

Culture Studies of *Alexandrium affine* (Dinophyceae), a Non-Toxic Cyst Forming Dinoflagellate from Bahía Concepción, Gulf of California

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Alexandrium affine (Inoue *et* Fukuyo) Balech, isolated from Bahía Concepción (Gulf of California), was studied to determine the effect of environmental factors on cyst germination and vegetative growth. *Alexandrium affine* was homothallic and isogamous, and formed cysts in nutrient-deficient (N- or P-limiting) medium. The maturation period of newly formed cysts varied between two weeks and three months, depending on the storage temperature, with colder temperatures prolonging the process. The rate of germination increased with increasing temperature, and was not significantly influenced by light. Germination experiments suggest a broad temperature window for *A. affine* cysts, ranging from 5 to 25 °C. The optimal vegetative growth rates were 0.25 to 0.34 day⁻¹ at 20–30 °C. No vegetative growth was observed below 15 °C or above 35 °C. With HPLC toxin analyses, we confirm that this species does not produce saxitoxins. These data on the dormancy, excystment, and growth characteristics seem to be regulated by the environmental constraints of this subtropical bay.

Introduction

Alexandrium affine (Inoue *et* Fukuyo) Balech is a coastal, bloom-forming dinoflagellate (Hallegraeff *et al.* 1991). The vegetative cell and cyst of this species were first described in Japan by Fukuyo *et al.* (1985), after the summer blooms of 1974, 1975, and 1977. *Alexandrium affine* typically forms chains of 2–8 cells long, but chains as long as 16 cells have been observed. The cysts are spherical, smooth-walled, and mucilaginous. The protoplasm contains many starch globules and one or two red-pigmented bodies. Several species of the genus *Alexandrium* are toxic and produce saxitoxin, which is associated with paralytic shellfish poisoning (PSP) (Anderson 1998), but *A. affine* is considered non-toxic.

As has happened with other blooming species after their first description, *A. affine* has recently been reported in numerous countries, such as Spain (Bravo 1986, Fraga *et al.* 1989), Korea (Lee 1990, Kim *et al.* 1990), Tasmania (Hallegraeff *et al.* 1991), Thailand (Balech 1995), Philippines (Balech 1995), Portugal (Balech 1995), Brazil (Balech 1995), Peru (Vera *et al.* 1999), Indonesia (Sidabutar *et al.* 2000), Vietnam (Yoshida *et al.* 2000), and Mexico (Gárate-Lizarraga *et al.* 2001).

The bloom dynamics of this species are poorly understood. *Alexandrium affine* commonly blooms with other chain-forming dinoflagellates such as *Gymnodinium catenatum* Graham (Bravo 1986, Fraga and Bakun 1993), *Cochlodinium* sp., and *Pheopolykrikos hartmannii* (Zimmerman) Matsuoka *et* Fukuyo (Kim

et al. 1990). It can form blooms in areas of downwelling (Fraga and Bakun 1993), and its presence has been related to an increase in the surface temperature, river runoff, and rainfall (Wagey *et al.* 2000). Fraga *et al.* (1989) also argue that the chain-forming ability allows dinoflagellates like *Alexandrium affine* to dominate in waters with strong flows that disperse weaker-swimming species.

Few studies have been conducted on cultures of this species. Flynn *et al.* (1996) determined the effect of low nitrogen and low phosphate medium on growth and physiology. Several cultures of *A. affine* have been used for genetic studies. On the basis of ribosomal RNA sequences, strains from France, Spain, and Thailand have been grouped together, demonstrating that this species has nucleotide sequences species-specific or a unique ribotype within the *Alexandrium* genus (Scholin *et al.* 1994, Leinonen-DuFresne *et al.* 2000).

Many studies have been undertaken to understand the effect of environmental factors on the life history stages of a variety of dinoflagellate species. Much of the work thus far, however, has been on toxic species from temperate waters. Data on tropical and non-toxic species are lacking. Here, we report results of a culture study on the response of a subtropical isolate of *A. affine* to environmental factors at different stages of its life cycle. Data are also presented to verify that it does not produce saxitoxins.

Material and Methods

Collection site and typical environmental conditions

Bahía Concepción (26°33′–26°53′N, 111°42′–112°56′W) is a semi-enclosed bay on the east coast of Baja California Sur, Mexico, with an area of 275 km² (Fig. 1). Mean depth is 22 m, with a maximum of 34 m. Sediment at the bottom of the bay is mainly biogenic (Shumilin *et al.* 1996). Mean surface temperature is 24.5 °C with a winter minimum of 16.6 °C (Góngora-González 2001) and a summer maximum of 34.8 °C (Mateo-Cid *et al.* 1993). Mean salinity is 35.3 ppm (Dressler 1981). In this bay, two hydrological scenarios take place. During late autumn and winter, the water column is well-mixed; during spring-summer, a strong stratification promotes a hypoxic and anoxic sulfur-reducing environment (Bustillos-Guzmán *et al.* 2000, Lechuga-Devéze *et al.* 2000).

Phytoplankton sampling

Phytoplankton samples were collected twice a month from February to August 2000 by vertical tows using a 20 µm net. The cell concentrate was sieved through a 60 µm mesh to eliminate larger organisms and was enriched with modified *f/2* medium (Anderson *et al.* 1984) in the laboratory. Vegetative cells of *Alexandrium affine* were isolated from the enrichments with micropipettes using an inverted microscope (Carl Zeiss, Axiovert 100, Oberkochen, Germany). The cells were transferred to 96 well plates with modified *f/2* medium and maintained at 21 ± 1 °C in a L:D cycle 10:14 at 150 µmol m⁻² s⁻¹. Cultures from these wells were later transferred into larger vessels for experimental purposes. All experiments on vegetative cells were based on a non-axenic culture of *A. affine* (AABCV-1) isolated in March 2000.

Identification

Light microscopy observations of cysts and motile cells was done with a digital camera (Media Cybernetics, Cool Snap-Pro cf, Silver Spring, U.S.A.). Epi-fluorescence observations were done with a Carl Zeiss MC80 microscope (Oberkochen, Germany). Cells were stained with 1% calcofluor (Fritz and Triemer 1985) for observation of plate tabulation.

Effect of temperature on growth

Temperature effects on growth rate were determined in a temperature gradient bar (Watras *et al.* 1982) ranging from 5 to 35 °C, at 20 different temperature levels. This device consists of an aluminium bar with a heat source connected at one end and a cooling device at the other. The bar contains holes for the insertion of 50 mL capacity test tubes arranged in 20 rows of four holes each between the two ends of the bar. Light was supplied by four cool-white fluorescent lamps set at a 10:14, L:D cycle at 230 µmol m⁻² s⁻¹, positioned under the test tubes (Rengefors and Anderson 1998). The test tubes were filled with 25 mL modified *f/2* medium. Prior to transfer into the temperature gradient bar, cultures were acclimated for two generations at or near each experimental temperature, with transfers made during exponential growth. Culture growth was determined by *in vivo* fluorescence monitored daily with a fluorometer (Turner Designs 10-AU, Sunnyvale, U.S.A.). The fluorescence data were used to calculate exponential growth rates according to Guillard (1973). The average growth rate at each temperature was determined from the average of four replicates.

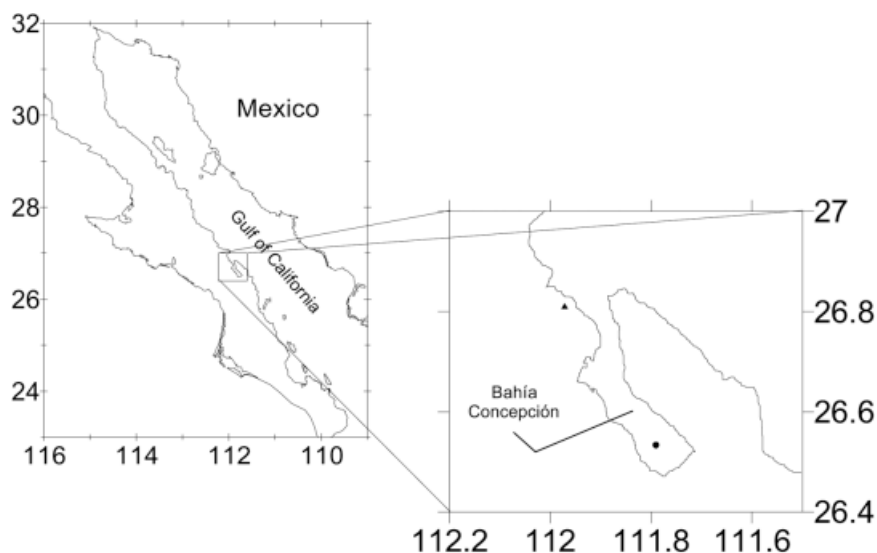


Fig. 1. Map of Bahía Concepción showing the collection site for sediment and phytoplankton.

Sediment collection

Sediment samples were collected by SCUBA diving in the southern part of the basin of Bahía Concepción at approximately 30 m depth (Fig. 1). The collections were taken monthly from May to August 2000. The surface layer of the sediment (top 1 cm) was collected in 500 mL amber glass containers. These were capped underwater and stored in darkness at 20 °C.

Cyst processing and enumeration

Aliquots for the sediment samples were diluted (1:5) with filtered seawater, sonicated for 60 s at 60 W (Branson Ultrasonics Corporation 250, Danbury, U.S.A.), and then sieved sequentially through 80 and 20 µm Nitex screens. The material retained on the 20 µm screen was resuspended in 9 mL of filtered seawater. Identification and counting of dinoflagellate cysts was done microscopically using a 1 mL Sedgwick Rafter slide.

Germination rate

Sediment was collected from a single batch collected 13 May 2000, 150 mL of sediment was combined with 500 mL of filtered seawater. From this slurry 5 mL was dispensed into 125 mL Erlenmeyer flasks, and 40 mL of modified *f/2* medium was added to each flask. The flasks were incubated at 5 different temperatures (5, 10, 15, 20, and 25 °C) under dark and light conditions. At each temperature 10 flasks were exposed under 10:14, L:D conditions and an additional 10 flasks were enclosed in a dark box. Cyst samples incubated under dark conditions were always processed in a dark room, with a red lamp providing the only light source to minimise any light-induced germination. To determine the number of *Alexandrium affine* cysts, the flasks were emptied, rinsed well with filtered seawater, and the contents sonicated and sieved to retain the 20 to 80 µm fraction. The material retained on the 20 µm sieve was resuspended in 5 mL of filtered seawater and dispensed on 1 mL Sedgwick-Rafter slides for quantification. Six randomly selected flasks were processed immediately to determine the initial cyst concentration for all treatments. Only cysts with full cytoplasm were counted. Those not matching this criterion were considered dead. Germination rates were determined by the decrease in the number of living cysts over time. The germination rate was calculated using the linear trend of the germination curve according to:

$$N_t = N_0 \exp(-kt)$$

where N_0 is the initial concentration of cysts, N_t is the concentration of cysts remaining at time t , and k is the specific germination rate with units of time^{-1} (Anderson *et al.* 1987).

In this experiment, samples from the various treatments were counted at different intervals, always by

harvesting replicate flasks, until 90% germination was achieved. A covariance analysis was used to determine significant differences in the germination rate between light and dark treatments at each temperature.

Sexual induction

For crossing experiments, 2 strains of *Alexandrium affine* were inoculated in 25 mL of *f/2* nitrate deficient medium (ca. 44 µM NO_3 , but all other major nutrients, trace metals and vitamins at *f/2* concentrations) at 25 °C and 150 µmol $\text{m}^{-2} \text{s}^{-1}$, L:D, 10:14 cycle (Anderson *et al.* 1984). The isolates were mixed together, and kept clonal to determine if *A. affine* is heterothallic or homothallic. After fifteen days, the cysts that were produced were transferred to 3 mL vials capped with a 20 µm filter. The vials were placed in a glass jar filled with anoxic sediment, which was tightly sealed and incubated in the dark at 15 °C. The 20 µm filter over the mouth of the vial allowed for gas and nutrient exchange between the sample and the sediment, which is necessary for the survival of laboratory-reared cysts.

Mandatory dormancy period

At least 90 cysts formed in the laboratory and stored as described above were harvested at different times and isolated with a micropipette into individual wells of a 96-well plate filled with modified *f/2* medium (Anderson *et al.* 1984). The plates were incubated at 20 °C, 10:14, L:D cycle and 150 µmol $\text{m}^{-2} \text{s}^{-1}$. Plates were checked after a week for empty (germinated) cysts under an inverted microscope. Dead and living cysts were recorded, and germination percentage was calculated, based on living cysts.

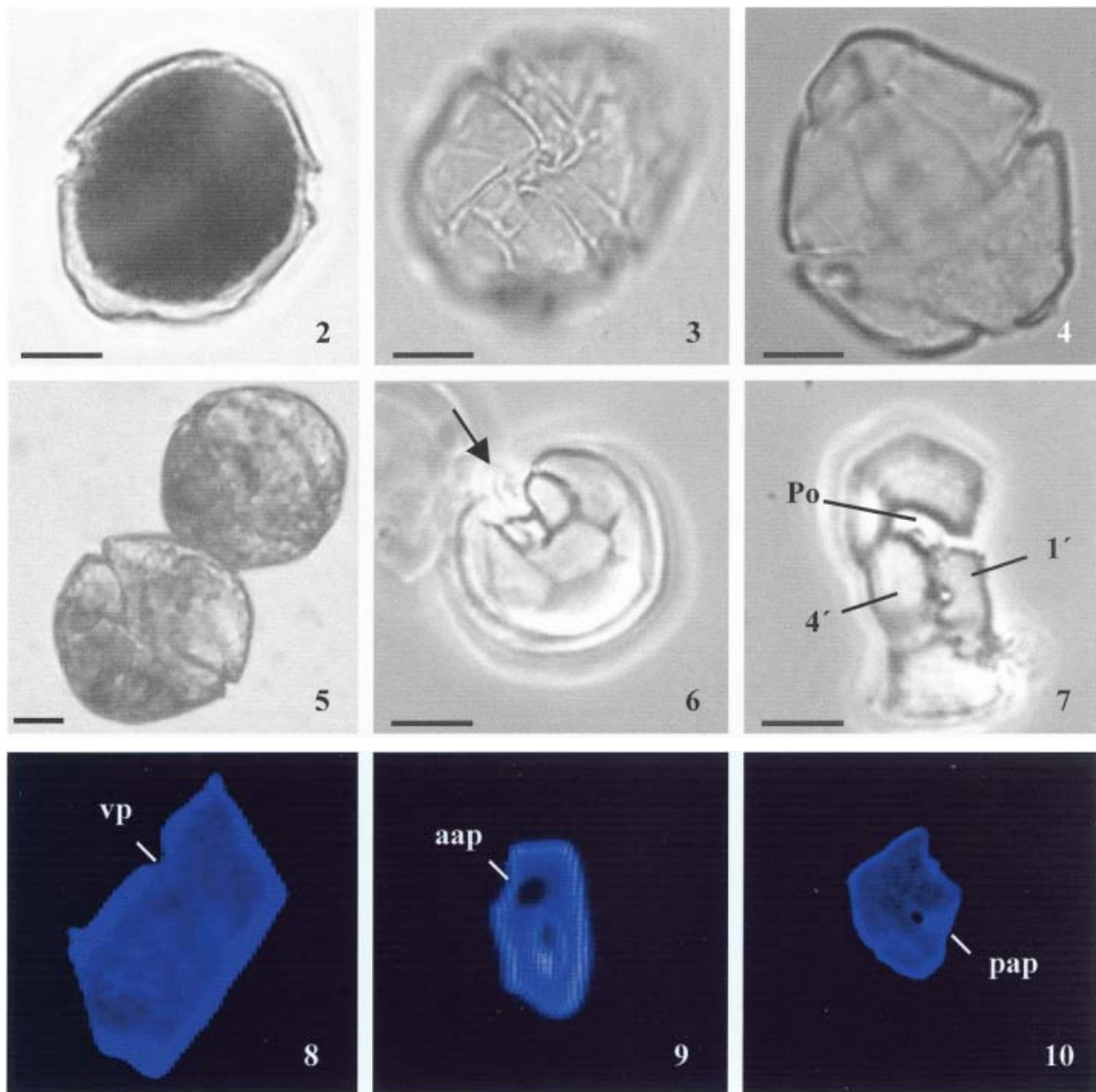
Toxin analysis

A volume of 15 mL of a mid-log culture was harvested by centrifugation at 5000 × g for 5 min. The supernatant was removed by aspiration and 1 mL of 0.05 M acetic acid was added. The cells were sonicated with an ultrasonic probe at 10 W for 40 s while on ice. A subsample was examined for remaining intact cells. The extract was frozen at -20 °C. Thawed samples were stirred and centrifuged to remove debris. Injection samples were prepared for high performance liquid chromatography. The samples were analysed for saxitoxin and its derivatives, following the method of Oshima (1995) as detailed by Anderson *et al.* (1994).

Results

Identity of the organism

Cultured cells are usually longer (29–66 µm) than wide (23–65 µm), the epitheca is rounded and the



Figs 2–10. *Alexandrium affine*.

Fig. 2. Light microscopy (LM) of single cell from culture, cell longer than wide with rounded epitheca. Fig. 3. LM. Ventral view. Fig. 4. LM. Lateral view. Fig. 5. LM. Cultured two-celled chain. Fig. 6. LM. Antapical view showing antapical concavity and plate arrangement. Fig. 7. LM. Detail of detached apical plates with apical pore plate (Po) and small ventral pore (vp) between 1' and 4' plates. Fig. 8. Epifluorescence microscopy (EM) of calcofluor stained 1' plate with vp. Fig. 9. EM. Detail of Po showing comma-shaped apical pore and anterior attachment pore (aap). Fig. 10. EM. Detail of posterior sulcal plate (Sp) with posterior attachment pore (pap). Scale bar 10 μ m.

sulcus is deeply indented (Figs 2–4). This species can form chains (Fig. 5). The hypotheca has an antapical wide concavity (Fig. 6). Plates are rather thick and have scattered pores. The plate tabulation (Figs 6–10) coincides with what has been reported by other authors (Fukuyo *et al.* 1985, Hallegraeff *et al.* 1991, Balech 1995). The first apical plate is directly connected with the apical pore plate and has a small ventral pore between the 1' and 4' plates (Figs 7, 8). The apical pore plate is sub-rectangular with a comma-shaped apical pore (Fig. 9), and a large round anterior attachment pore which can be

absent if the cells are not forming chains. The posterior sulcal plate has a posterior attachment pore (Fig. 10).

Cyst densities in natural sediments

Cysts of *Alexandrium affine* are spherical, with a diameter of 28–30 μ m (Fig. 11). They typically contain one or two red eyespots. Cysts are identical to those first described by Fukuyo *et al.* (1985). The abundance of *A. affine* cysts at a single station was measured monthly from May to August. Highest concen-

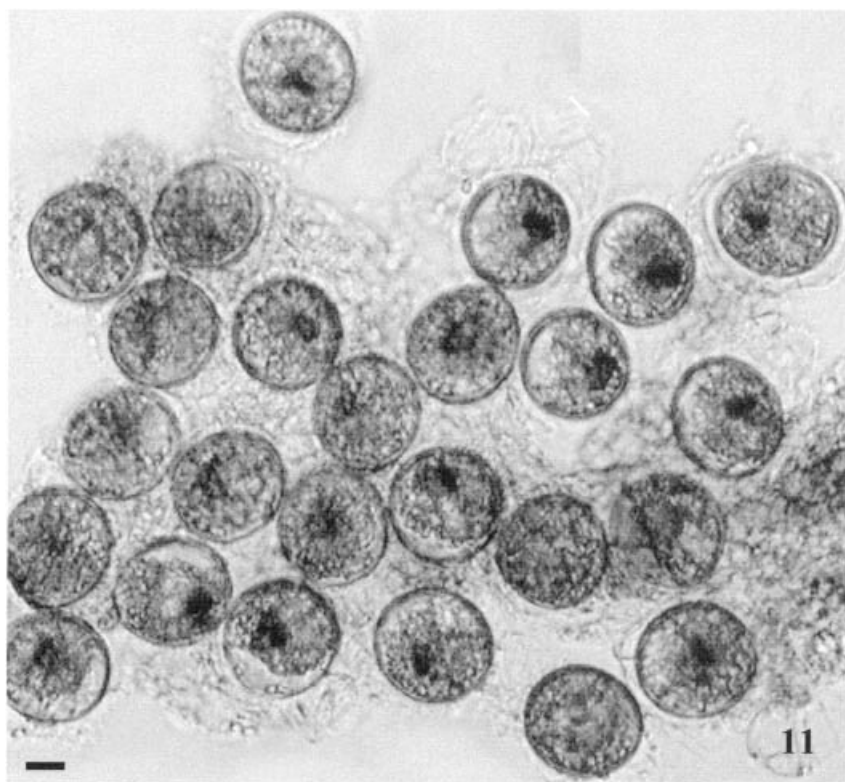


Fig. 11. Cysts of *A. affine* produced under low nitrogen conditions. Scale bar 10 μm .

Table I. Cyst densities of *A. affine* in surface sediments of Bahía Concepción from May to August 2000. Replicates are from a single sediment sample.

	Cysts mL^{-1} (average \pm SD)	Cysts g^{-1} (average \pm SD)
May	251 \pm 50	991 \pm 198
June	448 \pm 54	1848 \pm 215
July	360 \pm 14	1436 \pm 56
August	213 \pm 18	913 \pm 71

trations were found during June and July, with a density of 350–486 cysts mL^{-1} (Table I).

Sexuality and cyst formation

Based on observations of fusing cells, gametes of *A. affine* are morphologically indistinguishable from vegetative cells and are thus isogamous. Clonal *A. affine* cultures produce cysts when incubated in low phosphate and low nitrate medium. The percentage of cyst formation in phosphate deficient medium was lower (1%) than in nitrate deficient medium (8%).

Mandatory dormancy period

A small percentage (3–4%) of the cysts stored at

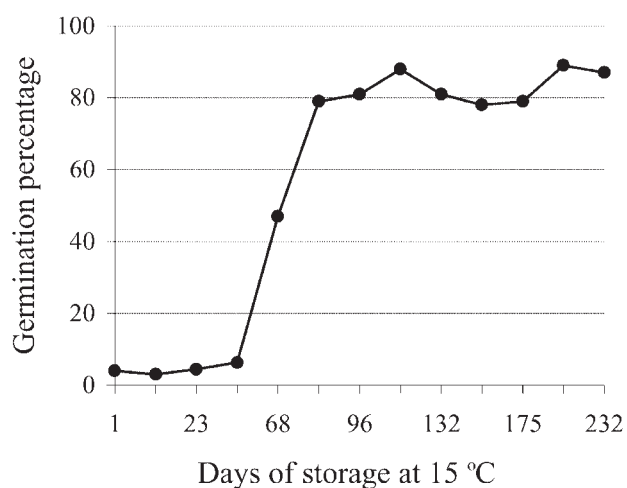


Fig. 12. Maturation and germination percentage of laboratory-induced cysts of *A. affine* stored in darkness at 15 °C.

15 °C started to germinate within 1–10 days of formation (Fig. 12). Germination remained insignificant until day 47, when 6% germination was recorded. By day 68, nearly 50% germination had occurred. Over 80% germination was achieved after 96 days of incubation at 15 °C. During these incubations, some of the laboratory-formed cysts died (typically 3–14% of those initially isolated). Dead cysts were not included in the calculations of germination success.

Germination rate

The rate of germination of mature cysts increased with increasing temperature. There was a significant difference between the light and dark treatment at 10, 15, 20, and 25 °C ($p > 0.05$). The germination rate in light varied between 0.02 day⁻¹ at 5 °C, and 0.13 day⁻¹ at 25 °C (Table II). With dark conditions, the germination rate varied between 0.03 day⁻¹ at 5 °C, and 0.01 day⁻¹ at 25 °C. Approximately 90 % of the *A. affine* cysts germinated following 15–20 days of incubation at 20–25 °C (Fig. 13 A, B), whereas at 5 °C and 10 °C, the germination rate was significantly slower, requiring 100 days to achieve ~90 % germination (Fig. 13 D, E).

Table II. Germination rates of *A. affine* cysts from natural samples (collected 13 May 2000) at different temperatures in light and dark incubations.

Temperature (°C)	Germination rate (day ⁻¹)	
	Light	Dark
25	0.127*	0.095*
20	0.158*	0.079*
15	0.125*	0.067*
10	0.040*	0.031*
5	0.026	0.026

* Slope significantly different between light and dark treatments ($p > 0.05$)

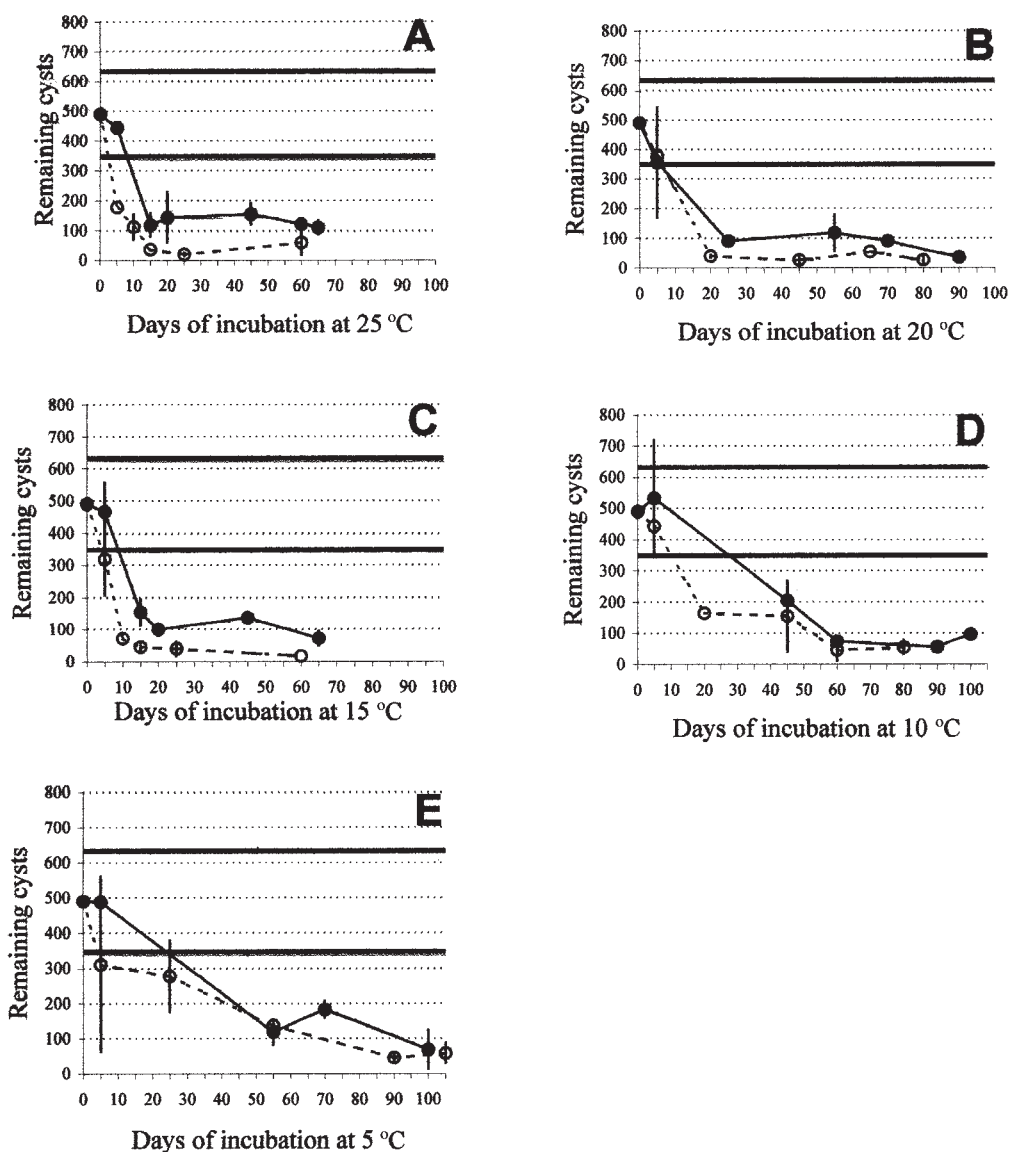


Fig. 13. Germination of *A. affine* cysts from natural samples (date of collection: 13 May 2000) with light (○) and dark incubation (●) at different incubation temperatures. The number of remaining cysts indicate ungerminated cysts in sediment. Dark lines represent the area of mean initial counts ± 1 SE. The difference between the mean and the final counts is assumed to represent germinated cysts.

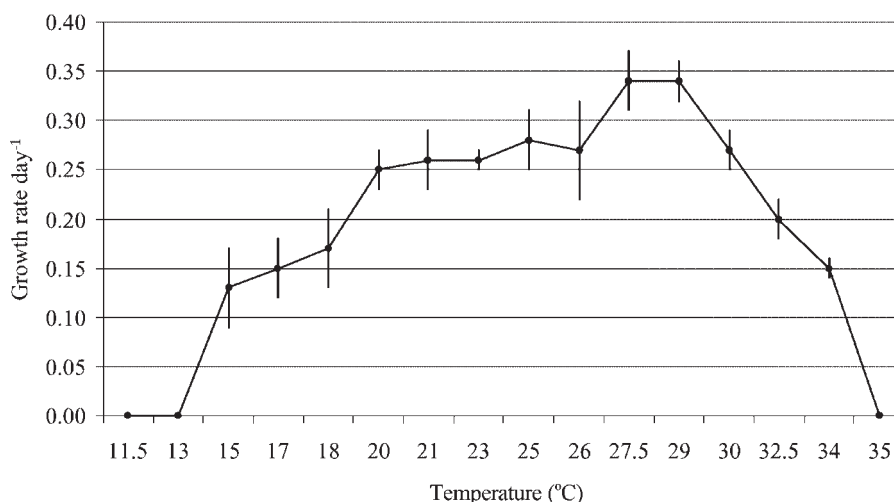


Fig. 14. Growth rates of *A. affine* at different temperatures.

Temperature effects on growth

The growth of *A. affine* at different temperatures is shown in Figure 14. Growth was observed between 15 and 34 °C, with optimal rates of 0.25–0.34 day⁻¹ between 20–30 °C. The highest rates were observed between 27.5 and 29 °C. Growth rate decreased rapidly at high temperatures and was zero at 35 °C.

Toxin measurements

Alexandrium affine clone AABCV-1 was analysed for STX toxins (STX, dcSTX, neoSTX), GTX toxins (GTX1, GTX2, GTX3, GTX4, GTX5) and C toxins (C1, C2, C3, C4). As expected *A. affine* did not show any detectable STX, GTX or C toxins (data not shown).

Discussion and Conclusion

The dinoflagellate genus *Alexandrium* includes a number of cyst-forming species that have been studied extensively because many are linked to dangerous and destructive blooms as a result of the toxins they produce (Anderson 1998). Given the importance of cyst and motile cell dynamics in the bloom process, there have been a number of studies of the encystment, dormancy, excystment, growth, and toxicity of many of these species (e.g., Watras *et al.* 1982, Anderson *et al.* 1983, 1984, Kita *et al.* 1993, Blanco 1995, Jensen and Moestrup 1997, Garcés *et al.* 1998). To date, however, studies of tropical or subtropical *Alexandrium* species are few, as are investigations of non-toxic *Alexandrium*. Here we describe the results of culture studies on *A. affine* from Bahía Concepción, a subtropical bay of the Gulf of California. The data that are now available on the dormancy, excystment, and growth characteristics of this strain of *A. affine* allow us to suggest its seasonal variability in

the context of the local hydrography of this subtropical bay.

Sexuality and cyst formation

In culture *A. affine* typically forms chains of 2–8 cells, although 16-cell chains have occasionally been observed. This species includes a cyst stage in its life history (Fukuyo *et al.* 1985), but little is known of cyst physiological characteristics or dynamics. Here we conclude that *A. affine* is homothallic, as we obtained thick-walled resting cysts or hypnozygotes from clonal cultures in nutrient deficient medium (Fig. 11). *Alexandrium affine* is isogamous, as gametes were indistinguishable from vegetative cells. The cysts are spherical, smooth-walled, and mucilaginous, as described by Fukuyo *et al.* (1985). The cyst protoplasm contains many starch globules and one or two red-pigmented bodies.

Commonly, dinoflagellate cyst production in culture is achieved by nutrient depletion (e.g., Pfiester 1975, Turpin *et al.* 1978, Anderson *et al.* 1984). However, this apparent linkage between nutrient limitation and encystment has not been consistently confirmed by field measurements. For example, *Alexandrium tamarense* (Lebour) Balech from the St. Lawrence Estuary (Castell-Pérez *et al.* 1998) and *Scrippsiella trochoidea* (Stein) Loeblich III from Korea (Kim and Han 2000) showed no apparent relationship between cyst formation and nitrogen or phosphate depletion; surface nitrate and phosphate concentrations were relatively high at the time most cysts formed. Likewise, Anderson *et al.* (1983) reported cyst formation in *Alexandrium tamarense* at relatively high nutrient concentrations in shallow salt ponds at Cape Cod. The only species of *Alexandrium* so far reported not to require environmental stress to induce sexual reproduction in laboratory cultures is *A. hiranoi* Kita *et al.* (Kita *et al.* 1993).

In Bahía Concepción, *A. affine* cysts form during the development of thermal stratification (Morquecho and Lechuga-Devéze 2002). At that time the surface layer (0–10 m) has low nutrient concentrations, whereas under the thermocline (below 20 m), the concentration of nitrates, nitrites, silicates, and phosphates is higher, and dissolved oxygen concentrations are lower (Bustillos-Guzmán *et al.* 2000, Góngora-González 2001). Low levels of oxygen in deeper waters might enhance sexual reproduction of *A. affine* by restricting motile cells to the upper, nutrient-depleted layers of the water column. Cells would experience a rise in temperature and nutrient depletion, leading to encystment. As Anderson *et al.* (1983) pointed out, *Alexandrium* growth increases with higher water temperatures, leading to a higher demand for nutrients that might not be satisfied in this upper layer compared to when temperatures (and growth rates) were lower. Without data on vertical migration, species-specific nutrient quota measurements or other measures of nutrient status, this aspect of bloom dynamics of *A. affine* remains unknown.

Cyst maturation

The successful production of cysts in laboratory culture made it possible to determine, for the first time, the duration of the mandatory dormancy interval for *A. affine*. Based on these studies, *A. affine* has a maturation period between two weeks and 3 months, depending on storage temperature. Cysts stored at higher temperatures germinated more rapidly than those stored at lower temperatures. At 25 °C, 90% germination was obtained within 15 days of incubation, while at 5 °C, 85–95 days were necessary to achieve comparable levels.

Only a few studies have explored the length of the cyst maturation interval for *Alexandrium* species. A similar temperature dependence to that observed for *A. affine* was also observed for *A. tamarense*, for which the duration of maturation was between 1–6 months, with the longest time interval associated with the coldest temperatures (Anderson 1980). *Alexandrium tamarense* from the St. Lawrence Estuary had a maturation period of 12–14 months at cold temperatures (Castell-Pérez *et al.* 1998).

Temperature effects on germination

Alexandrium affine cysts do not require a shift in temperature to initiate germination. Cysts incubated at the same temperature used for storage (20 °C), but in the presence of oxygen, germinated under both light and dark conditions. The rate of germination was faster at higher temperatures, but a change in temperature was not needed to trigger the excystment process. Most dinoflagellate germination studies performed on temperate marine species have shown that excystment occurs at specific temperature ranges or

after a temperature shift (Dale 1983, Pfiester and Anderson 1987). Some argue that excystment occurs within a permissive temperature window once maturation is complete, without the need of a shift upwards or downwards (e.g., Dale 1983, Anderson 1998). The results of our germination experiments suggest that the permissive temperature window for *A. affine* cysts is broad. Germination took place from 5 to 25 °C, which is similar to the germination window (5–21 °C) for *A. tamarense* (Anderson 1980). This would allow for germination in all seasons in this subtropical bay, since the bottom water temperature ranges from 16 °C in winter to 26 °C during summer. Our laboratory results suggest that *A. affine* cyst maturation lasts approximately 2 weeks in the temperature window, and that this species should be present in the water column throughout the year, if maturation timing and temperature were the only regulatory factors for germination. *Alexandrium affine* has a highly seasonal pattern of population abundance, with long intervals when it is not detected in the plankton. Clearly, other factors keep this species in a quiescent state in the sediments for extended periods of time.

Light effects

Light is not essential for germination of *A. affine* cysts ($p > 0.05$). However, at 10, 15, 20, and 25 °C, germination rates were significantly different between light and dark conditions ($p > 0.05$), whereas there was no difference at 5 °C. Though statistically significant, these differences are not large, and positive germination did occur at all temperatures in darkness. We conclude that light (or its absence) is not a major factor in the germination dynamics of *A. affine* in natural waters. In a similar manner, light was not required for germination of *A. tamarense* cysts from the St. Lawrence Estuary. Anderson *et al.* (1987) showed that light stimulation affected the rate of germination and the total number of cells excysted in five dinoflagellates, including *A. tamarense* from the Cape Cod area, though light was not an absolute requirement.

Culture growth rate

Growth rate varied significantly with temperature. *Alexandrium affine* can maintain vegetative growth between 15 and 34 °C, with optimal growth at 27.5–29 °C. No growth was observed below 13 °C or above 35 °C. The highest growth rates (0.25–0.34 day⁻¹) were at 20–30 °C. The temperature range for growth of the Bahía Concepción strain of *A. affine* is thus higher than for species of *Alexandrium* from temperate waters, as would be expected for a subtropical strain or species. *Alexandrium tamarense* from the Cape Cod area does not grow well below 7 or above 26 °C and has an optimum range between 11 and 22 °C (Anderson *et al.* 1984). The optimum temperature for cell division of *A. minutum* Halim from

Adelaide, Australia (Cannon 1993) is near 20 °C, with cells actively growing between 12 and 25 °C. *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen from Danish waters grew at temperatures from 11.3 to 23.7 °C (Jensen and Moestrup 1997). The maximum division rate for that species was 0.3 day⁻¹ at 20 °C, which falls within the range of growth rates typical of many dinoflagellates. Maximum rates in laboratory cultures of other species of *Alexandrium* are typically 0.3 to 0.7 day⁻¹ (Anderson 1998). In this context the maximum rate of growth we observed for *A. affine* (0.34 day⁻¹) falls at the low end of the range of other strains of *Alexandrium*. Culturing of *A. affine* using different seawater sources and nutrient enrichments did not increase the growth rate (data not shown).

Alexandrium affine seasonal variability

In Bahía Concepción, cells of dinoflagellates, including *A. affine*, are present during spring and early summer (Gárate-Lizárraga *et al.* 2001, Morquecho and Lechuga-Déveze 2002). The production rate of cysts of *A. affine* in the water column is highest at the beginning of spring, coincident with higher densities of *A. affine* vegetative cells in the water column (Morquecho and Lechuga-Déveze 2002). With a short maturation interval at permissive warm spring temperatures (18–25 °C), no absolute light requirement for excystment, and a permissive temperature window for germination that falls within the ambient temperature range, *A. affine* cyst germination should readily occur throughout the year. Water temperatures are also supportive of growth throughout the year, although they are most favourable in the spring–early summer. Even then, they are slightly lower than those that lead to maximal growth rates in laboratory cultures (21–30 °C). Other dinoflagellates also bloom in conditions that are sub-optimal for vegetative growth. For example *Gymnodinium nolleri* Ellegaard *et* Moestrup has optimal growth in the laboratory at 25 °C, whereas the temperatures of Danish waters rarely reach this level (Ellegaard *et al.* 1998). *Alexandrium tamarense* from the Cape Cod area showed optimum growth and encystment at 21 °C in the laboratory, yet blooms are generally over when the water temperature reaches 12–15 °C (Anderson *et al.* 1983).

In the absence of any other regulatory factors, we would expect the germination of *A. affine* cysts to occur throughout the year, resulting in recurrent blooms, or at least in the presence of *A. affine* cells in the plankton. This is not the case, however, and the answer to this apparent dilemma probably lies with the seasonal anoxia that develops in Bahía Concepción. All five marine dinoflagellate cysts tested by Anderson *et al.* (1987) required oxygen for germination. Likewise, Rengefors and Anderson (1998) demonstrated that anoxia inhibited the germination of the cysts of the freshwater dinoflagellate *Ceratium hirundinella* (Müller) Dujardin. These and other

studies indicate that under anoxic conditions, mature cysts do not germinate but remain quiescent until more favourable conditions occur. In Bahía Concepción, the particulate organic matter produced in the winter and spring accumulates in bottom sediments, and due to a strong thermocline and warm temperatures, hypoxia and anoxia develop in bottom waters (Bustillos-Guzmán *et al.* 2000, Lechuga-Déveze *et al.* 2000, 2001). This would prevent *Alexandrium affine* cyst germination, at least until the thermocline breaks down and oxygen is re-supplied to surface sediments in the winter. At that time, cyst germination may occur, but the development of blooms may be impeded by the lack of water column stability and lower water temperatures and light levels. Careful observations during the winter months might reveal some *A. affine* cells. These light, temperature, and stratification constraints disappear in the spring, when the first significant numbers of *A. affine* cells are observed.

Alexandrium affine does not produce paralytic shellfish poisons, confirming other reports (Hallegraeff *et al.* 1991).

In summary, the laboratory experiments reported here establish the basic physiology of *Alexandrium affine* from subtropical Bahía Concepción and explain in part its seasonal variability. In particular, the seasonal nature of the blooms suggests that the timing of cyst germination is probably regulated by anoxia in bottom waters. We believe the factors leading to cyst formation are related to low nutrients and rising temperatures in the stratified surface layer, coincident with hypoxia and anoxia in bottom waters. However, it may be that other, non-nutritional factors also regulate cyst formation. These and other unknowns need further study.

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