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Quantitative Response of *Alexandrium catenella* Cyst Dormancy to Cold Exposure

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Many dinoflagellate cysts experience dormancy, a reversible state that prevents germination during unfavorable periods. Several of these species also cause harmful algal blooms (HABs), so a quantitative understanding of dormancy cycling is desired for better prediction and mitigation of bloom impacts. This study examines the effect of cold exposure on the duration of dormancy in *Alexandrium catenella,* a HAB dinoflagellate that causes paralytic shellfish poisoning (PSP). Mature, dormant cysts from Nauset Marsh (Cape Cod, MA USA) were stored at low but above freezing temperatures for up to six months. Dormancy status was then determined at regular intervals using a germination assay. Dormancy timing was variable among temperatures and was shorter in colder treatments, but the differences collapse when temperature and duration of storage are scaled by chilling-units (*CU*), a common horticultural predictor of plant and insect development in response to weather. Cysts within Nauset meet a well-defined chilling requirement by late January, after which they are poised to germinate with the onset of favorable conditions in spring. Cysts thus modulate their dormancy cycles in response to their temperature history, enhancing the potential for new blooms and improving this species' adaptability to both unseasonable weather and new habitats/climate regimes.

Key words: Resting cyst; dinoflagellate; dormancy; chilling; germination; endogenous clock.

Introduction

The phenology of phytoplankton blooms at higher latitudes is seasonal, a direct consequence of the large changes in temperature and photoperiod that occur over the course of a year and directly affect species' growth. In response, many phytoplankton have evolved specific behavioral and life-cycle strategies for surviving seasonally-induced stress and exploiting periods that are more favorable for growth and reproduction (Ji et al. 2010). An especially common strategy among dinoflagellates is the formation of resting cysts. Dinoflagellate cysts can survive extended periods of unfavorable environmental conditions, and eventually germinate to enter the reproductive, planktonic phase of their life cycle when conditions improve (Dale 1983; Wall 1971). New resting cysts are formed as blooms decline and remain in bottom sediments until some germinate, providing an inoculum for subsequent blooms (e.g. Brosnahan et al. 2017).

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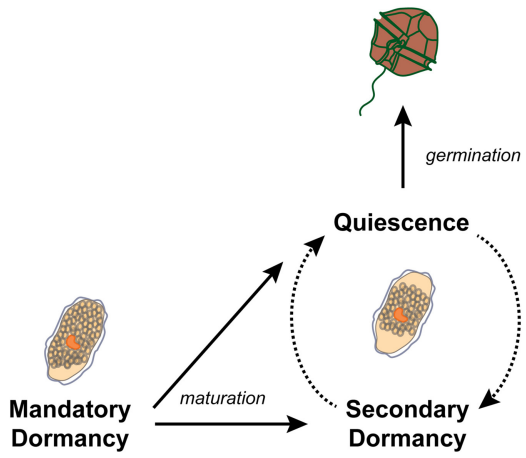

Figure 1. Schematic of dormancy–quiescence transitions in *Alexandrium catenella* cysts. Mandatory dormancy begins at cyst formation and inhibits germination for several weeks to months until cysts have matured (indicated by the loss of starch granules within the cyst on the right). At this point, mature cysts either become quiescent – a state in which they will germinate if environmental conditions are favorable – or may be induced into secondary dormancy by either internal and/or external factors, e.g., via endogenous rhythmicity, external temperature conditioning, or other as yet undiscovered mechanisms.

In many species, cyst germination is inhibited immediately after a bloom and during certain periods of the year because of dormancy (Anderson et al. 2003). Dormancy is an internal state that impedes germination under otherwise adequate environmental conditions and is directly comparable to the dormancy described in the seeds of higher plants (e.g. Bewley et al. 2013). On exit from dormancy, cysts are termed quiescent, a state in which they will germinate if environmental conditions are favorable (Pfiester and Anderson 1987). Among the factors that are essential for germination is temperature, which must be within an optimal range (e.g. Anderson and Morel 1979; Bravo and Anderson 1994; Huber and Nipkow 1923), and oxygen, which must be present (Anderson and Keafer 1987; Kremp and Anderson 2000; Rengefors and Anderson 1998). Two types of dormancy have been described (Fig. 1). The first, termed mandatory dormancy, begins at cyst formation and can last from several weeks to months until maturation is completed (e.g. Anderson 1980; Perez et al. 1998; Pfiester 1977). The second type (termed secondary dormancy) only occurs in mature cysts and can recur multiple times during a cyst's lifetime (Anderson and Keafer 1987; Matrai et al. 2005).

Many dinoflagellates are harmful algal bloom (HAB) species and therefore a quantitative understanding of the internal and environmental factors controlling cyst dormancy is needed to improve bloom prediction and mitigation of impacts. One example is *Alexandrium catenella* (= *A. fundyense*[1]), a species that causes paralytic shellfish poisoning (PSP) in temperate and subarctic coastal areas worldwide (Anderson et al. 2012). Mature *A. catenella* cysts from deep "seedbed" areas of the Gulf of Maine (GOM) cycle between dormancy and quiescence under control of an endogenous circannual clock (Anderson and Keafer 1987; Matrai et al. 2005). This rhythmicity ensures that dormancy is broken prior to the onset of favorable bloom conditions within the euphotic zone, even with dampened seasonal signals at depth. In contrast, dormancy cycling in shallow water populations seems to be determined by cysts' external environment and so has been frequently likened to the secondary dormancy of terrestrial plant seeds (e.g. Anderson and Keafer 1987; Matrai et al. 2005; Rathaille and Raine 2011). In seeds, secondary dormancy is induced by unfavorable environmental conditions and many different species annually cycle between secondary dormancy and quiescence (e.g. Batlla and Benech-Arnold 2010). Here, we examine an *A. catenella* cyst population from a shallow temperate estuary. In previous studies of dinoflagellate cysts, secondary dormancy and endogenous rhythmicity have been juxtaposed as opposing mechanisms of dormancy control, yet our results suggest that a common feature may underlie both behaviors – a chilling response detailed herein.

Chilling has previously been shown to shorten dormancy and improve the viability of dinoflagellate cysts (Montresor and Marino 1996; von Stosch 1967, 1973), suggesting that cysts may have a chilling requirement much like the seeds of many terrestrial plants (e.g. Vegis 1964). The goal of this study was to determine if *Alexandrium* cysts have such a chilling requirement for exit from dormancy, and if so, to determine how it regulates germination in the context of environmental temperature fluctuations. Here, we present the results of a series of experiments that applied different cold storage temperatures to naturally-formed *A. catenella* cysts.

[1] *Alexandrium catenella* and *A. fundyense* are conspecific, and "*catenella*" was determined to have nomenclatural priority (Prud'homme van Reine 2017).

Results

Monthly Observations of Vegetative Cell and Resting Cyst Abundance and Dormancy Status

Cyst abundance within the surficial sediments of Roberts Cove (part of the Nauset Marsh estuary, Orleans, MA USA; Fig. 2) was measured approximately monthly from March 2013 to June 2015 (Fig. 3a), where the cysts experienced bottom water temperatures ranging from −1 to 24 °C (Fig. 3b). During this period, a large bloom occurred in late April 2013 (depth-averaged maximum: 4.3×10^5 cells L^{-1}) and a very small bloom in early May 2014 (depth-averaged maximum: 3.6×10^3 cells L^{-1}; Fig. 3a). Termination of the 2013 bloom coincided with new cyst production, which was observed as a dramatic spike in cyst abundance to $6.4 \times 10^3 (\pm 0.8)$ cysts cm^{-3} on 02 June 2013 – the highest concentration recorded during the study. Cyst production was presumably very low during the small 2014 bloom, and thus cyst densities fell during the bloom and afterward, reaching a low of 7.1×10^2 (± 0.80) cysts cm^{-3} on 13 November 2014.

The dormancy status of the *A. catenella* population in situ was tested using monthly germination assays from July 2013 to June 2015 (see Methods). Cysts were considered quiescent when more than 50% germinated within the first week of the assay. In both years, the population was quiescent from approximately January to May (grey shading, Fig. 3c) and dormant from June through December.

Required Cold Storage Periods for Dormancy Breaking

To examine the effect of chilling on both newly-formed cysts and those that had already experienced one or more Nauset winters, mature, dormant cysts were collected in October of 2013 and 2014 (Fig. 3a) and stored in the laboratory at temperatures that occur naturally from late fall through early spring (2, 4, 6, and 8 °C) (Fig. 3b). Because the majority of the cysts in the 2013 and 2014 collections were formed following termination of the 2013 spring bloom, the latter had already completed a dormancy cycle in situ (i.e. during the winter of 2013–2014; Fig. 3c) prior to laboratory cold storage. Hereafter, we refer to these two collections as "first-cycle" and "repeat-cycle" cysts, accordingly. First-cycle cysts were stored at 2 and 6 °C, while the repeat-cycle cysts were stored at 4 and 8 °C. Approximately bimonthly, a subsample of cysts was removed from each of the storage temperatures and tested for germinability (see experimental design in Fig. 4). Cumulative germination of chilled cysts increased with days of cold storage at each temperature (Fig. 5; storage duration is indicated by the green to blue gradient) and with increasing days of incubation during germination assays (bottom axes). Control cyst cohorts that were not chilled did not germinate to any significant extent through the experiment (not shown in Fig. 5). The maximum cumulative germination (G_{max}) also increased with increasing time in cold storage for all treatments. For example, after 10, 67, and 190 days of cold storage, the G_{max} of cysts stored at 2 °C increased from 0%, to 63%, to 77%, respectively (Fig. 5, top left), while after 15, 64, and 180 days of cold storage, the G_{max} of cysts stored at 8 °C increased from 0%, to 4%, to 70%, respectively (Fig. 5, bottom right).

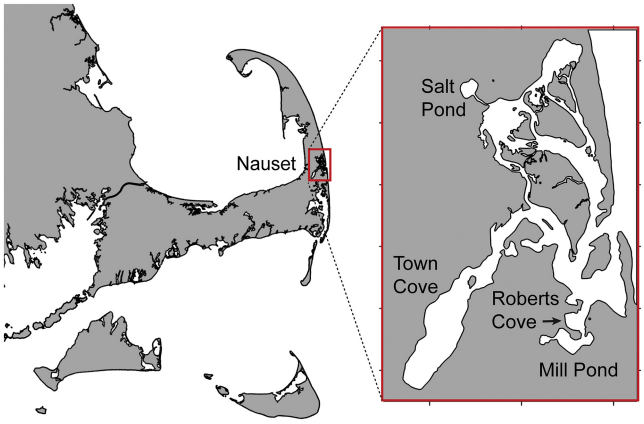

Figure 2. Location of Roberts Cove within the Nauset Marsh estuary on Cape Cod, MA USA.

Differences in peak G_{max} between storage temperatures were interpreted as differences in cyst viability. Non-germinating cysts often appeared blackened and/or showed green autofluorescence at the conclusion of dormancy assays, both indicators of cyst death (Fig. 6; Tang and Dobbs 2007). The highest G_{max} values were attained by cohorts that spent at least 180 days at 6 °C ($\bar{G}_{max} = 94\%$, $SD = 0.023$). The effect of chilling on dormancy similarly plateaued in other temperatures treatments but after different time periods, e.g. >56 days at 4 °C ($\bar{G}_{max} = 83\%$, $SD = 0.018$), >126 days at 2 °C ($\bar{G}_{max} = 77\%$, $SD = 0.018$), and >70 days at 8 °C ($\bar{G}_{max} = 68\%$, $SD = 0.020$). A one-way ANOVA comparison of the peak G_{max} values observed in different storage temperature treatments (highlighted by open circles in Fig. 5) showed a significant temperature effect ($F = 3, 14$, $p < 1 \times 10^{-6}$), with

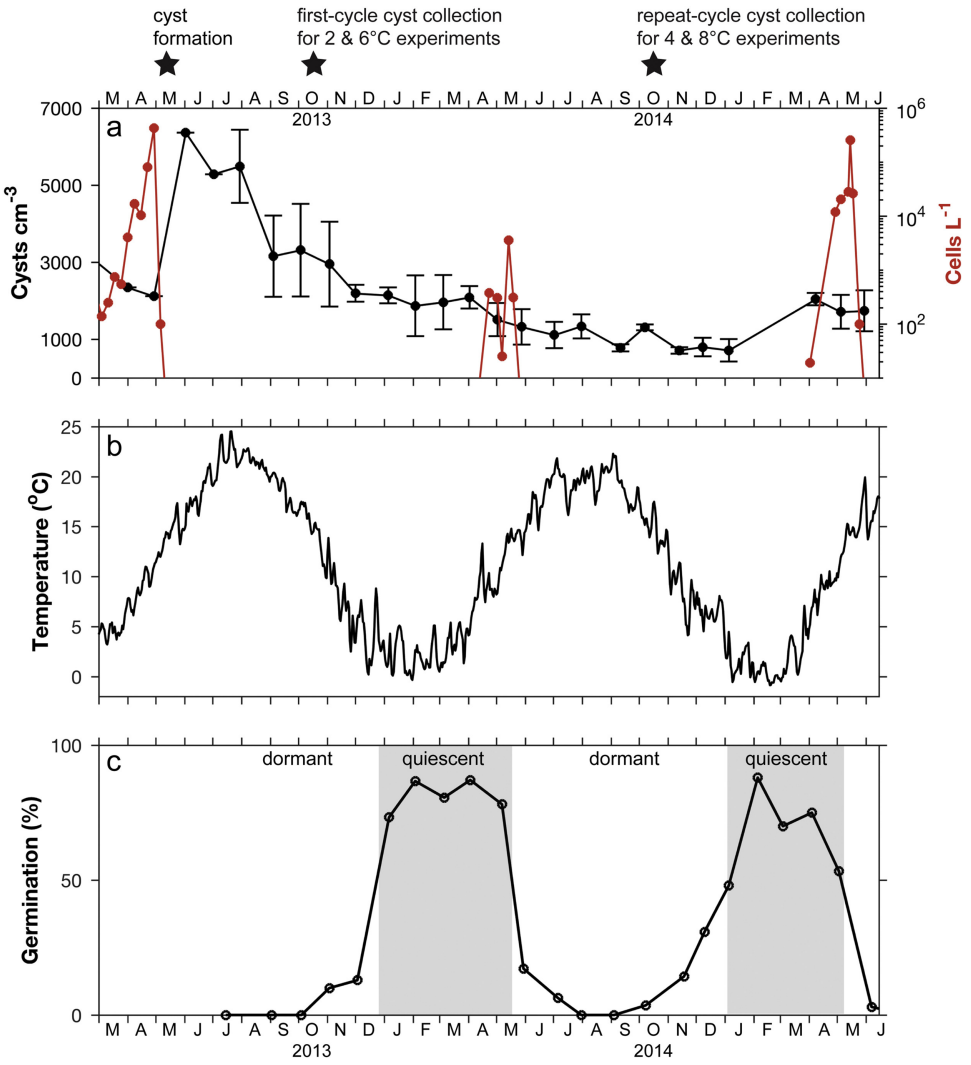

Figure 3. Seasonal dormancy status of the *A. catenella* cyst seedbed in relation to cell abundance and bottom water temperature from March 2013 to June 2015 in Roberts Cove, within Nauset. (**a**) Monthly triplicate counts of cyst abundance in the surface 1 cm layer and weekly vegetative cell abundance (depth-averaged). Stars highlight the timing of cyst formation following the 2013 spring bloom, and the collections of first- and repeat-cycle cysts, in October 2013 and 2014, respectively, for cold storage experiments. (**b**) Ambient bottom water temperatures experienced by cysts. (**c**) Cumulative germination of a cohort of freshly-collected cysts ($n = \sim 30$) incubated under optimal conditions (15 °C, light, and oxygen) for one week. Periods of majority quiescence (when cumulative germination exceeded 50%) are demarcated by grey shading.

6 °C cohorts germinating at greater peak rates than those from the other storage treatments (post-hoc Tukey test, $p < 0.05$). To control for these differences in cyst viability experienced during storage, cumulative germination percentages were normalized by the peak G_{max} values at each temperature, yielding G^* (see Methods, Equation (1)). The G^* statistic was used in all subsequent analyses that combined results from the respective temperature treatments.

Chilling-unit Scaling Collapses Differences Between Temperature Treatments

Generally, the colder the storage temperature, the fewer days of cold storage were required to break dormancy (Fig. 7a). A useful statistical descriptor of these differences is the duration of storage required for G^* to reach 50% (G^*_{50}). This metric indicates when half of the living cysts in a storage cohort has transitioned from dormancy to quiescence. The

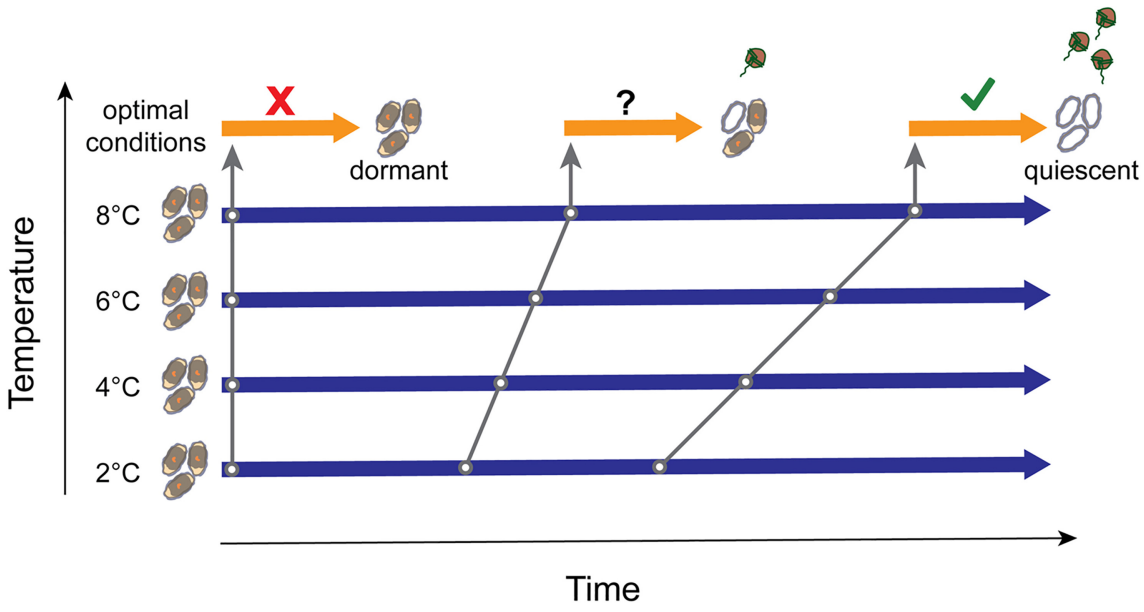

Figure 4. Germination assay of dormancy status. Dormant cysts were stored in anoxic, dark storage at 2, 4, 6, or 8 °C and cohorts were removed approximately bimonthly to examine their dormancy status. For each assay, approximately 30 cysts were isolated and incubated under optimal conditions (15 °C, light, and oxygen) for one week. The linked points on the four storage temperature timelines are equivalent steps of chilling accumulation – i.e., different durations at different storage temperatures were required to reach comparable levels of chilling.

colder the storage temperature, the sooner G^*_{50} was reached: 108 days at 8 °C, 93 days at 6 °C, 87 days at 4 °C, and 67 days at 2 °C (Fig. 7a). Thus, to take into consideration the combined effects of time and temperature on dormancy duration, scaling of these factors via a simple chilling-unit metric was investigated.

The chilling-unit calculation required specification of a threshold temperature below which *A. catenella* senses chilling (T_{max}, see Methods, Equation (2)). Values of T_{max} ranging from 2 to 30 °C were evaluated by fitting resultant chilling-units and G^* responses to sigmoidal response curves (e.g. $T_{max} = 15$ °C, Fig. 7b). Goodness of fit increased with increasing values of T_{max} up to 15 °C ($r^2 = 0.99$; Fig. 8). Measures of fit generally declined above 15 °C and were no longer ordered according to their relative temperatures (Fig. 8). Thus, 15 °C was chosen as the best approximation of T_{max} for *A. catenella* in Nauset and applied to all subsequent chilling-unit calculations.

Differences in G^*_{50} between the 2, 4, 6, and 8 °C treatments collapse when G^* is plotted versus chilling-units, rather than days of storage (Fig. 7). Across all temperature treatments, the threshold above which the majority of cysts transitioned from dormancy to quiescence (or the G^*_{50}) was approximately 800 *CU*, which is equivalent to ~10 weeks at 4 °C or ~16 weeks at 8 °C. A small percentage of the cyst population began to germinate upon attainment of approximately 500 *CU*, while others required much more than 800 *CU* to germinate, reflecting normally distributed variance in each cyst's chilling requirement, i.e., the specific amount of cold conditioning needed for transition to quiescence. First- and repeat-cycle cysts (see Fig. 3a) had similar responses to chilling, which is remarkable given that a large proportion of the former had never experienced wintertime chilling in the field, whereas the latter had. Using a permutation procedure detailed in the Methods, scaling by chilling-units was found to significantly reduce G^*_{50} differences among the respective temperature treatments ($p < 0.05$). No matter the cold storage treatment, all of the Nauset *A. catenella* cysts required approximately 800 *CU* to break dormancy.

The threshold behavior associated with the chilling requirement is visualized by incorporating the number of days of incubation under optimal conditions required for cyst cohorts to reach various germination percentages (Fig. 9). If cysts had less than 500 *CU*, only a limited number germinated, regardless of the number of days of incubation (G^* never exceeded 18%; light blue region). Cyst cohorts with 500 to 800 *CU* ultimately reached G^*_{50} but required a minimum of two weeks of incubation at 15 °C to do so (dark blue region). Once cysts had attained the 800 *CU* chilling requirement, they

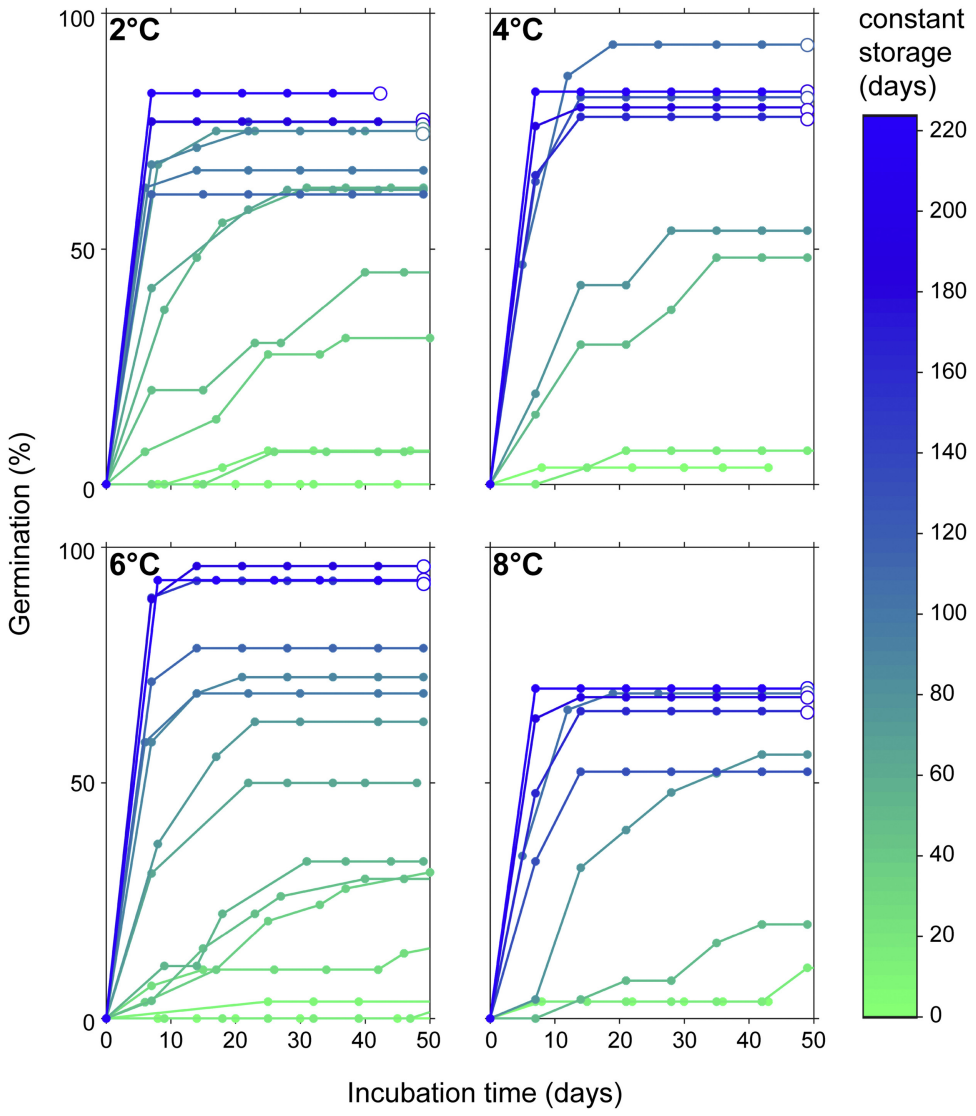

Figure 5. Germination time-course curves for *A. catenella* cyst cohorts exhumed from 2, 4, 6, and 8 °C storage treatments after different storage durations. A green to blue gradient indicates the duration of constant cold storage for each cohort – the bluer the color, the longer the duration. The open circles indicate the plateau-phase G_{max} values of cohorts that exceeded 85% of the maximum germination of each treatment – these were averaged to calculate the $\bar{G}_{max}$ for each treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

achieved or exceeded G^*_{50} within one week (purple region). Cysts with over 1200 *CU* attained G^* of ~100% (pink region).

Winter Chilling Experienced by Nauset Field Populations

The annual chilling of the cyst seedbed in Roberts Cove was calculated from temperatures recorded during the study period. Starting from cyst formation in May 2013, chilling did not occur until five months later in late October (i.e. temperatures did not fall below the 15 °C, T_{max}, prior to that time) (Fig. 10). Because the cysts collected during the first week of October 2013 were chilled immediately following their collection, they experienced chilling approximately three weeks before those that remained in Roberts Cove sediments (and would be collected the following October). The total annual chilling calculated in Nauset was 1674 *CU* and 1985 *CU* in 2013–2014 and 2014–2015 (Fig. 10), respectively, which was within range of

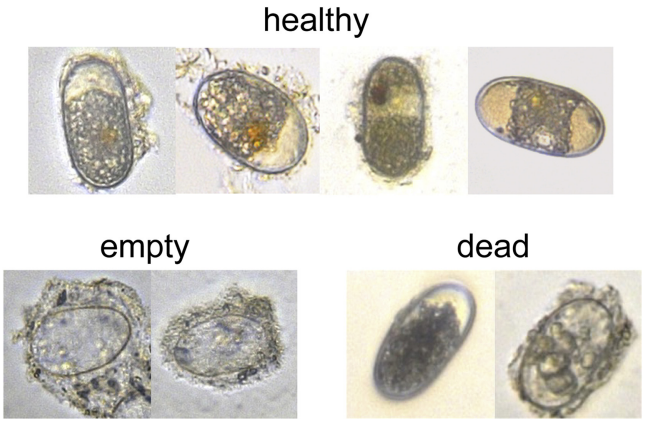

Figure 6. Light micrographs of *A. catenella* cysts. Top row displays examples of healthy cysts that were isolated for the experiments in this study. Bottom row shows empty cysts that were used as an indicator if germination had occurred and dead cysts.

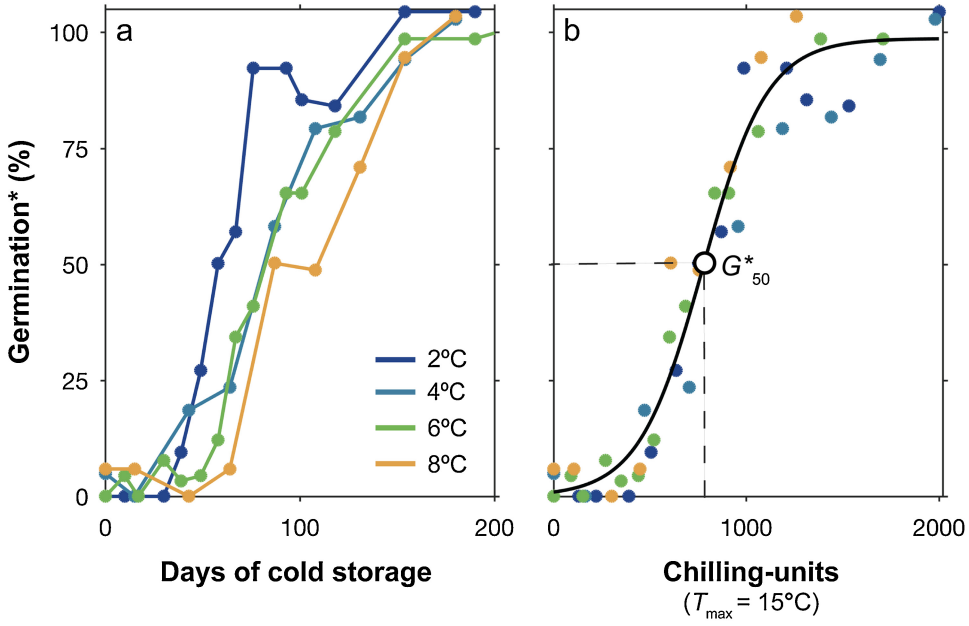

Figure 7. Cumulative percent germination of cyst cohorts that were removed from different cold storage temperatures after different durations and incubated under optimal conditions for one week. Data are shown as function of (**a**) days of cold storage and (**b**) accumulated chilling using $T_{max} = 15\,^{\circ}C$. A sigmoid curve function is overlaid (black line) to demonstrate the goodness of fit. Germination percentages are normalized to the $\bar{G}_{max}$ of each cold storage temperature treatment. The chilling requirement, or the amount of chilling associated with attainment of G^*_{50}, is demarcated.

the 2000 *CU* maximum exposure in this study (Fig. 7b). Thus, the natural *A. catenella* cyst population reached the 800 *CU* chilling requirement by late January in both 2014 and 2015 – well before the end of winter and the beginning of spring. The exceedances of this threshold also correlate well with dormancy–quiescence transitions observed through monthly field collections (Fig. 3c).

Discussion

Cold exposure shortened the duration of dormancy in mature *A. catenella* cysts. This inverse relationship between temperature and the rate of dormancy breaking is opposite to most other physiological rates including cell division, new cyst maturation, and germination – all of which proceed slower at lower temperatures. Results from this study demonstrate for the first time that *A. catenella* cysts must

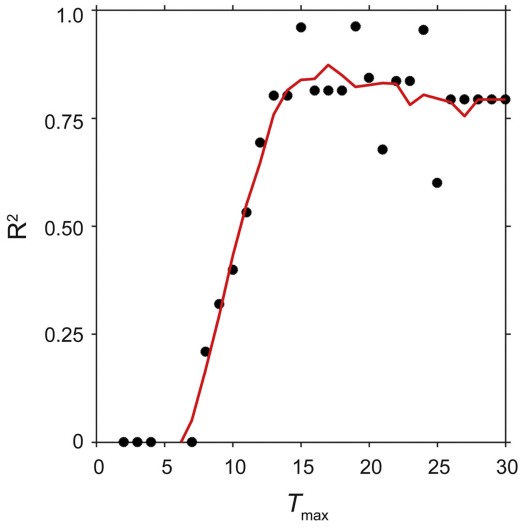

Figure 8. Testing the rigor with the T_{max} needs to be defined to quantify *A. catenella* cyst germination responses to chilling accumulation. Different permutations of T_{max} from 0 to 30 °C were tested to generate a range of chilling quantities associated with each germination percentage. The goodness of fit of the chilling and germination percentage to a sigmoid curve was assessed.

surpass a chilling requirement to break dormancy. Once dormancy is broken, cysts become quiescent and are able to germinate upon exposure to favorable conditions. Further, monthly observations of the *A. catenella* cyst population in situ reveal the presence of a persistent, annual rhythm of dormancy in the summer and autumn and quiescence in the winter and spring (Fig. 3c). This pattern reflects the temperature conditioning of *A. catenella* cysts in situ which modulates their dormancy cycling in a given year or habitat. Incorporating this dormancy response into existing models of bloom initiation will improve bloom forecasting, especially in the context of species biogeography, local weather phenomena, and climate change. Results from these experiments also suggest a way in which temperature may modulate dormancy cycling in deep water populations that are thought to be under the control of an endogenous circannual rhythm (Anderson and Keafer 1987; Matrai et al. 2005). These insights into cyst dormancy control by chilling are explained in detail below.

Two Physiological Parameters Predict Dormancy Duration: T_{max} and the Chilling Requirement

By scaling cold exposure using chilling-units, the timing of *A. catenella* cyst dormancy transitions can be predicted with considerable precision. Integral to this simple chilling model are two physiological parameters. The first is the threshold temperature, T_{max}, which determines when, and to what degree, a cyst population registers cold exposure. This study demonstrates that the Nauset *A. catenella* cysts have a T_{max} of 15 °C (Fig. 8). Interestingly, it is at this same temperature that vegetative *A. catenella* cells in laboratory cultures divide fastest (Watras et al. 1982). Similarly, germination of quiescent cysts proceeds most rapidly at this temperature (Anderson et al. 2005).

The second physiological parameter is the chilling requirement. The chilling requirement is the sum of chilling-units that must be accumulated for the majority of a population to transition from dormancy to quiescence. Across each of the tested cold storage temperatures (2, 4, 6, and 8 °C), >50% of viable cysts germinated within the first week of the germination assay only after attaining approximately 800 *CU* (Fig. 9). Beyond 1200 *CU*, essentially all viable cysts germinated within the first week of incubation. In contrast, cohorts with 500–800 *CU* required between two and seven weeks of incubation for 50% germination, and below 500 *CU*, very few cysts germinated. Thus, for Nauset *A. catenella* cysts, 800 *CU* is a critical threshold for rapid and complete germination to occur.

Characterization of both the T_{max} and the chilling requirement is needed for other populations of *A. catenella* in order to assess the extent to which these findings can be generalized globally, or instead are region- or population-specific. For instance, does the same T_{max} accurately describe *A. catenella* populations living in habitats that, unlike Nauset, have temperature regimes that seldom, if ever, reach or exceed 15 °C? Likewise, do chilling requirements vary among populations globally? If so, differences may help to explain how this organism can colonize widely varying habitats. Whether or not these specific parameters differ among *A. catenella* populations, dormancy control is clearly one of several physiological mechanisms that affects the occurrence of *A. catenella* blooms. Enhanced germination of other dinoflagellate species in response to chilling has been reported (Montresor and Marino 1996; von Stosch 1967, 1973), which suggests that dormancy may be regulated similarly in many other dinoflagellate species too.

Dormancy Cycling within the Nauset Cyst Seedbed

Though all *A. catenella* cysts collected were dormant at the start of the cold storage experiments, the number of times that they had previously experienced dormancy varied. The majority of "first-cycle" cysts collected in October 2013 formed after a large bloom in May 2013 and were approximately 5 months old when move to laboratory cold storage (Fig. 3a). Ongoing physical and biological sediment mixing in Nauset buried many of these May 2013-formed cysts into inhibitory, anoxic sediments such that they experienced a dormancy cycle through the 2013–2014 winter (Fig. 3c). Thus, essentially all "repeat-cycle" cysts collected in October 2014 were one or more years old and had experienced at least one natural winter prior their transfer to laboratory cold storage (Fig. 3c). In spite of these differences, similarity in the responses of the first- and repeat-cycle collections is revealing. Chilling accumulated within Nauset by older year-class cysts was somehow nullified or reset – otherwise the responses of the first- and repeat-cycle collections would have differed even after scaling by chilling-units (Figs 7b and 9).

Previous studies of *A. catenella* cysts have suggested that dormancy cycles are driven by one of two distinct mechanisms: either endogenous rhythmicity, in which dormancy periods are set by an internal biological clock, or secondary dormancy, in which cysts cycle in and out of dormancy in response to their external environment much like the seeds of many terrestrial plants (e.g. Anderson and Keafer 1987; Matrai et al. 2005; Moore et al. 2015; Rathaille and Raine 2011). Though invocation of the secondary dormancy concept in studies of dinoflagellates was intended to distinguish this type of control from an endogenous mechanism (Anderson and Keafer 1987), terrestrial seeds have been shown to enter and exit secondary dormancy via control of an endogenous rhythm (e.g. Baskin and Baskin 2014; Gutterman 1981; Handley and Davy 2005). The term secondary dormancy in plant studies thus includes this type of recurring dormancy and is used to distinguish the dormancy of fully matured seeds from "primary dormancy", which is associated with maturation of newly formed seeds and is directly analogous to mandatory dormancy in dinoflagellate cysts (e.g. Anderson 1980; Baskin and Baskin 2014). Thus, we propose that the definition of secondary dormancy in dinoflagellates conform with its definition in plants, i.e., to indicate the dormancy cycling of fully matured cysts after mandatory dormancy is complete. Under this definition, cysts might be induced into secondary dormancy by either internal and/or external factors, e.g. via endogenous rhythmicity or external factors such as temperature conditioning (Fig. 1).

Both deep and shallow water populations of cysts can experience secondary dormancy in their lifetimes, even if the events leading to this state in the two populations differ or are unclear. Histories leading up to establishment of dormancy also differed among cysts in the two collections of shallow water cysts examined in this study. Anderson (1980) previously reported that mandatory dormancy periods in these shallow, salt pond habitats range from one to four months and are shorter at the warmer temperatures typical of Nauset during the summer. In the present study, new cysts within the first-cycle cohort were dormant approximately five months before their collection, never achieving quiescence and instead transitioning directly from mandatory to secondary dormancy (Fig. 3). In contrast, all repeat-cycle cysts were induced into secondary dormancy after previously cycling between dormancy and quiescence. One conclusion drawn from these observations is that an intervening spell of quiescence is not required for induction of secondary dormancy (illustrated in Fig. 1). Unanswered is what factor(s) induced all of these different year class cysts to enter secondary dormancy.

Insights can be gleaned from common features in the temperature histories of the first- and repeat-cycle cohorts. Both groups were fully dormant when collected in October after experiencing the warmest months of summer. Temperatures in excess of 18.5 °C have been shown previously to inhibit excystment by quiescent *A. catenella* (Anderson and Rengefors 2006), and ambient water temperatures in Nauset were greater than this threshold from mid-June through mid-September in both years of the study. We propose that these warm summertime conditions induced secondary dormancy, effectively "resetting" the older cysts in both collections.

Temperature Determines the Length of Dormancy

Past studies of endogenous dormancy control are similar to the present study in that they have subjected test populations of *A. catenella* cysts to continuous storage conditions (Anderson and Keafer 1987; Matrai et al. 2005; Moore et al. 2015; Rathaille and Raine 2011). The most basic finding of the present study – that cysts stored at colder

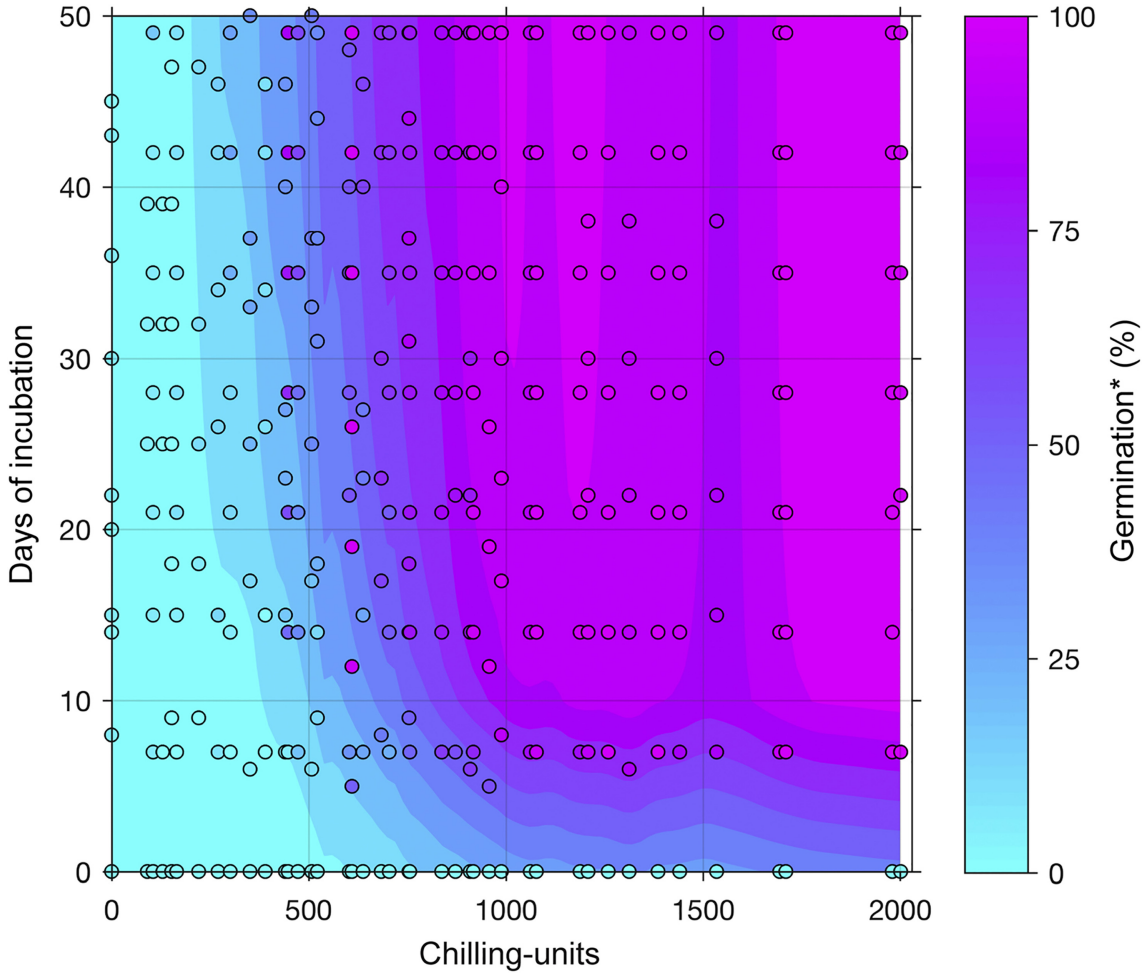

Figure 9. Relationship between the chilling accumulation of *A. catenella* cyst cohorts and the incubation time needed to reach various germination percentages. These are the same data as Figure 5 (open circles indicate sampling resolution) but plotted as a function of chilling-units using $T_{max} = 15\,^{\circ}C$ and normalized to the $\bar{G}_{max}$ of each cold storage temperature treatment. Differences in cumulative germination are indicated by the color gradient – light blue colors indicate mainly dormant cysts and light pink colors indicate mainly quiescent cysts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

temperatures attain quiescence faster – has direct implications for these earlier rhythmicity experiments.

A curious aspect of the endogenous circannual rhythms documented in GOM cysts is that their period is just 11 months, rather than a full year (Anderson and Keafer 1987; Matrai et al. 2005). In situ, such a short cycling period would pose a substantial ecological disadvantage to older year classes, especially considering that the lifetime of a cyst in the sediment can be several decades or more (Feifel et al. 2012; Keafer et al. 1992; Miyazono et al. 2012), and thus cysts can experience more than one dormancy cycle. The effect of chilling on dormancy provides an explanation. Past rhythmicity experiments stored cysts at 2 °C, somewhat colder than the average bottom water temperatures in the GOM (7.4 °C ±2.6; 2009–2016; http://www.neracoos.org/datatools). If the T_{max} and chilling requirement of GOM cysts are the same as those in Nauset (15 °C and 800 *CU*, respectively), 2 °C storage temperature would have shortened the dormancy period by a little over a month compared to the duration predicted under GOM conditions. Warmer GOM bottom water temperatures might therefore prolong the dormancy cycling period to a full year. If *A. catenella* cysts in the GOM have a comparable chilling response to those in Nauset,

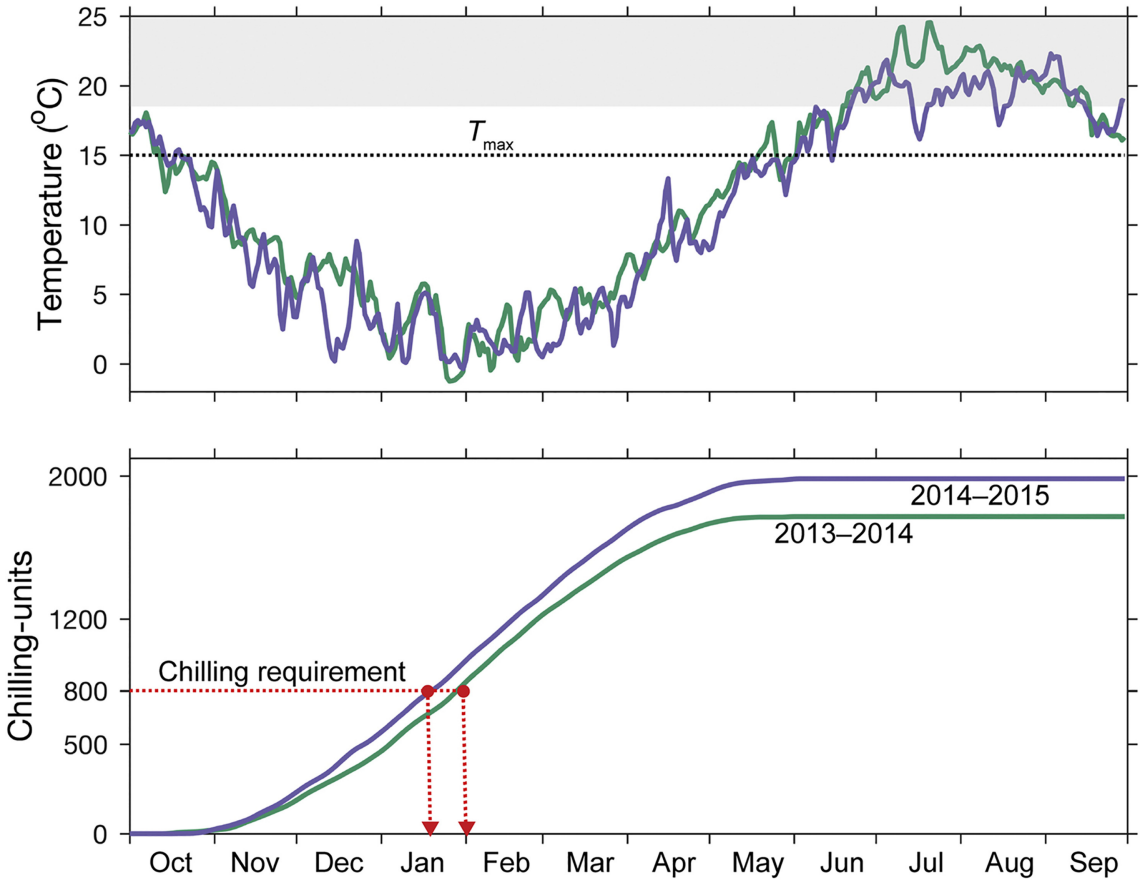

Figure 10. Ambient bottom water temperature and chilling-unit accumulation in the Nauset Marsh estuary from October 2013 to 2015. The grey shaded area indicates temperatures above the 18.5 °C threshold for *A. catenella* cyst germination specified by Anderson and Rengefors (2006). The 15 °C T_{max} is delineated to demonstrate the possible window of chilling accumulation in Nauset. The predicted attainment of the chilling requirement in late January 2014 and 2015 is indicated by red arrows and is roughly consistent with observations of the field population (Fig. 3c).

environmental temperature is likely to play a role in the entrainment of their circannual rhythmicity. Such environmental entrainment of the dormancy cycling rhythmicity in GOM cysts is being explored further (A.D. Fischer, unpublished results).

Ecological Role of the Chilling Response in Shallow Water Systems

The ecological role of the chilling response can be investigated through examination of the Nauset bottom water temperature record. Using our laboratory-derived T_{max} and chilling thresholds, the transition of the cyst seedbed from secondary dormancy to quiescence was predicted and compared to monthly observations of dormancy state in the Roberts Cove cyst seedbed. In the laboratory, the onset of quiescence occurred at 500 *CU*, the G^*_{50} was attained at 800 *CU*, and all cysts had fully transitioned to quiescence by 1200 *CU* (Fig. 9). In the Nauset system, chilling begins in late October, and the 500, 800, and 1200 *CU* thresholds were attained between December and February in both study years (Fig. 10). These observations are in line with the January onset of quiescence predicted by the chilling model (Fig. 10) and observed directly through a time series of Nauset cyst dormancy status (Fig. 3c).

Still, the onset of quiescence is not synonymous with cyst germination. Nauset blooms typically do not develop until April or May (Ralston et al. 2014). Once dormancy is broken, germination of the now quiescent cyst seedbed is inhibited by lingering wintertime cold. Quiescent cysts are poised to produce a pulse of germling cells as the system heats during periods of warm spring weather (i.e. temperatures become favorable for germination). Such a synchronous germling pulse may be ecologically

advantageous in habitats where *A. catenella* must establish itself quickly to form blooms (e.g., due to short growing seasons or competition with other species).

In terrestrial plant seeds and bulbs, chilling requirements prevent precocious emergence during short spells of favorable conditions in otherwise unfavorable seasons (e.g. Arora et al. 2003; Horvath et al. 2003; Rohde and Bhalerao 2007). Similarly, *A. catenella* cysts in Nauset are unresponsive to unseasonably warm conditions that sometimes occur between October and December. Without secondary dormancy, germination would occur throughout the year, including at times when physical and biological conditions do not support sustained bloom development. By quantifying the duration and severity of cold, *A. catenella* cysts track the passage of winter just like many plants, delaying germination until spring when the outlook for bloom development is more favorable.

The same chilling response may produce different effects in the context of other shallow, inshore habitats with different temperature regimes. The amplitude of seasonal temperature oscillations in Nauset is quite strong (-1–24 °C; Fig. 10) relative to some other areas that are also chronically impacted by *A. catenella* blooms. For example, bottom water temperatures vary seasonally from 8 to 13 °C in Puget Sound (http://green2.kingcounty.gov/marine-buoy/), another well-established *A. catenella* habitat (Moore et al. 2011, 2015). Temperatures in Puget Sound are therefore favorable for both germination and division year-round (Anderson 1998; Bill et al. 2016). If this population had a similar chilling response to cysts in Nauset, milder seasonal temperature swings would promote less-synchronous, more-dispersed inoculae for new blooms. Such a scenario is consistent with patterns of multiple small-scale blooms throughout the year in this region (Dyhrman et al. 2010).

Plasticity of the Chilling Response and Climate Change Implications

Dormancy breaking by chilling is one mechanism by which *A. catenella* and other dinoflagellates can match germination timing to their environment, thereby maximizing bloom potential. Normally-distributed responses reflect plasticity in the chilling requirements of individual cysts. It is the heterogeneity of these responses that likely allows populations to adapt to the temperature regime of their habitats. Cyst-forming dinoflagellates at higher latitudes where temperatures are colder might be expected to have lower T_{max} values, whereas those at lower latitudes where temperatures are warmer might be expected to have higher T_{max} values. High concentrations of *A. catenella* cysts have been observed as far north as the eastern Bering and Chukchi Seas (Gu et al. 2012; Natsuike et al. 2013), and in Greenland and Iceland (Richlen et al. 2016). Laboratory studies have demonstrated that these northern populations have physiological adaptations to their cold habitat – they can germinate at 1 °C and divide at 5 °C, though slowly in both cases (Natsuike et al. 2017). It may be that mechanisms regulating dormancy would be similarly adapted in these high latitude populations.

Shallow coastal waters and estuaries that have little thermal inertia will be some of the first to reflect climate change-induced warming of the atmosphere. Results from Nauset are therefore highly informative for examining how temperature thresholds may alter the phenology of *A. catenella* blooms. Under present conditions in Nauset, cysts first become quiescent in January when temperatures are typically too cold to support germination or cell division. Bloom initiation is therefore driven by the onset of spring warming that stimulates these processes, not the cysts' transitions to quiescence. It is for this reason that, counterintuitively, bloom development is expected to commence earlier in Nauset during anomalously warm years even while dormancy is prolonged due to the chilling requirement. Indeed, this warmth-induced advancement of bloom onset has already been observed (Brosnahan et al. 2015; Ralston et al. 2015). Our observations emphasize that climate responses of individual organisms are complex and not driven simply by single physiological mechanisms (Hallegraeff 2010).

More substantial climate warming, if severe enough, will eventually prevent most cysts from meeting their chilling requirements. Nevertheless, variability among individual cysts in their chilling requirements (e.g. Fig. 7) might enable populations to adapt over successive bloom cycles, tempering the effects of warming. The seedbed constitutes a species reservoir of genetic diversity (e.g. Alpermann et al. 2009) and can contain viable cysts up to 100 years old (Miyazono et al. 2012). Cysts that are able to meet their chilling requirement in extremely warm years will be more likely to bloom and produce new cysts with similarly reduced chilling requirements.

Methods

Study site: The Nauset Marsh system is a shallow estuary (Fig. 2) consisting of tidal channels within a central marsh area that connect three drowned kettle ponds – Mill Pond, Salt Pond, and Town Cove – each of which hosts independent, localized populations of *A. catenella* (Crespo et al. 2011; Ralston et al. 2015). Localization of blooms within the ponds occurs through an interaction between the swimming behavior of the *A. catenella* cells, stratification within the ponds, and the shallow central marsh area which acts as a sill, blocking passage of cells between ponds (Anderson et al. 1983; Anderson and Stolzenbach 1985; Ralston et al. 2015). In turn, retention promotes the local deposition of cysts that then inoculate new blooms each spring, leading to PSP shellfishing closures on a near-annual basis. An exception occurred during the second year of this study (2014) when no toxicity was recorded within Nauset through a weekly monitoring program that is overseen by the Massachusetts Division of Marine Fisheries. Cysts used in this study's germination experiments were collected from Roberts Cove, an area that is located immediately outside the inlet of Mill Pond. This site was selected due to its accessibility and high mean concentrations of *A. catenella* cysts in its surficial sediment (typically $>10^3$ cysts cm^{-3}).

Bottom water temperature in Roberts Cove was monitored for the duration of the study by a moored, internally-recording HOBO logger (Onset Computer Corporation), that was naturally buried in the surface layer of sediment due to its fluffy, mucky consistency (Fig. 3b). Nauset sediments are extremely porous, a fact that is demonstrated by constant pore water temperatures down to a depth of 100 cm (Nowicki et al. 1999). Thus, the HOBO loggers located within near surface sediments recorded the same temperatures that were experienced by Nauset cysts.

Weekly spring surveys of vegetative cell abundance: Approximately weekly during the spring months of 2013 through 2015, large-scale surveys of physical and biological conditions were conducted in Nauset as described elsewhere (Choi et al. 2017; Crespo et al. 2011; Ralston et al. 2014). Here we summarize those methods and focus on data collection in Mill Pond – adjacent to Roberts Cove, the site of cyst collection (Fig. 2). Surveys occurred around daytime high tides and began in the early spring and finished about 2 weeks after bloom termination: 14 surveys from 01 February to 06 May 2013, 6 surveys from 19 March to 19 May 2014, and 8 surveys from 02 April to 25 May 2015. *A. catenella* cells may have been present in Mill Pond earlier than the first sampling date. During each survey, water samples for *A. catenella* abundance was collected with 2.5 L Niskin bottles at multiple depths: 1 m, 3 m, 5 m, and 1 m above the bottom (7–8 m). Details on sample processing and quantification of *A. catenella* abundance are provided in Crespo et al. (2011) and Choi et al. (2017). In late April 2013, there was a large bloom (depth-averaged maximum: 4.3×10^5 cells L^{-1}) followed by significant cyst production that was visible in the sediments (Fig. 3a; Brosnahan et al. 2017). In contrast, 2014 did not have a significant bloom (depth-averaged maximum: 3.6×10^3 cells L^{-1}) and there was no apparent cyst production.

Monthly surveys of resting cyst abundance: To understand the timing of cyst formation relative to the dormancy status of the cyst seedbed, cysts were collected monthly from intertidal sediment within Roberts Cove, an area immediately outside of Mill Pond in the southern branch of the Nauset estuary (Fig. 2). Each month from March 2013 through June 2015, a hand corer was used to collect an undisturbed sediment core that was transported back to the laboratory for extrusion and sediment processing. All cores had a light-colored, oxygenated surface layer of a few millimeters overlaying anoxic sediment, which was identified by its black coloration and odor. During transportation, sediment cores were kept upright in a bucket filled with water maintained at the ambient surface sediment water temperature of Roberts Cove.

Once in the laboratory, headwater from each core was carefully aspirated and sediment was extruded. The surface 1 cm layer was sampled, in an effort to examine the cyst population most recently deposited and most likely to contribute to a bloom. Sediment samples from the top 1 cm layer were sonicated with a Branson Sonifier 250 at a constant 40-W output for 1 min, and sieved to yield a clean, 20–100 μm size fraction (Anderson et al. 2003). These fractions were fixed in 10% unbuffered formalin in seawater (v/v) at 4 °C for at least 30 min, then resuspended in −20 °C methanol for pigment extraction for at least 48 h. Cyst fractions were then resuspended in 200 μg mL^{-1} primulin in deionized water (Yamaguchi et al. 1995). After staining for 30 min, the sample was centrifuged at 4000 rpm (~3000 *g*) and decanted for the final time and brought up to 5 ml with deionized water. Finally, a 1 mL stained subsample was counted on a Zeiss Axioskop microscope under blue light epifluorescence at 160X magnification with a chlorophyll filter set (band pass 450–490 nm, long pass 520 nm). *A. catenella* cysts were easily enumerated due to positive staining and their characteristic pill shape, 55–60 μm long and 20–25 μm in diameter.

Seasonal dormancy status of the Nauset cyst seedbed: Monthly, from July 2013 through June 2015, the dormancy status of the cyst seedbed in Roberts Cove was determined from cysts collected within the same surface 1 cm section processed for cyst abundance. Following sonication and sieving according to the methods described previously, cysts were separated from same size particles with higher specific densities using density cushion centrifugation as described by Schwinghamer et al. (1991). Because *A. catenella* cysts range in densities from ~1.15 to 1.30 g cm^{-3}, heavy solutions of colloidal silica were adjusted to 1.40 g cm^{-3} through addition of sucrose to capture cysts at the cushion interface. Round-bottom step gradient tubes were centrifuged for 15 min at 3000 rpm (~1600 *g*), and then cysts and other organic material were removed from the cushion interface and overlaying suspension by Pasteur pipette. Cysts and other light debris were then concentrated and thoroughly rinsed with filtered seawater over a 20 μm sieve. Sieve contents were then backwashed into a Sedgewick-Rafter counting chamber from which cysts could be easily identified and isolated under a Zeiss Axioskop upright microscope.

For each monthly dormancy test, approximately 30 healthy-looking cysts (starch granules present, golden to brown coloration; Fig. 6) were individually isolated by micropipette and subjected to conditions known to induce rapid germination of quiescent cysts. Isolated cysts were placed in wells of a 96-well tissue culture plate, each pre-loaded with 200 μL of f/2 medium (Guillard and Ryther 1962). The plates were then sealed to limit evaporative loss of medium and placed into an incubator at 15 °C on a 14:10 light:dark cycle (150 μE m^{-2} s^{-1} photon flux density). Individual cysts were checked for germination on a weekly basis using an inverted Olympus IX-70 microscope at 100X and 40X. The presence of vegetative *A. catenella* cells was a clear indication that germination had occurred. If no swimming cells were found, the next best indication that a cyst had germinated was if it was located and found to be clearly empty (Fig. 6). If the germination percentage of total cysts after the first week was less than 50%, cysts were deemed dormant, and if the germination percentage exceeded 50%, cysts were deemed quiescent.

Cold storage experiment: A germination experiment was designed to examine the effect of winter chilling on dormancy-breaking in natural cyst populations (Fig. 4). Sediment cores

containing cysts were collected from Nauset in early October. Because a large proportion of the cysts analyzed through the study were formed following a significant *A. catenella* bloom during its first year, the effect of chilling on new cysts and cysts that had experienced a Nauset winter could be examined (first- and repeat-cycle cysts, respectively; Fig. 3a).

Both the first- and repeat-cycle cysts had almost certainly completed mandatory dormancy at the time of their collection. Anderson (1980) previously showed that the mandatory dormancy period of *A. catenella* cysts from another comparable shallow embayment on Cape Cod, ranged from 1 month when incubated at 22 °C to 4 months when incubated at 4 °C. Given the warm temperatures experienced from May to October 2013 (15–24 °C; Fig. 3b), mandatory dormancy of the 2013-year class is assumed to have been completed within ~1 month of formation.

For both collections, six undisturbed sediment cores were collected and processed following the procedure detailed in the seasonal dormancy status experiment above. The average concentration of cysts in the surface 1 cm of sediment was 3314(±1205) cm^{-3} in 2013 and 1314(±74) cm^{-3} in 2014 (Fig. 3a). After the 1 cm layer was subsampled, the sediment was combined with anoxic, cyst-free sediment from >15 cm depth in a 1:1 ratio to prevent oxygen absorption, and thus suppress germination. This mixture was dispensed into 5 mL amber, crimp-top, glass vials and when no headspace remained, vials were sealed and buried in the remaining anoxic sediment from the cores as outlined by Anderson and Keafer (1987).

The goal of this study was to examine the effect of natural chilling in Nauset on dormancy, so cold storage temperatures were selected to represent those that occurred naturally from late fall through early spring in Nauset: 2, 4, 6, and 8 °C (Fig. 3b). Each of the four 2-L glass containers was stored at a different cold temperature. The first-cycle cysts were stored at 2 °C and 6 °C, and the repeat-cycle cysts were stored at 4 °C and 8 °C. Although it would have been preferable to test the effects of all four temperatures on each cycle class, the significant amount of time and effort required to conduct these experiments made this impossible. Thus, a different pair of temperatures was tested each year. While it was feared that this could complicate data analysis, the temperature responsivity of the first- and repeat-cycle cysts was comparable, conveniently allowing all results to be combined. To control for the effects of chilling, first- and repeat-cycle cysts were also incubated at the ambient water temperature at the time of collection (18 °C) and their germination was monitored for 7 weeks.

Bimonthly for the first 6 months of cold storage (October–March), cysts were removed from 2, 4, 6, and 8 °C storage to test their dormancy status. To determine the dormancy state, single vials were removed from each cold storage container under a dim red photographic light to prevent stimulation of remaining cysts (Anderson et al. 2003; Binder and Anderson 1986) and special care was taken to maintain all contents at their given cold storage temperature. Vial contents were sonicated according to the previously detailed methods. For each bimonthly dormancy test, approximately 30 healthy-looking cysts (Fig. 6) were isolated and subjected to the same favorable 15 °C conditions as detailed in the methods above. Cysts were monitored for germination a total of seven weeks. Results from these dormancy tests were expressed as G, the cumulative fraction of total cysts germinated at each weekly check and G_{max}, the total fraction germinated at the end of seven weeks.

At the outset of these experiments it was not known whether cyst survival might differ between cold storage temperatures. In order to test for a temperature effect, values of G_{max} were compared between cold storage treatments. Initial assessments also showed that only longer-chilled cohorts from each storage treatment germinated readily. Therefore, G_{max} comparisons considered only tests from cohorts that reached at least 85% of the highest G_{max} of a given cold storage treatment (demarcated by open circles in Fig. 5). This subset of cohorts was then compared by one-way ANOVA. To account for storage temperature based differences in G_{max} among the cohorts, the term G^* is calculated from G:

$$G^* = \frac{G}{\bar{G}_{max}} \tag{1}$$

where $\bar{G}_{max}$ is the mean of G_{max} values derived from the subset of cohorts considered (>85% G_{max}). The effect of this normalization was to make the range of germinability across all of the cold storage treatments range from 0 to ~100%. In a small number of cases where G exceeds $\bar{G}_{max}$, the resulting G^* value exceeds 100%. From the G^* time-series, G^*_{50} was calculated for each temperature treatment, the point in cold storage time at which cyst cohorts attained a G^* of 50% within the first week of the germination assay.

Chilling-unit calculation: Chilling unit scaling was explored as a means to account for differences in the severity of cold exposure experienced during the 2, 4, 6, and 8 °C storage treatments. Initially devised for horticulture, chilling temperatures extend from the freezing point to the threshold temperature of an organism (T_{max}), which is the temperature below which "cold" is perceived (e.g. Luedeling et al. 2009; Penfield and Springthorpe 2012). This metric is similar to degree-days in that temperature and time are quantified together, but the former quantifies cold and the latter quantifies heat. In the simplest models, one chilling-unit is equal to one day's exposure to the chilling temperature, and these units are summed up for the period of interest. For this study, daily chilling-units (*CU*) were calculated from the average daily temperature (t_i) that cysts experienced during various cold storage treatments using the following formula:

$$CU\,(t) = \Delta t \sum_{i=t0}^{t} \begin{cases} T_{max} - t_i & \text{if} \quad T_{max} \geq t_i \geq 0 \\ 0 & \text{if} \quad \textit{otherwise} \end{cases} \tag{2}$$

To further illustrate this concept, imagine a species with a T_{max} = 10 °C. If one cohorts were exposed to 0 °C for 2 days and a second to 6 °C for 5 days, both would accumulate the same level of chilling (20 *CU*).

A T_{max} that is specific to *A. catenella* cysts in Nauset must be determined to calculate chilling-units for the experimental treatments in this study. From March 2013 through June 2015, Nauset bottom water temperatures ranged from −1 to 24 °C (Fig. 3b), so local cyst populations would be expected to have a T_{max} somewhere around this range. Our approach to estimate the most accurate T_{max} for Nauset *A. catenella* populations was to use T_{max} values from 2 to 30 °C to calculate chilling-units for each cold storage experiment. Next, for each value of T_{max}, the chilling-units associated with the G^* after one week of incubation (from all four temperature treatments) were fit to a sigmoid curve. Sigmoidal responses are commonly observed in terrestrial plant studies and reflect the normally-distributed variance of germination times among cysts in the experimental populations. The T_{max} was then selected by optimizing goodness of fit to this sigmoid curve.

To test the significance of the collapse in G^*_{50} differences observed between temperature treatments after chilling unit scaling, the following statistical model was devised. The null

hypothesis (H_o) was that the cold storage temperature had no effect on G^*_{50} timing and was evaluated against the alternative hypothesis (H_1) that there was an effect. Under H_o, the timing of the G^*_{50} is not mediated by the temperature of chilling and the difference among treatments reflects variability due to other untested factors. If H_1 is true, the variation in the number of *CU*s needed to attain G^*_{50} for each temperature treatment is small relative to a distribution constructed under H_0, i.e. with the assumption that storage temperature does not affect the *CU* variance. Our data consist of the G^*_{50} periods (in days) for each treatment (d_j, $j=2, 4, 6, 8\,^\circ$C) and the corresponding number of *CU*s ($c_j(d_j)$, $j=2, 4, 6, 8\,^\circ$C) accumulated through each cold-storage experiment. The observed range of *CU* required to reach G^*_{50} in each temperature and storage duration combination was used as a test statistic and significance was evaluated against a distribution constructed under the assumption of no temperature effect (from H_o). This distribution was approximated by re-assigning the values of d_j (d_2^*, d_4^*, d_6^*, d_8^* of d_2, d_4, d_6, d_8), forming the quantities $c_2(d_2^*)$, $c_4(d_4^*)$, $c_6(d_6^*)$, and $c_8(d_8^*)$ and then determining the resulting ranges of *CU* for each combination.

To compare our cold storage treatments to the natural chilling that cysts experienced during the same time period in Nauset, chilling-units were calculated from surface sediment water temperature records collected at Roberts Cove (Fig. 10). Calculations applied a start date in late May in 2013 and 2014, which coincides with the typical timing of new cyst formation within Nauset.

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