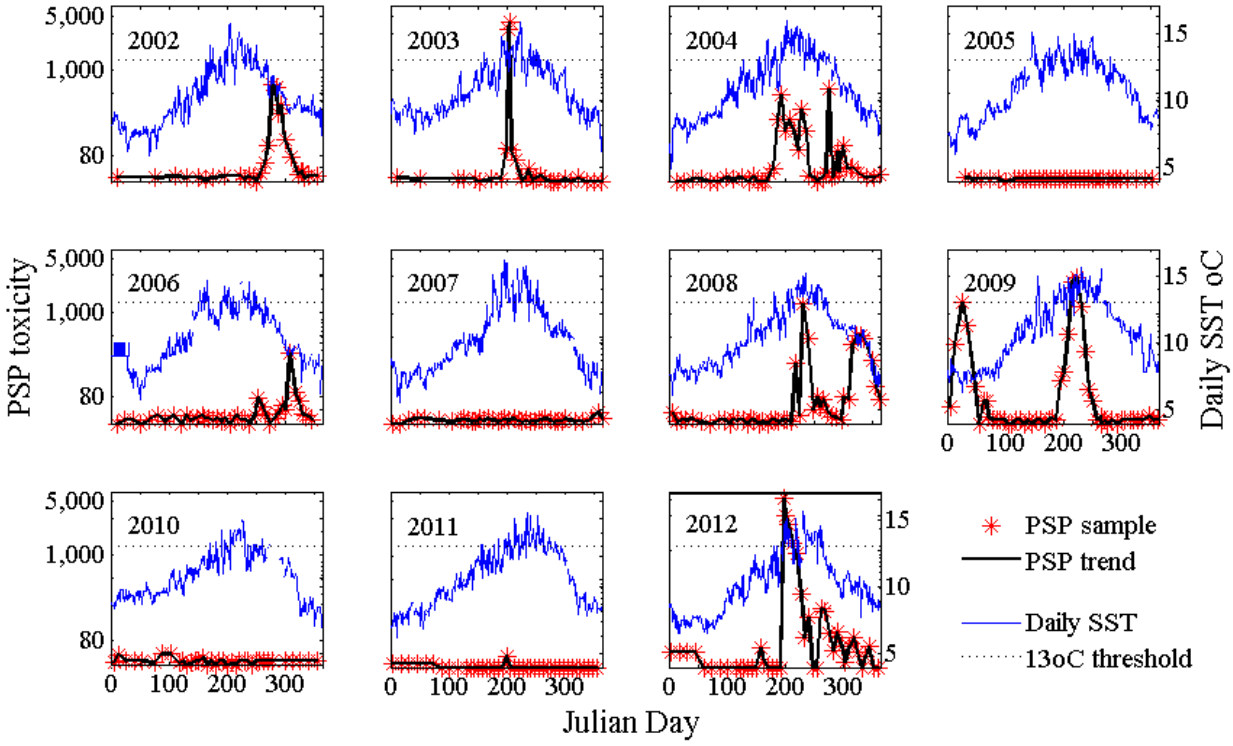


Figure 4.4 Comparison of cyst concentrations (black), yearly PST values (red), and a low-pass filtered PST record (blue). The inset box is a correlation plot of the interpolated cyst record and the low-pass filtered PST record (r = 0.8488, p = 0.0157).

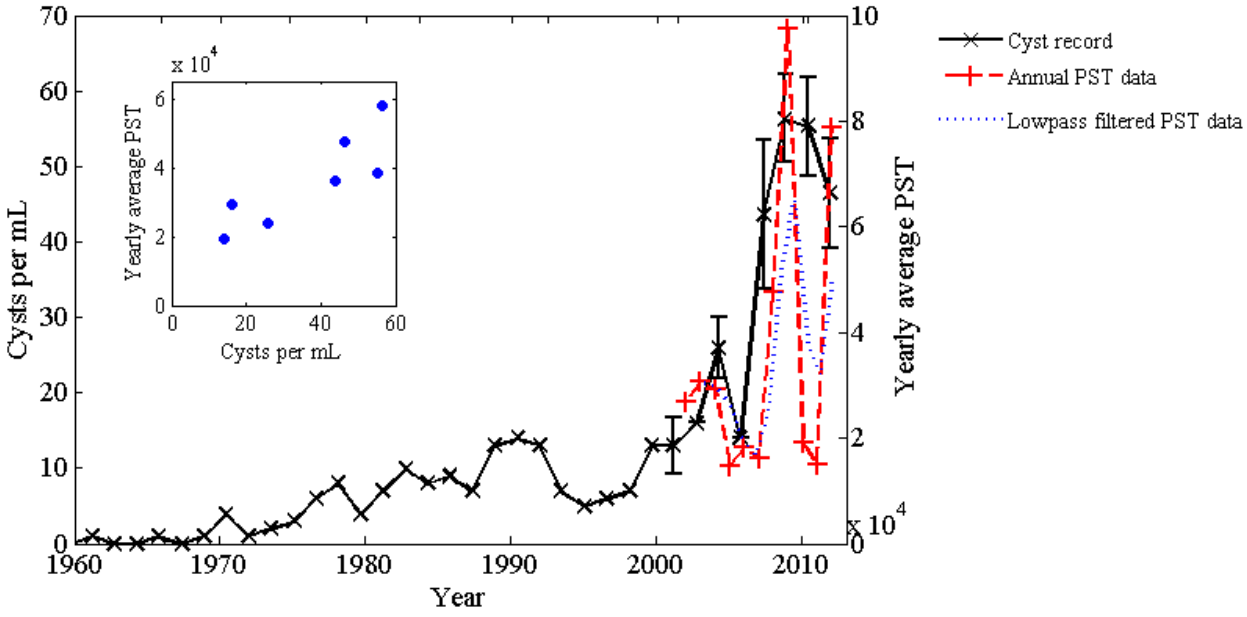
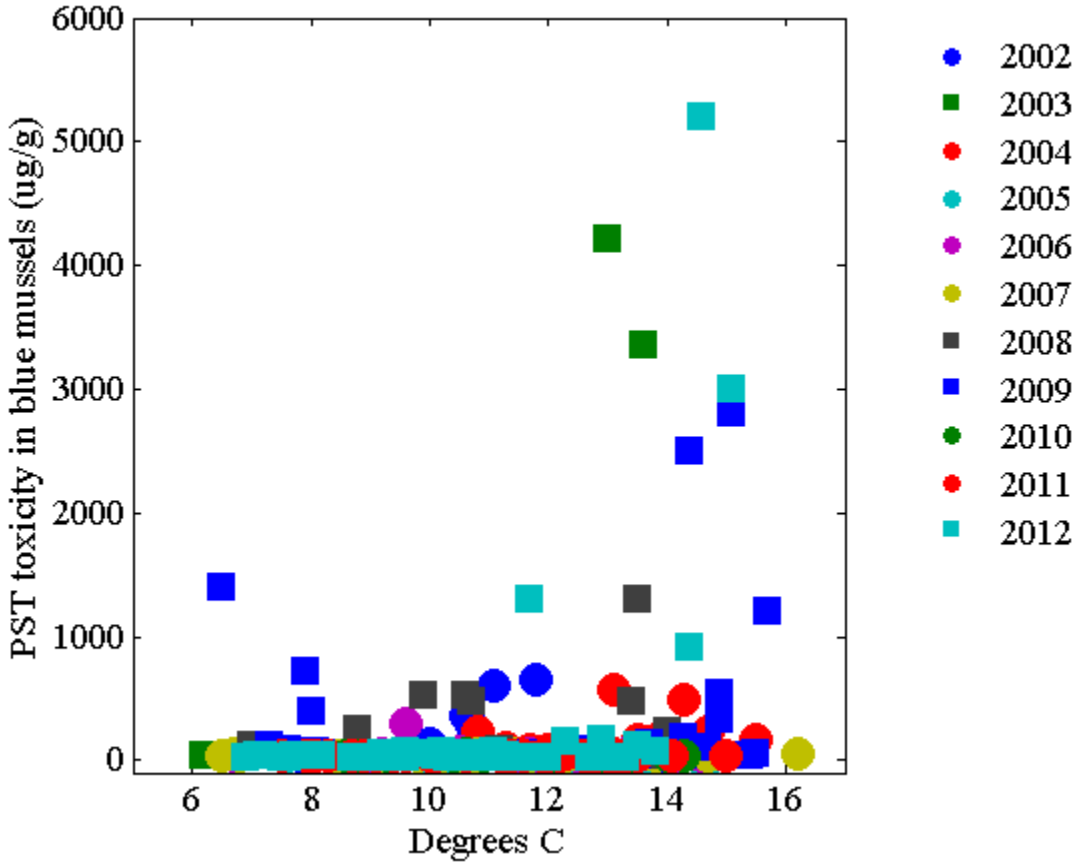


Figure 4.5 SST and toxicity records. This plot shows weekly to bi-weekly PSP toxicity values for years between 2002-2012 plotted against sea surface temperature as measured at Amphitrite Lighthouse.



5 Percent germination estimates of cysts from natural sediments: a modified plating method and a model for error estimation

Kirsten M. Feifel

5.1 Introduction

More than 10% of dinoflagellate species, including those in the toxic genus *Alexandrium* and non-toxic genus *Scrippsiella*, produce a benthic resting stage during their life histories (Bravo and Figueroa, 2014). Understanding the long term survival, percent germination and seasonal to decadal changes in the germination potential of surface and sub-surface *Alexandrium* cysts can have important management, human health and economic implications. For example, *Alexandrium* cyst germination potential, as measured in the laboratory by Anderson and Keafer (1987), and cyst concentrations in the field are primary inputs into a physical-biological model developed to forecast annual *Alexandrium* blooms in the Gulf of Maine (e.g. McGillicuddy et al., 2005). These types of forecast models are used to help shellfish managers, growers and health officials to better prepare for the potential negative impacts of harmful algal blooms.

Cyst germination is also a valuable indicator of long term cyst survival and viability in the natural environment. It had been assumed that *Alexandrium* cysts degrade quickly in the environment (Keafer et al., 1992) but new research suggests that *Alexandrium* cysts not only survive for decades or more in the sediments (Miyazano et al. 2012; Feifel et al., submitted), but

may also have the capacity to promote large, toxic blooms if re-suspended into the water column after a large disturbance (Kamiyama et al., 2014; Natsuike et al., 2014).

A variety of methods have been used to assess the percent germination of naturally-produced cysts including: cyst isolation and monitoring for germination and vegetative cells (e.g. Genovesi et al., 2009; Miyazono et al., 2012; Vahtera et al., 2014), applying live/dead stains to cysts (e.g. Gracia et al., 2013), and the most probable number method (MPN, e.g. An et al., 1992). While all of these methods provide quantitative estimates of percent cyst germination, each has limitations. Isolating cysts with a pipette under a microscope, while minimize counting error, can potentially damage cysts during sample preparation and from associated temperature fluctuations (Lundholm et al., 2011). The estimated percent germination from cyst isolations can be skewed by a human bias to select the easily identifiable, ostensibly 'healthy' cysts (McQuoid et al., 2002). Further, the cyst isolation method is most feasible when there are high concentrations of cysts in the sediment samples, whereas low concentrations can be typical.

In the live/dead stain method, cysts are stained with SYTOX green, a nucleic acid stain that will enter a dead cell that has a compromised membrane. When excited by a light wavelength between 450 and 490 nm, the dead cysts emit a green fluorescence. Using lab-produced cysts in a pure culture, Binet and Stauber (2006) showed that the results from this method compared well with germination results from the cyst isolation method. Gracia et al. (2013) applied the SYTOX green stain method to estimate germination in environmental samples; however, to date, no validation of the SYTOX green method with naturally-produced cysts has been made. The MPN method involves incubating serial dilutions of sediments and using statistical MPN tables to estimate percent germination. The MPN method, however, has been shown to not be reliable for cell densities less than 50 cysts per sample (Harris et al., 1998). At higher cell concentrations, the

MPN method is semi-quantitative, tends to underestimate the number of organisms present and has low precision (Ballantine, 1953; Harris et al., 1998; Lewis et al., 1999).

Other methods using minimally treated sediment or sediment trap samples, rather than isolated cysts, have been used to derive germination estimates. Kremp and Anderson (2000) used a sediment trap to collect *S. hangoei* cysts, sieved the sample, incubated sub-samples of the cyst suspension and counted the number of empty and full cysts over time under a microscope to estimate the percent germination. Though samples were minimally processed in this method, the relative purity of the sample (degree of potentially obscuring sediment particles or other cyst species) is critical to accurate counting of empty cysts. For many environmental samples, particularly when working with sediment cores, it is not possible to obtain a sample without extraneous particles. Tobin and Horner (2011) incubated untreated sediment in a total of 48 replicate, individual wells and checked wells for the presence of vegetative *Alexandrium* cells daily. Germination success was defined as the overall portion of wells that had vegetative cells present at some point over a 15 week study period. The germination success was analyzed relative to the time it took for 50% of the wells to have vegetative cells in them, termed '50% germination rate'. This method is hampered by its inability to quantify how many cysts germinated over time and instead, is best used to estimate seasonal timing and potential magnitude of cyst germination rather than precisely estimating a percent germination.

We present a modified well-plate method that provides a rapid, quantitative estimate of percent germination with minimally processed sediment samples. Using a relatively small sample volume, we are able to accurately estimate the percent germination from samples with low cyst concentrations. A computer simulation is used to assess associated errors throughout the experimental procedure to ultimately provide a precise, statistically accurate, quantitative

estimate of percent germination. This method can accommodate the presence of sediment particles and/or a variety of germinating dinoflagellate cysts; this increases its general application and appeal to a broader research audience.

Increasingly, efforts are being undertaken to assess long-term viability and germination potential of protist cysts in sediments, particularly those of harmful algal species. This research is hampered by time constraints, intrinsic errors in various methods, and the need to have relatively high concentrations of cysts in the sediment sample. Thus, there is a need for a rapid, quantitative method to more quickly ascertain the percent germination from cysts found in the environment at relatively low concentrations. Here, percent germination of naturally-produced cysts in minimally treated sediments was determined with multiple subsamples distributed across well plates and associated cyst concentration estimates using conventional microscope methods. A simulation model was used to determine error due to random distributions across well-plates and variable percent germination to help constrain and quantify error. Coupling germination plate results, cyst concentration counts and a simulation model we were able to estimate percent germination in minimally treated sediments to quickly and efficiently estimate survival of *Alexandrium* and *Scrippsiella* cysts in sediments.

5.2 Methods

5.2.1 Sediment cores

Two sediment cores from different locations were used in this study. The first core, 1 m long, was extracted from Sequim Bay, Washington, USA at 48.09 ^{o}N, 123.03 ^{o}W in a water depth of 27 m in December, 2011 using a kasten corer with a 10.2 cm diameter. The second core, 12.5 cm long, was taken from Effingham Inlet, British Columbia at 48^{o}59.77', 125^{o}11.31' in a water

depth of 86 m in February, 2013 using an Ocean Instrument MC200 Sediment multi-corer with 1 m long x 10 cm diameter coring cylinder. Both cores were stored in the dark at 2 ^{o}C until processing. The Sequim Bay/Effingham Inlet cores were processed 6/4 months after extraction dates and were sectioned into 2/0.5 cm intervals.

5.2.2 Initial sediment processing

To limit damage to resting cysts, sediment was minimally processed. For each section, 5 cc's of sediment were suspended in 30 mL of 2 ^{o}C filtered seawater (FSW), sonicated while in an ice bath, and sieved through a 20-90 µm filter set. The sediment and particles that were captured on the 20 µm filter were carefully rinsed and concentrated using FSW and then transferred into a 50 mL centrifuge tube and the volume was raised to 30 mL using 2 ^{o}C FSW. Two 7 mL subsamples were transferred into two clean 15 mL centrifuge tubes; one of them, the quantitative germination plate subsample, was concentrated to 5 mL using a centrifuge.

5.2.3 The quantitative germination plate method (QGP)

Twenty µL of the well-mixed 5 mL sediment slurry sample was added into each of 50 wells of a 96-well plate containing 80 µl of f/2-Si media for a total plated volume of 100 µL per well. The 15 mL centrifuge tube was vortex mixed 2-3 times throughout the plating process to help homogenize the sample. In sum, a total of 1 mL of the sediment slurry sample was distributed across 50 wells for each sediment sample. For the Effingham Inlet core, two germination plates were made for every one sediment slurry sample.

The germination plate edge was wrapped with plastic wrap to reduce evaporation. Plates were incubated at 13 ^{o}C, 20 µE m^{-2} s^{-1} photoperiod, 12 light:12 dark for one week. After one week,

germination plates were examined using an inverted microscope (Zeiss, West Germany, Axiovert 35). Each well was checked for the presence or absence of *Alexandrium* and *Scrippsiella* vegetative swimming cells. The number of swimming cells was not estimated because it was impossible to ascertain if the individual cells came from one cyst germinating and subsequent cellular division or, if the individual cells came from more than one cyst germination. The vegetative cell that is produced after cyst germination can quickly divide making it difficult to quantify how many cysts germinated solely by counting the number of vegetative cells.

5.2.4 Cyst concentration estimates

The remaining 4 mL (Sequim Bay core) and 3 mL (Effingham Inlet core) of the 5 mL sediment slurry sample used for the germination well plates was preserved and stained following methods outlined by Feifel et al. (2012). In short, the sediment was fixed with dilute formalin, soaked in methanol overnight, and then stained with primulin. A Sedgewick Rafter Counting Chamber (Hausser Scientific, Blue Bell, PA, USA) was used to enumerate the *Alexandrium* and *Scrippsiella* dinoflagellates cysts found in a 1 mL sub-sample under epiflourescent light. Counts were done in triplicate for the Sequim Bay sediment core. The Effingham Inlet samples were counted in duplicate.

5.2.5 Cyst isolation

The second 7 mL sediment subsample was treated with a modified density centrifugation method using a 1.3 g cm^{-3} sodium polytungstate (SPT) solution following Bolch (1997) to concentrate and separate cysts from their surrounding sediments. Three mL of the SPT solution were carefully layered underneath the 7 mL sediment slurry for a total volume of 10 mL; the sample was centrifuged (International Equipment Co., Needham Hts., MA, USA, International Clinical

Centrifuge Model 42283M-6) for 10 minutes at 2,000 rpm (relative centrifugal force of 313 g). Three layers were present after centrifugation: a clear top layer, a cloudy thin layer at the 3 mL mark, and a sediment pellet at the bottom of the centrifuge tube. The particles caught at the SPT density interface were pipetted into a clean 15 mL test tube and the volume was increased to 3 mL using FSW. The cyst concentrate was stored in the dark at 2 ^{o}C prior to cyst isolation. Only the Sequim Bay samples were used to for cyst isolation.

Within 24 hours, *Alexandrium* and *Scrippsiella* cysts were isolated from the concentrate. The 3 mL sample was vortexed and 1 mL was pipetted into a Sedgewick-Rafter counting slide (Hausser Scientific, Blue Bell, PA, USA). Individual cysts were isolated using a mouth pipetted drawn-out capillary tube under a standard microscope (Zeiss, West Germany, 47 30 28) into wells of a 96-well plate filled with 100 µL of f/2 –Si media. The well plate was kept on ice during the isolation process. The well plate edges were taped to minimize evaporation and plates were incubated at 13 ^{o}C on a 12 dark:12 light (20 µE m^{-2} s^{-1}) photoperiod. The well plates were visually examined for cyst germination on days 2, 7, 14 and 21 following Lundholm et al. (2010). Successful germination was recorded if the cyst either was empty of internal contents (ECNC) or if a vegetative cell was found swimming in the well (Y, see Chapter 3 for a discussion).

5.2.6 Model

A model was developed to simulate our experimental design. In the simulation model, any number of cysts (*j*) can be distributed into any of 50 wells (*i)* with a multinomial probability of 1/50 for any number of trials (*d)*. Every cyst has an equal probability of going into any one well; any one well has an equal probability it will get any one cyst. The model creates virtual well

plates with cysts ($N_{d,j}$) distributed into 50 wells. The model then goes into each well ($N_{d,j,i}$); if one or more 'cyst' is present in the well, the model will 'germinate' each individual cyst according to a random variable (either 0 or 1) drawn from a Bernoulli distribution with a prescribed probability, the percent germination (*go)*. If the random variable drawn is a 1, the cyst has 'germinated'; if it is a 0, it has not. The model then sums the total number of cysts that 'germinated' ('actual germination', $AG_{d,j}$) and the number of wells that had one or more germinations in it ('experimental germination', $EG_{d,j}$). For example, if 3 cysts were in the i^{th} well and all three 'germinated', $AG_{d,j,i} = 3$ but $EG_{d,j,i}=1$.

5.3 Results

5.3.1 Cyst concentration and germination

In this study, results for *Alexandrium* germination plates/cyst isolations are limited to those that were done between the months of May through September to minimize any potential influence seasonality may have on cyst percent germination. Results for *Scrippsiella* germination plates are from the months of May through December and the following January. Successful germination of *Alexandrium* cysts was documented in both cores. There were no *Scrippsiella* cysts present in the Effingham Inlet core and hence, no germinations were recorded. Sample counting error was commensurate with cyst concentrations (Table 1).

Based on a Pearson's correlation, the average number of *Alexandrium/Scrippsiella* cysts counted was significantly correlated to the number of wells with germination present ($r = 0.61/0.83$, $N = 111/52$, $p < 0.001$); the average number of cysts counted predicted 18/56 % of the variability in the number of wells with germination in them (Figure 1). This shows that the number of cysts

counted in the preserved and stained sample can be used to predict the number of expected germinations for the same sample.

The average percent germination of *Alexandrium* and *Scrippsiella* was estimated by regressing the mean number of cysts counted/isolated per depth and the number of wells with germination in them (Figure 1). Linear regression demonstrated a significant, positive relationship between the mean number of cysts counted/isolated and the number of wells with germination in them for *Alexandriu/Scrippsiella* ($F(1,109) = 64.702$, $p < 0.001$; $F(1,52) = 114.5$, $p < 0.001$). Thirty-four/thirty-one percent of *Alexandrium* cysts counted/isolated germinated ($b = 0.3416/0.307$) and 65/57% of *Scrippsiella* cysts counted/isolated germinated ($b = 0.6484/0.5721$). The percent germination estimate for both *Alexandrium* and *Scrippsiella* using the germination plates was 10 and 12% higher respectively to the estimates derived from cyst isolations. There was no correlation in percent germination with depth for *Alexandrium* ($F(1,95) = 1.44$, $p = 0.233$). *Scrippsiella* had a weak, but significant decline in percent germination with depth ($F(1,50) = 6.14$, $p = 0.017$). For every 2 cm increase in depth, the *Scrippsiella* percent germination decreased by <1% ($b = -0.006$; $r = 0.331$, $N = 50$, $p = 0.017$).

5.3.2 Plating error assessment

To accurately interpret our experimental results it was necessary to use the computer model to help quantify the chance that one or more cyst was plated into an individual well ($N_{d,j,i} > 1$, 'plating error'). We could not assume that the number of individual swimming cells recorded in the well plates was correlated to or representative of the number of cysts originally distributed in the well plate. Two swimming cells could be from two cysts that germinated or from one cyst that germinated and subsequent cellular division.

To assess the plating error associated with our method, the computer model was run with *go*=1 (100% germination) for varying cyst concentrations (N_j=2:100) for 1,000 trials (*d)*. All of the N_j cysts that were distributed in each trial (*d*) germinated. The difference between $AG_{d,j}$ and $EG_{d,j}$ represents the number of germinations that would have been unaccounted for in our experimental design (shaded area, Figure 2). Once cysts densities are 6 cysts mL^{-1} or higher, 5% or more of the cyst germinations would not be accounted for in the experimental design. For example, 20 cysts mL^{-1} will, on average be distributed into 17 of the 50 possible wells but could be distributed to as few as 14 or as many as 20 wells 95% of the time (red lines, Figure 2). At cyst concentrations higher than 25 cysts mL^{-1}, on average 20% or more of the cysts (i.e. 5 cysts when N=25) would be distributed into wells with another cyst; at 40 cysts mL^{-1}, on average 30% (i.e. 12) of the cysts will be plated into wells with other cysts; at 80 cysts mL^{-1}, on average 50% of the cysts will be duplicate (Figure 2).

5.3.3 Probability of germination and error assessment

The number of cysts that were counted in sediment samples was relatively low. *Alexandrium* cyst counts ranged from 0-29 cysts mL^{-1} while *Scrippsiella* cyst counts ranged from 0-69 cyst mL^{-1} (Table 1). Indeed, it is common to have relatively small samples sizes when estimating percent germination but it is difficult to assess compounding errors with low sample sizes and a given probability of germination.

The model was used to assess the error associated with using relatively small sample sizes to estimate percent germination by holding the number of cysts (N_j) constant for 1,000 trials (*d)* and varying the probability of germination (*go* = 0:1). As the sample size of cysts (N_j) increases, the precision of the estimated percent germination (g_e) also increases. For example, if 10 cysts

were isolated, monitored and 4 of them germinated, the estimated percent germination would be 40% (g_e). However, a 40% germination of 10 cysts could occur with a germination probability ranging between 18-68% (g_o) based upon the 95% confidence interval (Figure 3). If the sample size were increased to 100 cysts and 40 of them germinated, again the estimated percent germination would be 40% (g_e) ranging from 30-50% (g_o, Figure 3). Conversely, if 30 cysts were isolated and had a known probability of 40% germination (g_o), the actual number of cysts that germinate could range from 2-18 with a mean (± STD) of 9 (± 2.45, Table 2) and the experimental percent germination could range from 22-58% (g_e, Figure 3). As the sample size increases, the estimated error due to probability of germination decreases exponentially (Figure 4).

5.3.4 Using germination plates to confirm percent germination and estimate error

The maximum estimated concentration of *Alexandrium* in our sediment cores, 29 cysts mL^{-1}, suggests that at most, 22% (i.e. 6) of the cysts would be unaccounted for in the germination plates assuming 100% germination; for *Scrippsiella*, as many as 46% (32) of the cysts could be unaccounted for as cyst concentrations near 70 cysts mL^{-1} (Figure 2). Because the estimated percent germination for both *Alexandrium* and *Scrippsiella* is less than 100% (from regression analysis), the error due to potential unaccounted for cyst germinations is also reduced relatively. For example, at 40% germination (g_o), the percent of cysts that germinate and would be unaccounted for is less than 5% (2 cysts) for cyst concentrations up to 35 cysts mL^{-1} (Figure 2).

The simulation model was used to virtually run 1,000 experiments for $N_{d,j}$ cysts (i.e. cyst counts at various depths from each core) with an estimated germination probability (g_e) of 0.35 for *Alexandrium* and 0.65 for *Scrippsiella* (Figures 5,6). The duplicate germination plate samples

from Effingham Inlet cyst counts and germination plate results were combined to reduce error from small sample sizes (solid diamonds, Figure 5). Four of the 98 experimental observations (4.1%) of *Alexandrium* observed cyst germinations fall outside of the range of results predicted by the model. The potential error due to duplicate cysts in any one well is less than 5% for cyst concentrations up to 45 cysts mL^{-1}. The mean cyst concentration of *Alexandrium* cysts from both cores (Sequim Bay + composite Effingham Inlet) was 17.8 ± 10.1 cysts. Thus we can estimate that the percent germination for *Alexandrium* from both cores was 35% ± 10.5% (Figure 4).

Eight of the 53 experimental observations (15.1%) of the *Scrippsiella* experimental observations were outside of the range of results predicted by the computer model with a 65% germination (Figure 6). Five of the outliers were above the 1:1 line indicating that cyst counts potentially underestimated the number of cysts that were plated. The potential error due to duplicate cysts in any one well is less than 5/10% (1/3) for cyst concentrations up to 14/28 cysts mL^{-1}. The estimated percent germination for *Scrippsiella* in the Sequim Bay core was 65% ± 7.8% (Figure 4).

5.4 Discussion

The quantitative germination plate method can be used to estimate percent germination from minimally processed sediments with low concentrations of naturally-produced cysts. Assuming 100% germination, the method is accurate to within ±20% at cysts concentrations below 24 cysts mL^{-1}. If there is not 100% germination in the well plate, the plating error is dramatically reduced. At 40% germination, cyst concentrations as high as 76 cysts mL^{-1} would, on average, have less than 10% error (Figure 2). To reduce the error from using small sample sizes to

estimate percent germination (i.e. less than 30 cysts mL^{-1}) but increase the accuracy of the quantitative germination plate method, multiple germination plates from the same sample should be produced. Results from individual plates can be combined to increase the sample size, thereby statistically justifying the percent germination estimate.

There are many advantages to using the quantitative germination plate method to estimate percent germination of cysts in sediments. It is relatively quick and efficient to set up and therefore a larger volume of samples can be processed in a short amount of time. Human bias is reduced because this method does not require the operator to visually identify and manually isolate cysts. Further, cysts are less likely to be damaged because sediments are minimally processed relative to other methods. Both *Scrippsiella* and *Alexandrium* exhibited higher percent germination estimates when using the germination plate method vs. cyst isolations (Figure 1).

The errors that cannot be accounted for in this method are potential counting errors when estimating cyst concentrations and the degree of inhomogeneity of the sediment slurry when preparing germination plates. Five *Scrippsiella* observations were above the 1:1 line suggesting that in some cases the number of cysts counted and hence, estimated number of cysts plated, is lower than what was placed into the germination plate or the sample was not homogenous (Figure 6). The germination plate method is only suitable to estimate the percent germination of cysts that produce a vegetative cell capable of surviving a week in the 96-well plate. Thus, it is highly likely that many more cysts 'germinated' but did not produce a long-lived, swimming vegetative cell (Vahetera et al., 2014; Feifel et al., submitted). This cannot be accounted for using this method.

The long term survival of resting cysts produced by the harmful genus *Alexandrium* in natural sediments has been suggested to be 100+ years (Miyazono et al., 2012). They concluded that the percent germination decreased from 100% to 0% as the cysts aged based upon isolations of 7-20 cysts from each of a total of 8 depths in a 31 cm sediment core but there was no error estimate associated with their results. The relatively small sample sizes used in their analysis lessens the statistical significance of results. Sample sizes of 7-20 cysts would have a 20 and 10% standard deviation respectively on any percent germination estimate (Figure 4). This suggests that the statistical error in the sampling strategy could be relatively large, particularly at the lowest N values where there is an increased probability of none to a few cysts germinating despite a high percent germination (Table 2). This type of error could be reduced by increasing the sample size or repeating the experiment.

Using a simulation model to virtually replicate quantitative germination plate allows one to examine the range and type of data that could be statistically generated from any given experiment. The simulation model can help detect statistical outliers from the expected range of model results and confers the ability to comprehensively assess synergistic interplays from varying sample sizes and a range of percent germination. This method is quick and can complement conventional cyst counts using a microscope. There is a recognized need to have a method that can quickly, accurately and reliably estimate percent cyst germination in natural solutions. The quantitative germination plate method and simulation model presented here can help to meet this need.

5.5 References

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Table 5-1 Cyst counts and germination success in sediment cores.

	Sequim Bay core		Effingham Inlet core		
	Alexandrium	*Scrippsiella*	*Alexandrium*		*Scrippsiella*
N	29	53	46	23	46
# of wells with germination	0-10	0-40	0-11	1-19	0
Maximum depth with germination	77 cm	63 cm	12.5 cm		N/A
Range cyst counts	0-29	0-68	3-25	7-46	0
Mean cyst counts	12 ± 2	32.3 ± 5	12.5	25.1	0

Table 5-2 Results from 1,000 model simulation of N cysts under g_o percent germination. The number listed is the average number of cysts that would be expected to germinate ± STD. The numbers in the parentheses indicates the range of the model.

	Percent germination								
# of cysts	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**
5	1 ±0.68 (0-3)	1 ±0.88 (0-4)	1 ±1.02 (0-5)	2 ±1.10 (0-5)	3 ±1.13 (0-5)	3 ±1.07 (0-5)	3 ±1.00 (0-5)	4 ±0.88 (1-5)	4 ±0.67 (1-5)
10	1 ±0.95 (0-5)	2 ±1.27 (0-7)	3 ±1.42 (0-8)	4 ±1.54 (0-9)	5 ±1.66 (1-10)	6 ±1.50 (1-10)	7 ±1.41 (3-10)	8 ±1.26 (3-10)	9 ±0.94 (5-10)
30	3 ±1.67 (0-10)	6 ±2.14 (0-13)	9 ±2.45 (2-18)	12 ±2.73 (4-21)	15 ±2.72 (6-23)	18 ±2.76 (10-26)	21 ±2.41 (15-28)	24 ±2.19 (17-30)	27 ±1.72 (21-30)
70	7 ±2.46 (1-16)	14 ±3.38 (4-26)	21 ±3.92 (9-33)	28 ±4.20 (15-42)	35 ±4.03 (23-50)	42 ±4.29 (29-54)	49 ±3.93 (34-61)	56 ±3.39 (43-68)	63 ±2.41 (55-69)
100	10 ±3.04 (2-22)	20 ±4.02 (8-34)	30 ±4.63 (17-44)	40 ±5.12 (23-59)	50 ±5.32 (33-69)	60 ±4.69 (46-76)	70 ±4.52 (53-83)	80 ±3.99 (66-93)	90 ±3.05 (80-98)
300	30 ±5.21 (15-48)	60 ±7.0 (38-83)	90 ±7.94 (63-115)	120 ±8.61 (96-152)	150 ±8.53 (124-177)	180 ±8.63 (155-207)	210 ±7.85 (185-234)	240 ±6.87 (208-264)	270 ±5.30 (251-286)

Figure 5.1 Top row: Number of cysts counted in sediment sample compared to results from the 'quantitative germination plate' from the same sample - the number of wells that had a vegetative cell in it after one week. Bottom row: Number of cysts isolated by hand compared to the number of cysts that germinated. The color bar indicates the sample depth (cm) in the sediment core.

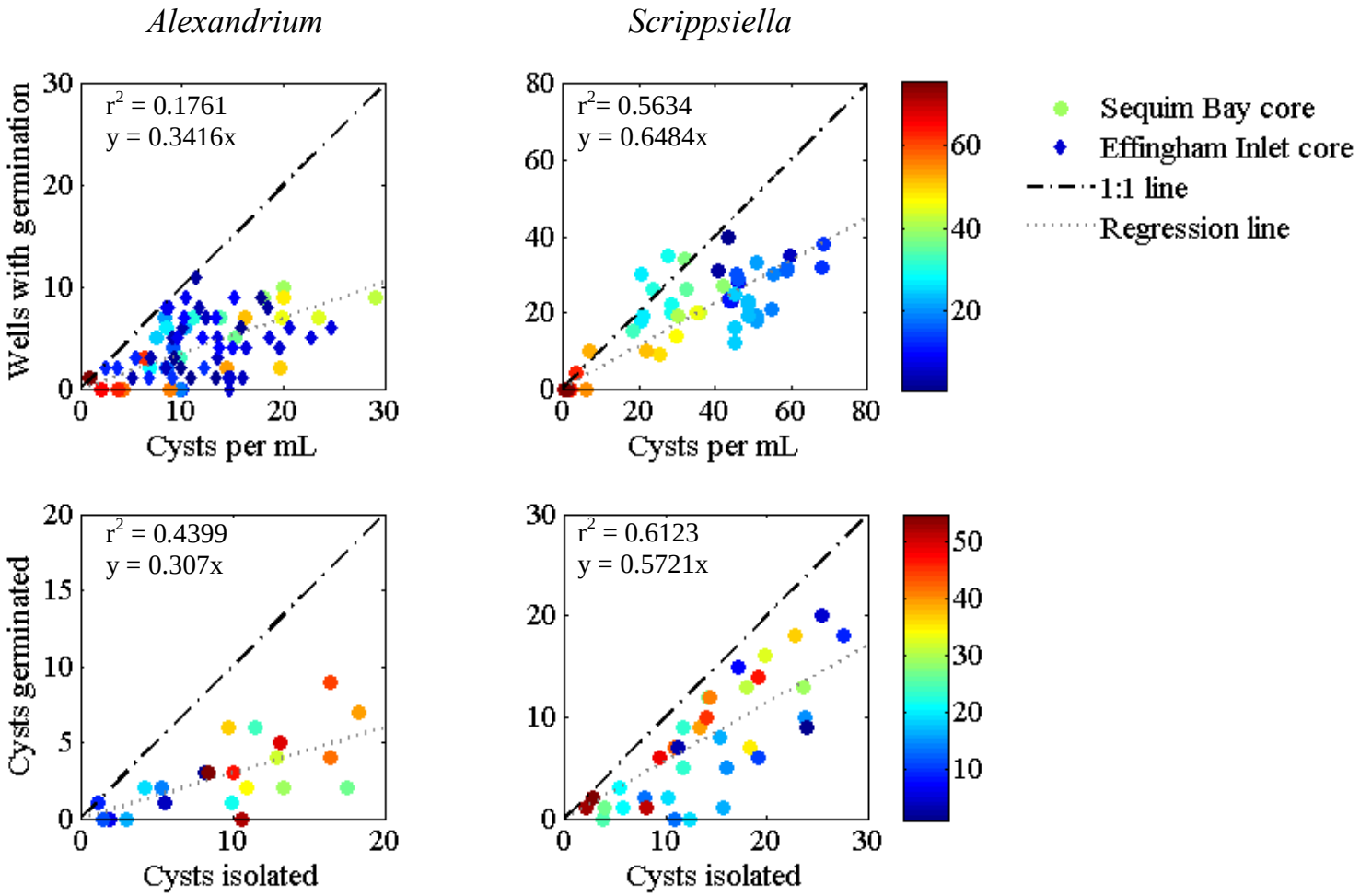

Figure 5.2 The percent of uncounted germinations is estimated by taking the average number of germinations uncounted (shaded area) divided by the number of cysts plated (N). As the percent germination (g_o) decreases, the percent of uncounted germinations decreases. Presented results are from 1,000 simulations.

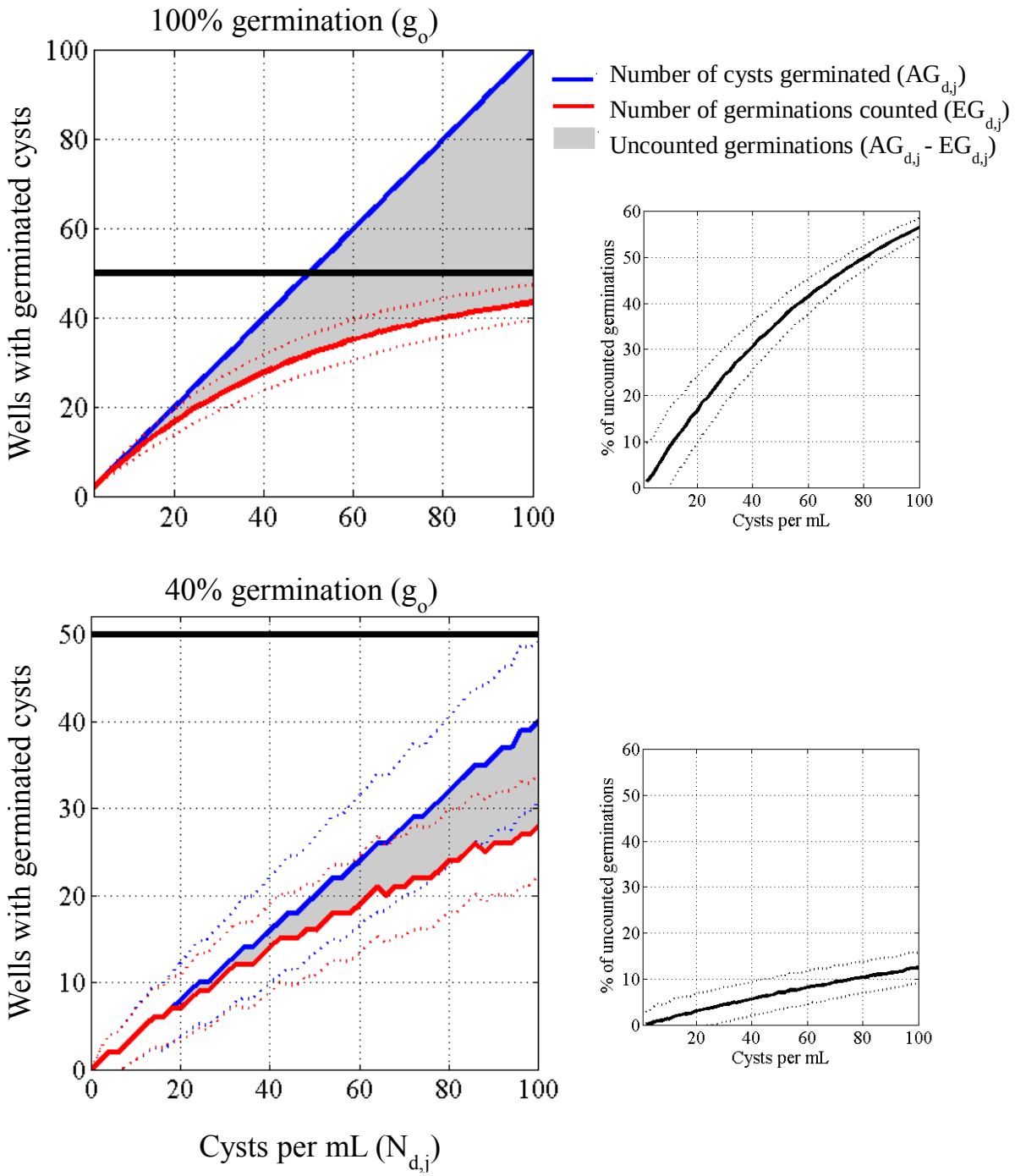

Figure 5.3 Estimating error due to sample size. The shaded polygons are the 95% confidence intervals for their respective sample size.

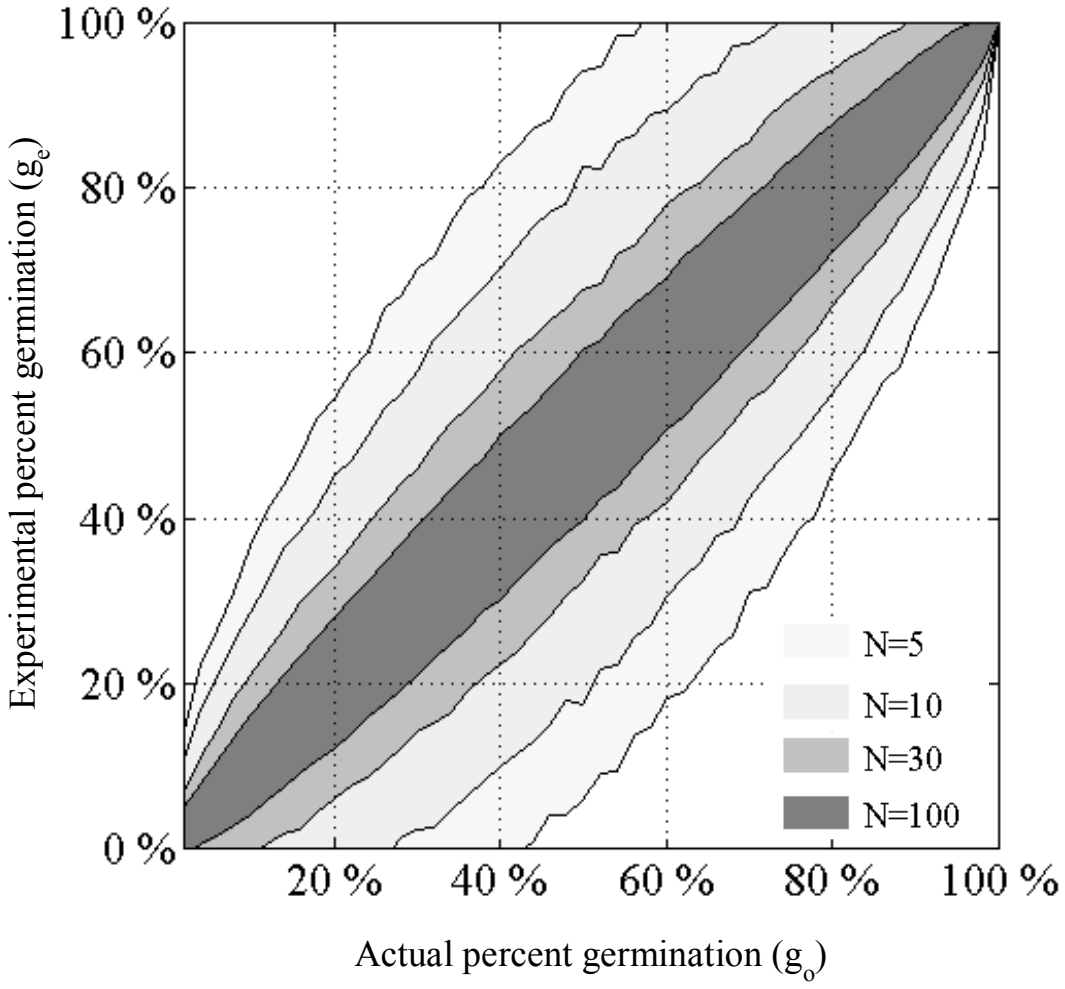

Figure 5.4 Estimating standard deviation for sample sizes. This graph estimates the standard deviation (± STD) for any cyst germination experiment for a given sample size (N).

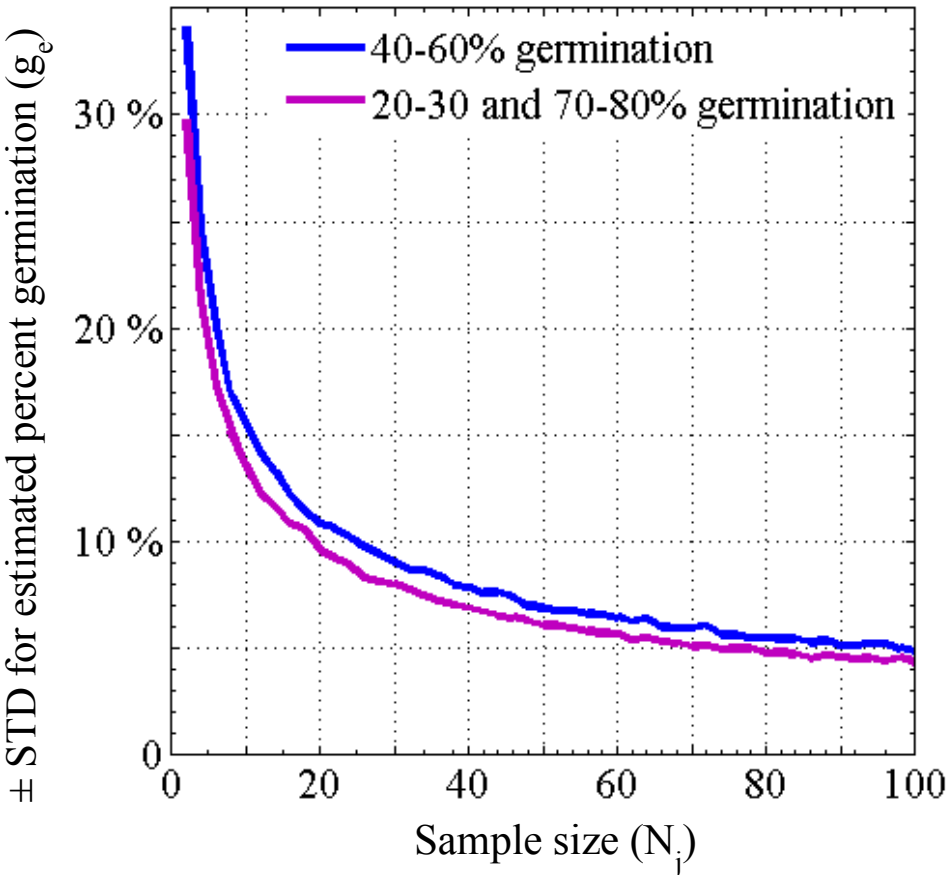

Figure 5.5 Results from 1,000 simulations for 35% germination. The results from the quantitative germination plates for *Alexandrium* are overlaid for all depths from both sediment cores.

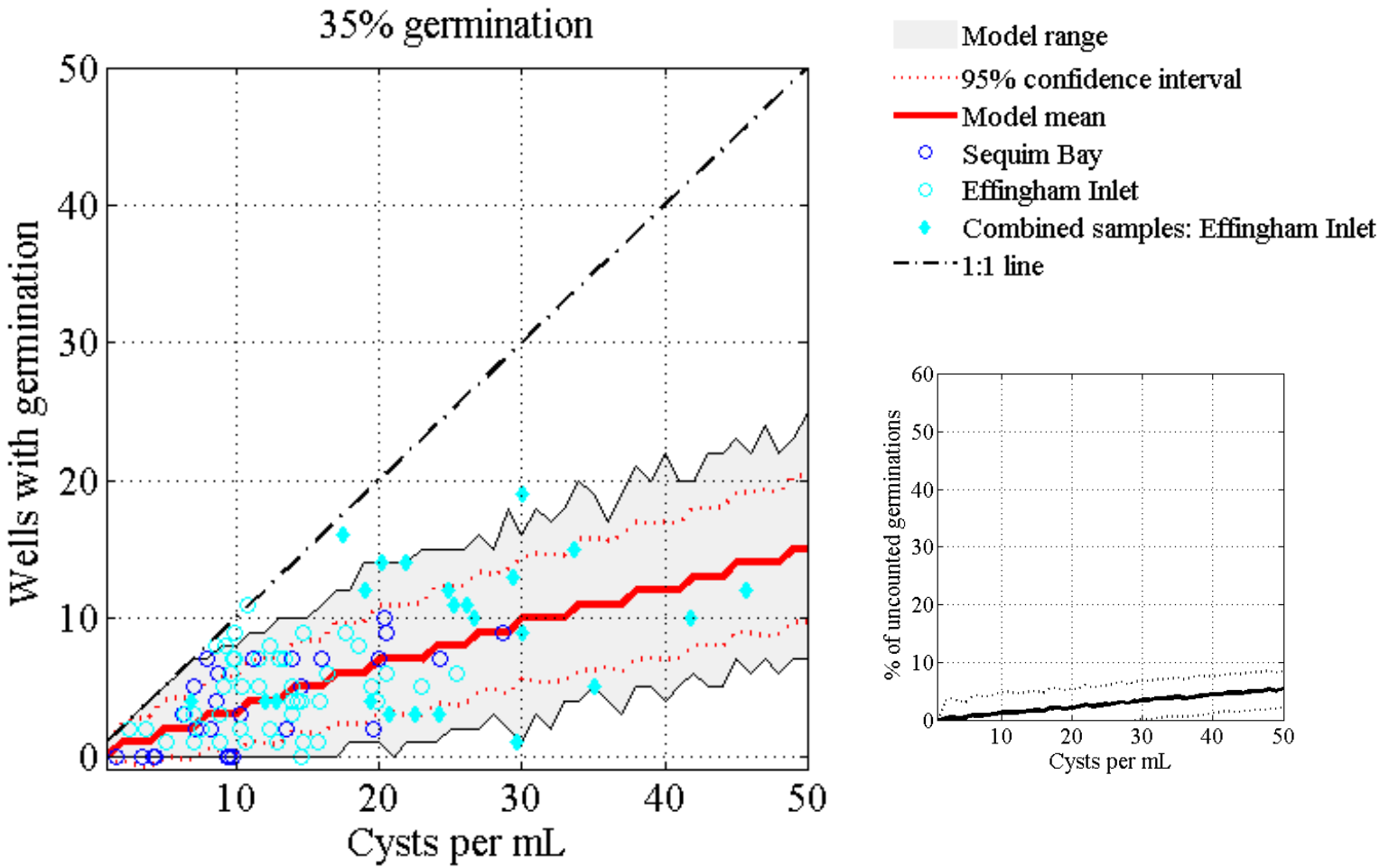

Figure 5.6 Results from 1,000 simulations for 65% germination. The results from the quantitative germination plates for *Scrippsiella* are overlaid for all depths from the Sequim Bay sediment core.