Alexandrium spp. and *Scrippsiella* spp. cyst germination success and cytoplasmic fullness in a
60-cm sediment core from Sequim Bay, WA

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Abbreviations:

FSW: filtered sea water, HAB: harmful algal bloom, PSP: paralytic shellfish poisoning, SD:
standard deviation, SPT: sodium polytungstate

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 to provide the inoculum for subsequent
blooms or, be buried by sediment deposits over time and entrained into the sedimentary record.

Buried cysts can be resuspended by mixing events (e.g. wind and storms) or other disturbances (e.g. dredging). It is not clear how long the cysts can survive while buried in the sediments and still be capable of germinating. Here, the germination success of cysts produced by the potentially toxic dinoflagellate genus *Alexandrium* and the non-toxic dinoflagellate genus *Scrippsiella* is reported from a 60-cm sediment core collected in Sequim Bay, WA, in December 2011. Cysts of *Alexandrium* spp. and *Scrippsiella* spp. were isolated from 2-cm sections of the core, placed in individual wells of a 96-well plate with growth medium, imaged, incubated at favorable conditions and monitored for germination. An image analysis program, DinoCyst, was used to quantitatively measure the amount of granular storage products (presumed energy stores) inside the cytoplasm to test the hypothesis that older cysts located deeper in the sediment core will have a smaller amount of energy stores available and will be less likely to germinate. An index of the area of the cytoplasm occupied with granular storage products relative to cyst size, termed 'cytoplasmic fullness', and age, based on ^{210}Pb dating of surrounding sediments, were compared with germination success or failure. This research indicates that cysts of *Alexandrium* spp. and *Scrippsiella* spp. can remain viable in sediments for 60 years or longer, show little visual evidence of cytoplasmic deterioration over this timescale (as measured by cytoplasmic fullness), and that germination success is statistically similar for cysts isolated from 0-60 cm deep in the sediment core. These results suggest that a cyst's cytoplasmic fullness is not indicative of viability and that cysts located as deep as 60 cm in the sediments are as likely to germinate as surface cysts given favorable conditions.

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48 1.1 Introduction

49 Many planktonic protists produce benthic resting stages in their life histories but little is known
50 about their long-term survival while in the sediments. Resting cells, cysts and spores serve as a
51 seed bank to help a species withstand adverse environmental conditions and repopulate the water
52 column once conditions suitable for vegetative growth arise (Wall, 1971). It has been postulated
53 that resting stages evolved to aid in long-term survival of plankton during the prolonged periods
54 of reduced solar transmission that may have occurred during ice ages and/or after catastrophic
55 meteor strikes (McMinn and Martin, 2013; Ribeiro et al., 2011). This suggests that some
56 dinoflagellate cysts could survive for long periods in the sediments. A variety of approaches
57 have been used to try to better constrain the lifetime of cysts in the natural environment. Previous
58 studies have estimated cyst degradation and survival rates based on mathematical models (Keefer
59 et al., 1992; Shull et al., 2014), physical examination of a cyst's structural integrity (Head et al.,
60 2006), available energy reserves and respiration rates (Binder and Anderson, 1990) and long-
61 term viability experiments using both stored and natural sediments (Lewis et al., 1999; Ribeiro et
62 al., 2011).

63

64 The potentially toxic genus *Alexandrium* is a cyst-forming dinoflagellate found worldwide in
65 temperate coastal waters (Anderson et al., 2012). Many species within the genus can produce a
66 suite of toxins known to cause paralytic shellfish poisoning (PSP). The presence of PSP-
67 associated toxins in shellfish above regulatory limits for human consumption can cause shellfish
68 harvest closures. These closures result in economic losses to the aquaculture industry and coastal
69 communities dependent upon recreational shellfish tourism as a source of income (Anderson et

al., 2000; Hoagland et al., 2002). Because of the human health and economic impacts of PSP, there is broad interest to better understand how long the benthic cyst stage of *Alexandrium* spp. remains viable in the sediments. This is especially true for areas with a history of PSP that experience sediment disturbance events – managers need to know if there is an increased risk of *Alexandrium* spp. blooms due to resuspension of “buried” cysts.

The *Alexandrium* species found in western Washington waters historically has been identified as *Alexandrium catenella* (Whedon & Kofoid) Balech (Nishitani and Chew, 1984). However, other species of *Alexandrium* have been reported in the Puget Sound and along coastal Washington (Horner et al., 1997). Recently, it has been proposed that *A. catenella* is part of the Group I strains within the *A. tamarense* species complex (John et al., 2014a) and that it be referred to as *A. fundyense* (John et al., 2014b). In light of continued taxonomic questions in the *A. tamarense/catenella/fundyense* complex it will be referred to here by its genus. In the U.S. Pacific Northwest, cysts produced by the non-toxic dinoflagellate genus *Scrippsiella* commonly co-occur in the sediments with *Alexandrium* spp. cysts (Feifel, unpublished data). Similar to *Alexandrium*, defining a species within the genus *Scrippsiella* is difficult (Montresor et al., 2003; Zinssmeister et al., 2011). Historically, the species of *Scrippsiella* found in the waters of coastal western Washington has been identified as *Scrippsiella trochoidea* (Tomas, 1996) but it will be referred to by its genus here. *Scrippsiella* spp. cysts tend to be smaller than *Alexandrium* spp. cysts and shaped more like a teardrop.

The cyst stage of dinoflagellates has an important role in seeding annual blooms. Once a newly-formed cyst has completed a mandatory dormancy period and is matured, it can remain in a

quiescent stage on the seafloor and is able to germinate under favorable environmental conditions (Anderson, 1980; Bravo and Anderson, 1994). Depending on species and environmental conditions, dormancy requirements have been estimated to range from <15 to 180 days for *Alexandrium* spp. (Anderson, 1980; Genovesi-Giunti et al., 2006) and 25 days to 6 months for *Scrippsiella* spp. (Binder and Anderson, 1987; Kremp and Anderson, 2000). Upon germination, a motile vegetative cell is released from the cyst that is able to divide, and in the case of some *Alexandrium* spp., form potentially toxic blooms. However, some mature dinoflagellate cysts do not germinate and instead become buried by sediment deposits and entrained into the sedimentary record. If *Alexandrium* spp. and *Scrippsiella* spp. cysts are buried below the depth that oxygen can penetrate, the cysts cannot germinate (Anderson et al., 1987; Kremp and Anderson, 2000). Presumably over some length of time, the dinoflagellate cysts in the anoxic sediment deteriorate to the point that germination becomes impossible even if reintroduced into surface sediments with favorable conditions.

Understanding the long-term viability of *Alexandrium* spp. and *Scrippsiella* spp. cysts in subsurface sediments will help to elucidate the potential for older cysts that are disturbed and mixed to the sediment surface to germinate and initiate blooms (e.g. Butman et al., 2014). Estimates of how long *Alexandrium* spp. cysts remain viable while buried in the sediments vary considerably. Keafer et al. (1992) suggest a half-life of 2-10 years for *A. tamarense* cysts buried in a shallow salt pond in Massachusetts. However, Miyazono et al. (2012) germinated an estimated 100-year old *A. tamarense* cyst from Funka Bay, Hokkaido, Japan. *Scrippsiella* spp. cysts have been identified in sediments from Koljö Fjord, Sweden that were deposited 90 years ago and cysts estimated to be 40 years old were able to germinate (Lundholm et al., 2011). In the

U.S. Pacific Northwest, *A. catenella* cysts have been identified 1 m deep in sediments estimated to have been deposited 120 years ago (Feifel et al., 2012), but it is not known if these cysts were able to germinate.

Most photosynthetic protist resting stages are thought to lose their ability to photosynthesize and survive in the sediments by reducing respiration rates and relying on internal energy stores in the cytoplasm for survival. The internal contents within a dinoflagellate cyst cytoplasm have been referred to as “storage compounds” (Binder and Anderson, 1990), “intracellular storage products” (Bravo and Figueroa, 2014; Genovesi et al., 2009), and “granular cytoplasm” (Vahtera et al., 2014). There has been scant research to confirm that the amount and content of material inside the cytoplasm represents the amount of energy available in the cyst to germinate and produce a dividing cell. Binder and Anderson (1990) found that cysts produced by *S. trochoidea* displayed a 60-fold reduction in cyst metabolism compared to its vegetative stage and that newly-formed cysts were primarily composed of lipids and carbohydrates. There has been little follow-up work to assess the chemical composition of energy stores inside cysts produced by other dinoflagellate species. Light microscopy work has shown that the presumed energy storage products found in the cytoplasm of dinoflagellate cysts tend to be granular in appearance but there can be quite a bit of inter and intra-species variation (Bravo and Figueroa, 2014). Here, the golden-brown, granulated intracellular contents in the cytoplasm of cysts produced by *Alexandrium* spp. and *Scrippsiella* spp. will be referred to as “granular storage products”. The amount of granular storage products and the presence of an accumulation body in the cytoplasm are commonly cited by researchers in cyst isolation work as a potential indicator of cyst health and viability (e.g. Lundholm et al., 2011; Moore et al., 2015).

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140 The cytoplasm of *Alexandrium* spp. and *Scrippsiella* spp. cysts can range from almost entirely
141 full of granular storage products to almost empty with only a few, colorless vacuoles (Genovesi
142 et al., 2009; Miyazono et al., 2012). Genovesi et al. (2009) suggested that the internal cellular
143 contents of laboratory-produced *A. tamarense* cysts systematically evolved from a newly-formed
144 resting cyst with a cytoplasm full of golden-brown granular storage products to a final-stage cyst
145 that exhibits transformation of colored granular storage products to colorless vacuoles in the
146 cytoplasm as the cysts aged over one year. In contrast, Binet and Stauber (2006) found that the
147 viability of laboratory-produced *A. catenella* cysts could not be predicted based upon cyst size,
148 natural fluorescence, or the internal cell structure found in the cytoplasm. They did not find a
149 correlation between a cyst's physical appearance and its ability to germinate. These studies
150 examined laboratory-produced cysts; there have not been any studies that examine the amount of
151 granular storage products in the cytoplasm of *Alexandrium* spp. and *Scrippsiella* spp. cysts
152 produced in the environment (termed 'naturally-produced' cysts) in relation to cyst viability or
153 age.

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155 In this study, the long-term viability of dinoflagellate cysts produced by *Alexandrium* spp. and
156 *Scrippsiella* spp. in a 60-cm sediment core was assessed. The following hypotheses were tested:
157 (1) cysts buried deeper in natural sediments and hence, older relative to surface cysts, will have
158 fewer granular storage products inside the cytoplasm as energy stores are respired over time; (2)
159 older cysts will be less likely to germinate compared to cysts isolated from the sediment surface;
160 and (3) the amount of granular storage products in the cytoplasm can be used as a primary

indicator of cyst viability. This research will help to better constrain the long-term viability of *Alexandrium* spp. and *Scrippsiella* spp. cysts in natural sediments.

2.1 Methods

2.1.1 Sediments

A 1-m sediment core was extracted from Sequim Bay, WA (48.09 °N, 123.03 °W) at 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 and discarded. Each interval was transferred to a plastic bag, sealed with no air spaces, and placed in the dark at 2 °C for 6 months. The 6 month storage time allowed any immature cysts present in our samples to complete a mandatory maturation period (Anderson, 1980). Core sections for the cyst isolation work 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 Octete PC, both with ULTRA series detector model BU-017-450-100), a naturally produced radioactive granddaughter from ^{222}Rn (Nitttrouer et al., 1979b). Estimates of background levels of ^{210}Pb were taken from previous work on a sediment core from Sequim Bay (Feifel et al., 2012).

To isolate the dinoflagellate cysts, a 5 cm³ subsample was diluted into 30 mL of 2 °C 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 °C FSW. The

sediment captured on the 20 μm filter was then concentrated, washed back into a 50 mL centrifuge tube using 2 °C FSW and the volume was raised to 30 mL using 2 °C FSW. The 30 mL sediment slurry was vortexed for one minute and one 7 mL subsample was taken and transferred into a clean 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. 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 cysts and associated 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 2 °C FSW. Cysts that were still adhered to heavier sediment particles after sonication may have passed through the SPT density gradient. The SPT treatment is not meant to be a quantitative process; cysts that were not captured at the density-gradient interface were not accounted for. The cyst concentrate was stored in the dark at 2 °C prior to cyst isolation.

Raw sediment incubations were done in tandem to the cyst isolation work. One mL of the remaining 23 mL of FSW+sediment slurry was evenly distributed across 50 wells of a 96-well plate and incubated at the same conditions described below for the isolated cysts. The raw

sediment incubations were checked for signs of *Alexandrium* spp. and *Scrippsiella* spp. cyst germination after one week.

2.1.2 Cyst isolation

Within 24 hours, *Alexandrium* spp. and *Scrippsiella* spp. cysts were isolated from the cyst 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 by mouth pipette using a drawn-out capillary tube under a standard microscope (Zeiss, West Germany, 4730 28) into wells of a 96-well plate filled with 100 μ L of f/2 –Si media. This process was repeated for the remainder of the cyst concentrate. 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 °C on a 12 dark: 12 light (20 μ E 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). The entire volume of each well was checked by focusing from the bottom to the top of the well multiple times and carefully examining the well's perimeter. If the cyst remained intact, no excystment (N) was recorded. If there was a live cell swimming that had undergone mitosis and divided at least once

in the well plate, successful germination (Y) was recorded. If the cyst was emptied 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; Vahtera et al., 2014).

2.1.3 Image analysis

Cyst images were analyzed using a custom-designed image analysis program called DinoCyst. DinoCyst is a plug-in which utilizes the freeware ImageJ software (Schneider et al., 2012) to determine the dimensions, cyst area and the area of granular storage products in the cytoplasm using a photo image. For each cyst image, 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 area of granular storage products is calculated by color thresholding the masked cyst image (Figure 2c). The cell wall is then subtracted from the estimated area of granular storage products. The DinoCyst version used in this study does not differentiate between the types of contents in the cyst (i.e. accumulation body vs. starch granules); it identifies contents based upon their color and then images were manually cross-checked to assure that the cytoplasmic contents that DinoCyst identified were either granular storage products and/or the accumulation body. A conversion factor of pixels to micrometers was calculated by taking an image of a micrometer ruler under the same magnification as the photo images and using ImageJ to count the number of equivalent pixels per micrometer.

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254 To ensure that DinoCyst was accurately identifying and masking the cysts, a method to estimate
255 image analysis error was developed. To estimate DinoCyst's image analysis accuracy, a polygon
256 was created based on the cyst mask by drawing straight lines around the outermost pixels (Figure
257 2b). The resulting polygon (Figure 2e) was then compared to the cyst mask image (Figure 2b). If
258 there was perfect overlap between the two polygons, the confidence that DinoCyst is accurately
259 identifying the cyst would be 100%. The percent difference between the confidence polygon
260 (Figure 2e) and the cyst mask (Figure 2b) allowed us to estimate the confidence that the picture
261 was of high-enough quality to be included in further analysis. Low image quality was commonly
262 associated with out-of-focus images and when particles or mucilage were attached to the external
263 perimeter of the cysts (see Figures 1, 2). For the image analysis portion of this paper, only data
264 from images that had an image accuracy above 85% for *Alexandrium* spp. and 90% for
265 *Scrippsiella* spp. are presented. For example, data from the *Alexandrium* spp. cysts seen in
266 Figures 1a and 2 were not appropriate for image analysis because their image accuracies were
267 below 85% due to the mucilage attached to the perimeter of the cyst and the focus.

268

269 3.1 Results

270 3.1.1 Cyst isolations

271 A total of 285 *Alexandrium* spp. and 508 *Scrippsiella* spp. cysts were isolated and observed for
272 germination from May, 2012 to January, 2013. Sediment sections from 11-30 cm deep in the
273 core were processed both in May-June and again in December-January (Table 1).

274 Contemporaneous cyst counts in each 2-cm sediment section using epifluorescence microscopy

techniques (Yamaguchi et al., 1995) found the highest densities of *Alexandrium* spp. cysts between 41-51 cm deep (Feifel, unpublished data).

3.1.2 Chronology

Lead-210 analysis indicated that the mixed layer was well-mixed and extended to 21 cm (Figure 3). The mixed layer is identified by the vertically 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 (Nittrouer et al., 1979a). The best-fit line to the decaying ^{210}Pb record ($r^2 = 0.82$, $p < .05$, $N = 9$) was used to estimate the sediment accumulation rate of 0.69 cm y^{-1} . This accumulation rate agrees with published sedimentation rates in Sequim Bay of 0.6 cm y^{-1} (Cox et al., 2008). As such, every 2-cm interval in the core represents roughly 2.89 years of accumulated sediment.

The maximum residence time of any particle in the mixed layer was estimated to be 31.1 years. Assuming steady-state conditions over the timescale captured by the length of the sediment core examined in this study, minimum cyst age can be estimated from the depth in the sediment and the accumulation rate. The minimum cyst age was estimated by the length of time since cyst burial below the mixed layer. This was calculated by subtracting the mixed layer depth (21 cm) from the depth the cyst was isolated at and multiplying the difference by the accumulation rate. For example, any cyst isolated from 30 cm depth would have been buried below the mixed layer 13 years prior to core extraction. However, a cyst at 30 cm depth could be as young as 13 years or as old as 44+ years considering the 31 year average residence time in the mixed layer. Here, minimum cyst age is conservatively reported with the notable caveat that any cyst below the

mixed layer could be 30+ years older than the ages presented herein due to the effects of mixing. Cysts isolated from the upper 21 cm, the mixed layer, are assumed to be composed of a mixed-age assemblage between <1-31 years old.

3.1.3 Image analysis

Of the 285 images of *Alexandrium* spp. cysts, 152 were of high-enough quality (i.e. not blurry, no particles attached to mucilaginous layer) to be accurately processed using DinoCyst.

Sediment sonication was done to loosen particles and mucilage that may be adhered to the dinoflagellate cysts but often times the *Alexandrium* spp. cysts were still coated with a mucilaginous layer after isolation (see Figures 1a, 2).

Two morphotypes of *S. trochoidea* cysts have been identified in sediment samples (Janofske, 2000; Wang et al., 2007). ‘Spiny-type’ cysts have short, calcareous spines extending from the cyst perimeter while ‘naked-type’ cysts lack the spines. The presence of spines would complicate image analysis using DinoCyst. More than 98% of the *Scrippsiella* spp. cysts isolated from the Sequim Bay sediment core were classified as ‘naked-type’ (see Figure 2). Of the 508 images of *Scrippsiella* spp. cysts, 361 were appropriate for image analysis (i.e. no spines, not blurry, no particles attached to perimeter).

The mean ($\pm$ standard deviation [SD]) dimensions of the imaged *Alexandrium* spp. cysts were $44.1 \pm 0.7 \times 27.6 \pm 0.5 \mu\text{m}$ and $32.5 \pm 0.6 \times 22.6 \pm 0.4 \mu\text{m}$ for *Scrippsiella* spp. cysts. There was no statistically significant difference between the cytoplasmic fullness of cysts that germinated (Y), excysted but had no live cell (ECNC) and did not germinate (N) as determined by one-way

ANOVA for either *Alexandrium* spp. ($F(2, 149) = .080, p = .923$) or *Scrippsiella* spp. ($F(2, 358) = .632, p = .532$; Figure 4). There was no significant trend in *Alexandrium* spp. cyst cytoplasmic fullness with depth in the sediment core ($r^2 = 0.005, p = .393, N = 152$; Figure 5). There was a weak, but statistically significant negative trend with *Scrippsiella* spp. cytoplasmic fullness and depth in the sediment core ($r^2 = .023, p = .004, N = 361$; Figure 5). *Scrippsiella* spp. 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.

3.1.4 Germination success in the sediment core

Successful germination was considered to occur if a live, swimming cell was observed in the wells or if an opening in the cyst was observed but a swimming cell could not be found in the well (i.e. ECNC+Y, Binder and Anderson, 1987; Vahtera et al., 2014). The germination success of *Alexandrium* spp. cysts isolated between the months of December and January was 7%, lower than the average germination success of 26% recorded during the months of May-November (Table 1). *Alexandrium* spp. surface cysts isolated from Quartermaster Harbor, WA, located approximately 100 km south of Sequim Bay, also show reduced germination success in December and January, potentially part of a weak annual oscillation (Moore et al., 2015; Tobin and Horner, 2011). It remains unclear if *Alexandrium* spp. cysts in Puget Sound possess an endogenous clock (Anderson and Keafer, 1987) but, out of precaution, *Alexandrium* spp. germination analysis within the sediment core is limited to data from cysts that were isolated between May and November. This reduces the *Alexandrium* spp. sample size suitable for germination analysis to 243 cysts. To date, there has not been any research that has conclusively documented the presence of an endogenous clock in *Scrippsiella* spp. In this study, there was

reduced germination in December and January (Table 1). Hence, *Scrippsiella* spp. germination analysis was also limited to the 408 cysts isolated between May and November.

Germination of *Alexandrium* spp. cysts in each 2-cm sediment interval varied from 0-60% with a mean of 26%; *Scrippsiella* spp. germination success varied from 9-88% with a mean of 57%. Depth had no influence on the likelihood that a cyst would germinate for *Alexandrium* ($X^2(5, N = 242) = 9.674, p = .085$; Figure 6). Of the 63 *Alexandrium* spp. cysts that germinated, 12-41% produced a germling cell that successfully divided in the wells. In contrast, germination success of *Scrippsiella* spp. cysts was not constant with depth ($X^2(5, N = 408) = 24.926, p < .01$). This result is primarily driven by the relatively low germination success seen between 11-20 cm depth ($X^2(5, N = 408) = 16.152, p < .01$; Figure 6). There were too few *Scrippsiella* spp. cysts isolated from depths 51-60 cm to detect any significant decrease in germination despite its relatively low germination success relative to other depths (Table 1). Zero to 55% of the 233 *Scrippsiella* spp. cysts that germinated produced dividing germling cells.

4.1 Discussion

In this study, *Alexandrium* spp. and *Scrippsiella* spp. cysts isolated from a 60-cm sediment core, estimated to be between <1-60+ years old, did not show any significant evidence of deterioration in cytoplasmic fullness or germination success with depth/age (Figures 5, 6). The cytoplasmic fullness of a cyst did not indicate its age or its ability to germinate and/or produce a dividing cell (Figures 4, 6). Germination success was statistically similar for *Alexandrium* spp. and for most of the *Scrippsiella* spp. cysts throughout the depth of the sediment core (Figure 6). In raw sediment incubations from sediments taken from 77 and 63 cm deep respectively, *Alexandrium* spp. and

Scrippsiella spp. cysts successfully germinated and produced live germling cells (Feifel, unpublished data). Based upon the sediment accumulation rate, these cysts' ages are conservatively estimated to be 81 and 61 years or older and were most likely entrained in the sediment during the 1930s and 1950s or earlier. Germination of *Alexandrium* spp. and *Scrippsiella* spp. cysts from various depths could occur throughout most of the calendar months in which cysts were isolated although there was decreased germination in December and January (Table 1).

If the cytoplasmic fullness is a reliable indicator of energy stores, it would be expected to shrink due to respiration as a cyst ages. Thus, a cyst with less granular storage products in its cytoplasm would be predicted to be older and have less energy available to successfully develop a dividing cell given favorable conditions for germination. There is no evidence of this in the data (Figure 4). Further, the cytoplasmic fullness of cysts from natural sediments does not decrease with cyst age (Figure 5). Taken together, these results argue that the cytoplasmic fullness of a cyst is not a primary indicator of viability or age over the timescales examined in this study. Presumably there is some lower threshold where a cyst does not have enough energy stores to create a dividing cell. The relatively small sample size of *Alexandrium* spp. 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* spp. ($N = 73$), data would suggest that a cyst needs to have a minimum cytoplasmic fullness of 47% in order to produce a dividing germling (Figure 4). However, no *Scrippsiella* spp. cysts were isolated and imaged that had a cytoplasmic fullness of less than 37% so a lower cytoplasmic fullness threshold cannot be definitively defined.

390 The mean germination success (ECNC + Y) of cysts of *Alexandrium* spp., 26%, and *Scrippsiella*
391 spp., 57%, throughout the core are comparable to other estimates of viability from surface cysts
392 in estuarine sediments. Gracia et al. (2013) used the live/dead stain SYTOX green to determine
393 that on average, 28% of the *A. tamarense* resting cysts in the St. Lawrence estuary, Canada were
394 viable although there was a high degree of site heterogeneity. *Scrippsiella* spp. cysts isolated in
395 surface sediments from a fjord in Sweden displayed germination rates of 50-60% (Lundholm et
396 al., 2011). Of the cysts which germinated throughout the sediment core used in this study, on
397 average 29% and 37% of the *Alexandrium* spp. and *Scrippsiella* spp. cysts respectively, produced
398 cells able to undergo mitosis and divide at least once (Y, Figure 6). The results for *Alexandrium*
399 spp. from this study are similar to others who have found that 27% of naturally-produced *A.*
400 *tamarense* cysts (Genovesi et al., 2009) and 47% of *A. fundyense* (Vahtera et al., 2014) were able
401 to germinate and produce cells capable of dividing at least once. These results are an important
402 reminder for any cyst germination experiment that uses the number of empty cysts present as an
403 estimate of the number of cyst germinations which produce a vegetative cell able to promote a
404 bloom. In this study, most of the cysts which “germinated” (ECNC+Y) did not produce a cell
405 capable of dividing (Figures 6). If our results translate to the environment, than a majority of the
406 cysts that are capable of germinating do not actually produce germlings able to divide and
407 populate a bloom (Vahtera et al., 2014). The low rates of germling survival from environmental
408 samples have been suggested to be evidence of a mixed-age assemblage of cysts, inferring that
409 older cysts on the sediment surface are less viable because they have fewer energy reserves
410 available to support the metabolic demands of germination (Genovesi et al., 2009; Vahtera et al.,
411 2014). Provided the amount of granular storage products inside the cyst is a relative indicator of
412 the energy reserves available for the germling, it can be concluded that the loss of energy

reserves as a cyst ages cannot fully account for the relatively low survival of dividing cells in the lab. An alternative explanation could be that parental strain crosses and environmental conditions experienced during cyst formation influenced the long-term survival and viability of cysts and germling cells (Brosnahan et al., 2010; Figueroa et al., 2005). More research is needed to better constrain the factors that influence survival and viability dinoflagellate cysts in the environment.

Few other studies have compared germination success or other measures of viability in *Alexandrium* spp. and *Scrippsiella* spp. cysts with different depth layers of a sediment core. In Funka Bay, Japan, Miyazono et al. (2012), while able to germinate a 100-year old cyst, reported that *A. tamarense* germination success decreased linearly with depth. However, the small number ($N = 7-20$) 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 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 germination success of *Alexandrium* spp. cysts was from depths between 41 and 50 cm (Figure 6). Others have found that after 20 years in the sediments, *Scrippsiella* spp. germination success decreased but despite this, cysts that were an estimated 40 years old remained viable (Lundholm et al., 2011). To the best of our knowledge, the estimated 60-year old *Scrippsiella* spp. cyst which germinated in this study is the oldest known.

This study shows that *Alexandrium* spp. and *Scrippsiella* spp. cysts are able to survive in the sediments and remain viable for decades. However, the mechanisms that support long-term survival of dinoflagellate resting stages in the sediments are not well known. Binder and

436 Anderson (1990) measured the respiration rate of *S. trochoidea* cysts and compared it to the
437 amount of energy stored inside the cyst's energy reserves. They estimated that *S. trochoidea*
438 cysts stored at 3 °C would deplete their energy reserves within 240 days. The authors recognized
439 that this was an underestimate of cyst survival because a previous study observed *S. trochoidea*
440 cyst germination after 350 days (Binder and Anderson, 1987). They hypothesized that either
441 respiration rates decrease with cyst age or that other lipid-based energy reserves became
442 important over time and could be generated via internal anabolism. Subsequently, a study found
443 that *S. trochoidea* cysts were able to accumulate P and possibly N during both dormancy and
444 quiescence (Rengefors et al., 1996). Sediment and pore water sources of P and N may be an
445 underappreciated source of energy for dinoflagellate resting stages and may help to explain how
446 cysts at depth are able to remain viable. This study was not designed to test these hypotheses but
447 results do suggest that *Alexandrium* spp. and *Scrippsiella* spp. cysts can survive in sediments for
448 over 60 years, exceeding conventional expectations.

449

450 The data presented herein is most likely reflective of mature *Alexandrium* spp. cysts in a
451 quiescent stage. The mandatory dormancy period for *Alexandrium* spp. cysts in Puget Sound is
452 unknown but, the maximum mandatory dormancy period recorded for *Alexandrium* spp. is 180
453 days (Anderson, 1980). The 6 month cold, dark storage period in this study minimizes, but does
454 not entirely negate, the likelihood that the germination of cysts in the surface sediments was
455 influenced by a high concentration of immature, dormant cysts. The viability of cysts in this
456 study could have been influenced by the oxygenated storage conditions. Some have argued that
457 oxidative stress influences germination success (Binet and Stauber, 2006; Genovesi et al., 2009;
458 Ni Rathaille and Raine, 2011). The maximum amount of exposure to oxygenated conditions for

cysts in this study was just over one year after core extraction. There was reduced germination success during the winter after sediments had been in cold, dark storage for 11+ months (Table 1). However, the increase in germination success from May to July suggests that storage conditions did not systematically reduce cyst survival over the duration of this experiment. Future studies should be designed to parse out the relative influence of cold, dark storage, exposure to oxygen, month isolated, and depth.

The variable germination success of *Alexandrium* spp. and *Scrippsiella* spp. cysts at different depths in the sediment core could be a result of the environmental conditions experienced by individual cysts while entrained in the sediment. The survival of cysts from different depths in the sediment core could have been influenced by water column conditions present during encystment (Figuerola et al., 2005) and their subsequent burial histories including variable ingestion rates by different benthic infauna (Laabir et al., 2007; Persson et al., 2006; Tsujino and Uchida, 2004), temperature, pore water chemistry, and light conditions. The most *Alexandrium* spp. cysts were isolated from a depth range of 41-50 cm but, it is difficult to extrapolate how the environment influenced the *Alexandrium* spp. cysts that survived in the deeper sediments and/or what proportion within each depth interval decayed over time. Conditions that promote or prevent the decay of *Alexandrium* spp. cysts in the sediments are poorly understood. The proportion of the empty, fossilized *Scrippsiella* spp. cysts (Head et al., 2006) relative to the full cysts could be used to approximate cyst survival. In this study, very few *Scrippsiella* spp. cysts with prominent calcareous spines were found and the calcareous remains of empty *Scrippsiella* spp. cysts were not identified during cyst counts (Feifel, unpublished data). The lack of 'spiny-type' *Scrippsiella* spp. cysts in the samples could be an artifact of the 6-11 month storage time

and/or low pH conditions within the sediment samples (Shin et al., 2013). Thus, *in situ* cyst degradation cannot be assessed with this study and we are left with inferences based upon viability and physical appearance only.

Our results suggest that any disturbance able to resuspend sediments from a natural event such as periodic storms (Kremp, 2001), tides and currents (Anglès et al., 2010) or human activity in the marine environment, such as dredging or piling removal, could be a source of *Alexandrium* spp. blooms. It has been demonstrated that the shear stress caused by waves from storms or tidal currents can resuspend the top millimeter of sediments and associated cysts depending on sediment type and bottom depth (Butman et al., 2014). These types of natural events and bioturbation (Keafer et al., 1992) could episodically introduce cysts from deeper in the sediments to the sediment surface, serving to increase the numbers of cysts that are able to potentially germinate and promote a bloom. Indeed, Kamiyama et al. (2014) hypothesized that disturbance to deeper sediments (20-100 cm deep) from the Great East Japan Earthquake and subsequent tsunami resuspended *Alexandrium* spp. cysts into the water column. They found an up to 10-fold increase in maximum *Alexandrium* spp. cyst densities from 2005 compared to 2011 in Sendai Bay, Japan post-tsunami. Of similar concern, towed demersal trawl fishing gear has been shown to disturb and resuspend dinoflagellate cysts in the sediments (Brown et al., 2013; Pilskaln et al., 1998). Thus, care should be taken when disturbing the seafloor because cysts located as deep as 60 cm below the sediment surface can remain viable.

In the Puget Sound, surface cyst bed locations have been mapped (Horner et al., 2011) but it is generally unknown if the subsurface cyst distributions mirror surface cyst concentrations or, if

subsurface cysts reside at locations where surface cysts are currently absent. It would be prudent to look for subsurface *Alexandrium* spp. cysts prior to disturbing sediments as part of a risk assessment for planned dredging or other activities that disturb benthic sediments. The results from this study also assist efforts to model and forecast blooms of *Alexandrium* spp. in Puget Sound. The viability of quiescent cysts as deep as 60 cm in the sediments was similar to those mature cysts found at the sediment surface. Thus, predictive HAB models in Puget Sound can assume that mature cysts that find their way to the surface of the seafloor behave similarly, negating the need to account for differing values of germination success for old versus young cysts in biological models. However, biological models should consider that a large portion of the cysts capable of germinating in natural sediments may not produce a vegetative cell that has enough energy to divide and actually promote an algal bloom.

5.1 Conclusion

Image analysis was used to assess the cytoplasmic fullness of cysts of the potentially toxic *Alexandrium* spp. and non-toxic *Scrippsiella* spp. Neither depth nor the cytoplasmic fullness could be used as an indicator of cyst viability. The germination success of cysts was relatively constant throughout the 60-cm sediment core. These results suggest that *Alexandrium* spp. and *Scrippsiella* spp. cysts are resilient and able to survive multiple decades in the natural environment. Older cysts that are resuspended and brought to the surface via bioturbation or other disturbances have the same germination potential as younger cysts that have completed their dormancy requirements. These results suggest that cyst resuspension due to natural and human-induced sediment disturbances could help be a source of harmful algal blooms caused by *Alexandrium* spp.

528

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537

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695

696 Table captions.

697

698 Table 1. Depth bins, month(s) isolated, sample size and germination success (ECNC+Y) for

699 *Alexandrium* spp. and *Scrippsiella* spp. cysts.

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702 Figure captions.

703

704 Figure 1. Examples of *Alexandrium* spp. cysts which excysted but did not produce a dividing cell

705 (ECNC). Top panel (A/B) cysts were isolated from a depth of 56-58 and 54-56 cm respectively.

706 Lower panel (C/D) shows germling cells that were not moving when imaged and whose

707 pigments faded over the three weeks that wells were monitored. Cysts categorized as ECNC

708 would not be capable of producing a cell able to divide and promote a bloom in the water

709 column.

710

711 Figure 2. Steps in image analysis using DinoCyst. Top row is an *Alexandrium* sp. cyst isolated

712 from a depth of 44-46 cm. Bottom row is a *Scrippsiella* sp. cyst isolated from a depth of 30-32

713 cm. Panel (A) is the original image, (B) is the image mask created by DinoCyst, (C) is the

714 granular storage products identified by DinoCyst, (D) is the cyst's width and length dimension

715 estimate, and (E) is the confidence polygon. The *Alexandrium* sp. cyst has an 80% image

716 analysis confidence based on the percent overlap between images B and E and was thus,

717 excluded from image analysis. The *Scrippsiella* sp. cyst has a 98% confidence and was included

718 in the image analysis.

719

Figure 3. Profile of log-transformed ^{210}Pb activity at the study site. The vertical distribution of ^{210}Pb from the surface to 21 cm indicates that the sediment was well mixed by physical or biological processes. The dashed line at 21 cm indicates the beginning of the permanent record. The linear fit of ^{210}Pb decay in the permanent record is used to estimate the site's sedimentation rate to obtain a chronology. Horizontal bars show counting error, vertical bars represent the 2-cm sediment core intervals.

Figure 4. Cytoplasmic fullness of *Alexandrium* spp. (solid line) and *Scrippsiella* spp. (dashed line) based on germination outcome. 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 5. Comparison of cyst germination outcome and cyst cytoplasmic fullness as a function of the depth the cyst was isolated from. Each circle represents an individual cyst. Black circles represent a successful germination and a dividing cell found in the well (Y), gray circles represent a successful germination but no dividing cell (ECNC), and the white circles represent no germination (N). The dashed vertical line indicates the boundary between the mixed layer and permanent record.

Figure 6. Minimum cyst age (based upon time since burial below the mixed layer), depth and germination success are displayed as a solid bar for successful germination (Y) and as a striped bar for the proportion that did not produce a dividing germling (ECNC). Error bars were calculated based upon the relative sample size within each depth bin (Table 1).

742

743 Table 1.

Depth	Month isolated	<i>Alexandrium</i> spp.		<i>Scrippsiella</i> spp.	
		Cysts isolated (N =)	Germination success	Cysts isolated (N =)	Germination success
0-10 cm	Nov.	35	17 %	105	66 %
11-20 cm	May / Dec.	18 / 28	28 / 0 %	58 / 74	40 / 16 %
21-30 cm	June / Jan.	23 / 14	22 / 14 %	73 / 26	63 / 42 %
31-40 cm	July	65	25 %	92	69 %
41-50 cm	July - Aug.	70	42 %	67	74 %
51-60 cm	Aug.	32	25 %	13	31 %

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745 Figure 1.

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Figure 4.

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