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The effects of temperature, growth medium and darkness on excystment and growth of the toxic dinoflagellate *Gymnodinium catenatum* from northwest Spain

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Abstract. The chain-forming dinoflagellate *Gynmodinium catenatum* Graham causes recurrent outbreaks of paralytic shellfish poisoning (PSP) in the Galician Rias Bajas (northwest Spain). A sediment survey in Ria de Vigo in April 1986 indicated that the highest concentrations of cysts of this species were located in the middle sections of the ria, with maximum abundance of 310 cysts cm^{-3}. The effects of temperature, growth medium composition and irradiance on the germination of laboratory-produced resting cysts were investigated. Newly formed cysts required very little time for maturation, as excystment was possible within 2 weeks of encystment. Growth media did not affect germination success. In contrast, the excystment rate was retarded signifiantly in darkness. Germination was also strongly affected by temperature, with ~75% excystment success at 22–28°C and little or no germination below 11°C after 1 month of incubation. In culture, the optimum growth rate of vegetative cells was between 22 and 28°C, the highest rate being 0.53 divisions day^{-1} at 24°C. Growth did not occur at temperatures <11°C or >30°C. These results are important with respect to the different hypotheses proposed to explain the initiation of *G.catenatum* blooms in the Galician Rias Bajas and Northern Portugal. The pattern of *G.catenatum* bloom development along this coast has been related to seasonal upwelling in the area, with major blooms occurring during the autumn as warmer offshore surface water is transported towards the coast when upwelling relaxes. The landward transport of established offshore populations of *G.catenatum* with the warm surface layer remains a viable explanation for the observed blooms within the rias, but alternatively, our data suggest that cysts within the rias can provide the inoculum population at times conducive to growth and bloom formation. Even though newly formed *G.catenatum* cysts have a very short maturation time and can germinate in darkness across a wide temperature range, bloom development will be significant only during the late summer and early autumn, since in other months light levels at the sediment surface and temperatures throughout the water column are too low for significant germination or growth.

Introduction

The toxic, chain-forming dinoflagellate *Gymnodinium catenatum* has been responsible for episodes of paralytic shellfish poisoning (PSP) in Spain (Estrada *et al.*, 1984), Mexico (Morey-Gaines, 1982; Mee *et al.*, 1986), Portugal (Franca and Almeida, 1989), Tasmania (Hallegraeff and Sumner, 1986) and Japan (Ikeda *et al.*, 1989). In Spain, the most important blooms of this species occur in Galician coastal waters (northwest Spain). These blooms have recurred nearly every year since 1985, the first year PSP in shellfish was conclusively linked to *G.catenatum* in Spain (Fraga *et al.*, 1988; Anderson *et al.*, 1989).

Two mechanisms have been advanced to explain *G.catenatum* bloom dynamics in the Galician rias. Fraga *et al.* (1988) hypothesized that established blooms of *G.catenatum* are carried into the rias with warmer offshore surface waters when the normal upwelling-favourable northerly winds change to downwelling-favorable southerly or southwesterly winds. When this occurs, a marked increase in water temperature is observed in the ria. A link between blooms, increases in surface water temperatures and the relaxation of upwelling has been observed in the years since the initial 1985 outbreak (Fraga *et al.*, 1990, 1993).

A second mechanism for bloom formation was suggested by Figueiras and Pazos (1991), who examined phytoplankton abundance in Ria de Vigo before and during a *G.catenatum* bloom. On the basis of principal component analysis, they rejected the hypothesis of bloom transport into the ria via downwelling surface waters. They suggested that sediment resuspension and cyst germination near the mouth of the ria provided a sufficient inoculum for bloom initiation.

The importance of dormant cysts in the bloom dynamics of marine dinoflagellates has been demonstrated in species such as *Alexandrium tamarense* (Anderson and Wall, 1978; Anderson and Morel, 1979; Anderson, 1980; Anderson *et al.*, 1983), *Gonyaulax polyedra* (Lewis, 1988) and *Gyrodinium uncatenum* (Tyler *et al.*, 1982). The present work describes the presence of *G.catenatum* cysts in the Galacian rias, as well as their dormancy and germination characteristics, and lends credence to the hypothesis that cysts play an important role in the initiation of blooms of this species. Nevertheless, the absence of a dormancy period in *G.catenatum* (Blackburn *et al.*, 1989) forces us to consider carefully the factors which control the observed seasonal timing of these blooms.

Temperature is one of the factors most frequently mentioned with respect to regulation of the timing of excystment (Huber and Nipkow, 1923; Wall and Dale, 1968; Fukuyo *et al.*, 1982; Endo and Nagata, 1984; Binder and Anderson, 1987). However, other factors can over-ride favorable or permissive temperatures and prevent germination, such that cysts remain in the sediments even when the temperature is within an optimal range (Huber and Nipkow, 1922; Anderson and Morel, 1979; Heaney *et al.*, 1983; Anderson *et al.*, 1983; Lewis *et al.*, 1985). For example, low light and low oxygen conditions can retard or inhibit excystment (Anderson *et al.*, 1987), and an endogenous annual clock has even been described as a regulatory factor (Anderson and Keafer, 1987). These responses, especially light requirements, vary among species (Anderson and Wall, 1978; Endo and Nagata, 1984; Anderson *et al.*, 1987).

Here we examine the effects of temperature, light and growth media on *G.catenatum* cyst germination and vegetative cell growth, and discuss the manner in which these factors can mediate the timing and magnitude of toxic populations in the Galician rias. Our results suggest that cyst germination and rapid cell growth within the rias are only likely during the late summer and fall, which is the interval when toxicity in shellfish and *G.catenatum* blooms are in fact commonly observed.

Method

Sediment cores from the Ria de Vigo (Figure 1) were taken with a gravity corer in April 1986 and sectioned at 1–2 cm intervals for cyst enumeration (Anderson *et al.*, 1982). Sediment was processed using an ultrasonic probe for 45 s at 4 A

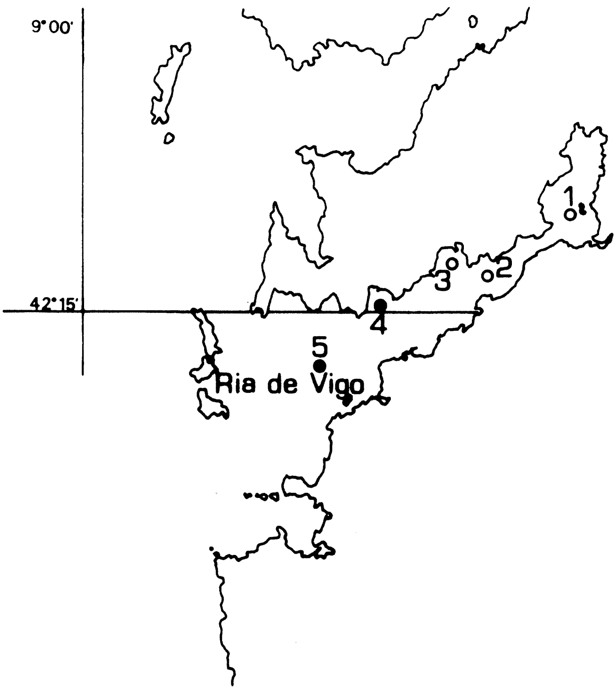

Fig. 1. Map of the area where sediment samples were taken. Circles denote station locations. Open circles (○) signify the absence of *G.catenatum* cysts and filled circles (●) their presence.

(Branson Instruments), followed by size fractionation of the slurry through sieves of 20 and 80 μm. Particles washed off the 20 μm sieve were examined microscopically using Sedgwick–Rafter slides. The volume examined represented 0.1 ml of the original sediment. Only live cysts were counted.

To study the effect of temperature on germination, cysts were produced from net plankton tows using methods similar to those of Anderson *et al.* (1988). The tows were collected during a toxic bloom of *G.catenatum* that occurred in November 1985 in the Galician Rias Bajas, and immediately taken to the laboratory and resuspended in 10 l of seawater enriched as in f/2 medium (Guillard and Ryther, 1962), but with no added NO_3^- and PO_4^{3-}. The samples were maintained at room temperature (12–18°C) under a 16/8 h light/dark (L/D) cycle with gentle aeration for 20 days. These cysts were resuspended in f/2 medium in 30 ml tubes and placed in the lighted temperature gradient bar (described below) for 29 days. Empty and live cysts were counted before and after the experiment to determine excystment success (Anderson *et al.*, 1987).

To determine the length of the mandatory dormancy period, cysts were produced from vegetative cells collected in a plankton net tow during a November 1986 bloom. Samples were incubated during 12 days in the same conditions as described above. Newly formed cysts were stored in the dark in the original medium at 5 and 20°C. The germination experiment started on day 13 (for cysts at 20°C) and day 18 (for cysts at 5°C). A minimum of 30 cysts were isolated each day by micropipetting from a microscope slide, individually placed in 100 μl of f/2 medium in 96-well tissue culture plates and incubated at 20°C on a 16/8 h L/D cycle with cool white illumination at 150 μE m^{-2} s^{-1}. The appearance of swimming *G.catenatum* cells indicated that germination had occurred. Germination was recorded after 5 days of incubation and again after 35 days.

Another experiment was performed to study the effects of irradiance and growth media on germination. Ten milliliters of exponential phase culture in K medium (Keller and Guillard, 1985) were inoculated into 15 ml of K medium lacking either N and P. As a result, the encystment culture was in K medium with the levels of N and P reduced to the proportions added with the inoculum. Cultures were incubated at 20°C for 15 days under a 14/10 h L/D cycle at 150 μE m^{-2} s^{-1}. Four different cultures (GC9V, GC21V, GC19V and GC7B) were crossed for the encystment studies; all were non-clonal and isolated from Galician waters. Cysts were collected by centrifugation and stored at 5°C. After 5 days of storage, the effects of irradiance and nutrients were investigated using 12 flasks, six of which contained cysts and 10 ml of filtered seawater, and six of which contained cysts in 10 ml of K medium. Three flasks from each group were illuminated (14/10 h L/D, 150 μE m^{-2} s^{-1}) and the other three were maintained in complete darkness. These cysts were exposed to light only at the beginning of the experiment and subsequent samples were taken in a dark room with low levels of red light. Empty and live cysts were counted weekly for 3 months.

Temperature effects on growth rate were determined using culture tubes (30 ml of medium) in an aluminium temperature gradient bar (Watras *et al.*, 1982). Heating one end and cooling the other resulted in a temperature range of 8–35°C. Light levels were ~150 μE m^{-2} s^{-1}. Cultures were acclimatized for at least three generations at or near each experimental temperature, with transfers made in late exponential growth. The growth rate was monitored in quadruplicate at seven temperatures. *In vivo* fluorescence was measured daily with a Turner Designs Model 110 fluorometer. Growth rate (divisions day^{-1}) was calculated from a semilog plot of relative fluorescence units versus time during exponential phase using a regression technique. ANOVA, using the test of Scheffe, was applied to the data on germination success after 5 and 35 days of incubation, respectively, and on germination in the dark and in the light.

Results

Cyst distribution

Gymnodinium catenatum resting cysts were only found at the outermost stations of the Ria de Vigo (Stations 4 and 5, Figure 1), with the highest concentrations at Station 4 (Table I). Cysts were most abundant below the sediment surface, with concentrations as high as 310 cysts cm^{-3} at 3–4 cm depths at Station 4 (Figure 2), representing ~90% of the total *G.catenatum* cysts at that station. Totaled over the entire core, *G.catenatum* cysts represented 11% of all cysts

Table I. Cyst concentrations in Ria de Vigo sediments, April 1986 (cysts cm^{-3})[a]

Species	Station no.				
	5	4	3	2	1
Gymnodinium catenatum	8	130	0	0	0
Gonyaulax polyedra	287	176	15 260	2706	6331
Scrippsiella spp.	280	493	127	403	120
Gonyaulax spinifera	56	57	33	103	107
Others	1270	273	37	1380	0
Total	1901	1129	15 457	4592	6558

[a] Averaged over the top 6 cm.

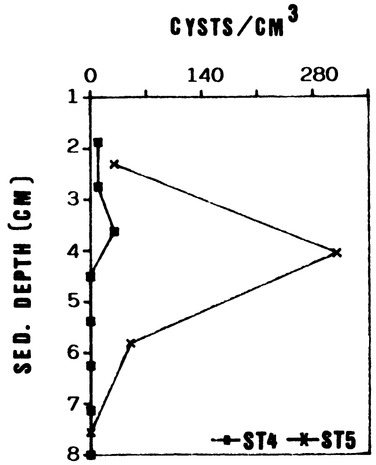

Fig. 2. Vertical distribution of *G.catenatum* cysts in Stations 4 and 5 in the Ria de Vigo.

present, with *Scrippsiella* spp. and *G.polydra* cysts as the most abundant forms. In the innermost stations (Stations 1, 2 and 3), *G.catenatum* resting cysts were not found, although the area was a rich depositional site for the cysts of other dinoflagellate species, especially *G.polyedra*, which represented 97% of all cysts present at Station 1. At Station 3, *G.polyedra* cysts were present at the very high concentration of 31 720 cysts cm^{-3} 3–4 cm deep in the core.

Mandatory dormancy interval

Cysts produced by incubation of plankton net tow material were stored at 5 and 20°C, and periodically isolated and tested for viability. Figure 3 shows the germination results of cysts stored in their original medium and incubated at 20°C in the light. At the warmer temperature, germination success ranged between 70 and 100%, with no apparent trend through time. Cysts germinated as readily at the outset of the experiment as they did nearly 3 months later. Even in the initial isolation, which was started 1 day after the experiment commenced

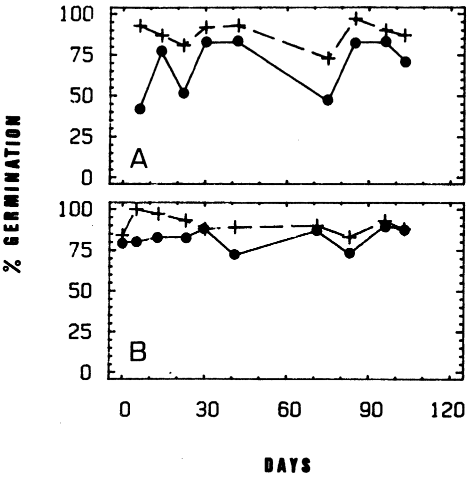

Fig. 3. Excystment success for cysts stored at 5°C (**A**) and 20°C (**B**) for the indicated time prior to incubation at 20°C in the light. Germination percentage after 5 days (——) and 35 days (----).

(with cysts that could not have been more than 12 days old), the germination rate exceeded 80%. Storage at 5°C reduced overall viability slightly, as between 40 and 80% germination was observed upon isolation and incubation (Figure 3A). Again, no trend was evident through time, indicating that the mandatory dormancy interval had been exceeded by the time of the first incubations.

Figure 3 also shows that germination success increased with longer incubation time. The difference between this long-term germination success (total viability after 35 days of incubation) and the 5 day results was significant ($P < 0.05$) at both temperatures. Nevertheless, this difference was higher (10–15% more) under storage at 5°C.

Irradiance and growth medium effects

Growth media seemed to have no effect on excystment of *G.catenatum* cysts, as germination rates and the overall percentages of successful germination were the same in enriched and unenriched medium (Figure 4). In contrast, the excystment rate was retarded significantly in darkness. Fifty percent germination success was achieved in ≤10 days in the light, but required nearly 50 days in the dark. Germination in the dark was significantly lower than in the light during the first 40 days, regardless of medium composition, but also significantly different from zero ($P < 0.05$). Thus, darkness retards germination significantly, but does not prevent it.

Temperature effects

Temperature effects on cyst germination and vegetative growth were examined between 6 and 34°C in the temperature gradient bar apparatus. The growth rate was optimal between 22 and 28°C with division rates of ~0.5 divisions day^{-1}

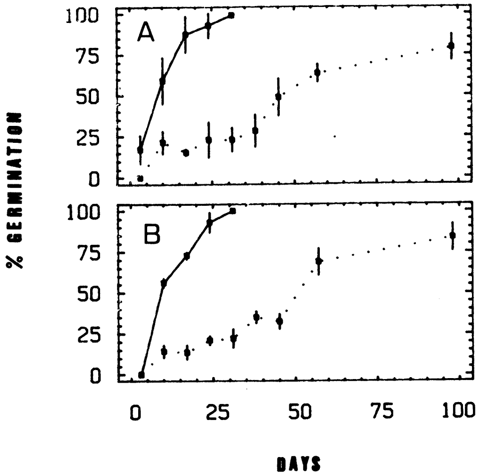

Fig. 4. Germination of *G.catenatum* cysts in nutrient-replete K medium (**A**) and in unenriched seawater (**B**); in darkness (...) and in light (——).

(Figure 5A). Growth was also quite rapid from 17 to 22°C, averaging ~0.4 divisions day^{-1}. Below 17°C, the growth rate decreased rapidly with decreasing temperature, and was negligible at 11°C. A sharp decrease from maximal growth at 28°C to no growth at 30°C was evident.

The germination success of cysts followed a similar pattern, with optimal germination (~75%) between 22 and 28°C, slightly less germination between 17 and 22°C, and little or no germination <11°C or 32°C (Figure 5B).

Discussion

The data presented here are the first published on the geographic distribution and germination characteristics of *G.catenatum* cysts from Spanish waters. In a study of dinoflagellate cysts in Galician coastal waters, Blanco (1988) found cyst concentrations of *G.catenatum* of up to 102 cysts ml^{-1} of sediment. He studied an area further north than that of the present work, where blooms of *G.catenatum* are less frequent.

One striking feature of the germination studies that was also seen with Australian *G.catenatum* (Blackburn *et al.*, 1989) is that newly formed cysts can germinate rapidly, so the mandatory dormancy or maturation interval lasts a few days at most. In the absence of other regulatory factors, newly formed cysts should germinate and resume a motile extistence soon after every bloom, providing the inocula for a nearly continuous motile population of *G.catenatum* throughout the year and leaving few ungerminated cysts in the sediments at any time. In the Ria de Vigo, neither of these characteristics are observed; *G.catenatum* cysts are abundant in the sediments (Table I) and the water column is devoid of this species for many months of the year. Here we demonstrate that seasonal fluctuations in the levels of temperature and light can be important regulatory factors for the cysts and vegetative cells of this species.

Germination and growth characteristics

Many newly formed dinoflagellate cysts cannot germinate for a particular interval of time even if external growth conditions are optimal (e.g. Anderson, 1980; Binder and Anderson, 1986; Blackburn *et al.*, 1989). Termed true dormancy (Pfiester and Anderson, 1987), this endogenous controlled resting state is distinct from the subsequent interval when the mature cyst is capable of germination, but is held in the resting state by unfavourable external conditions (quiescence). From one point of view, *G.catenatum* cysts from Ria de Vigo have no detectable true dormancy interval, as germination of cysts stored at 20°C was possible within the first few days of incubation. However, given the logistical difficulties of determining the absolute age of newly formed cysts produced in a plankton tow that was incubated for 12 days, cysts used in our experiments could have been nearly 2 weeks old when germination was first assayed. To be conservative, we suggest that the maturation of *G.catenatum* takes ≤ 2 weeks at 20°C, consistent with the reports of Blackburn *et al.* (1989) on *G.catenatum* from Tasmania.

New cysts stored at 5°C prior to being isolated and incubated in the light were also capable of germination within 5 days of the beginning of the experiment, although overall germination viability was slightly, but significantly ($P < 0.05$), reduced relative to the 20°C results. Low storage temperature thus did not appreciably alter the maturation or mandatory dormancy interval for *G.catenatum*. This is similar to results for *Scrippsiella trochoidea* (Binder and Anderson, 1986), but in marked contrast to those for *A.tamarense* cysts which took 60–90 days to mature at 5°C, but only 20 days at 20°C (Anderson, 1980).

Effect of light

Light enhanced the rate of cyst germination in *G.catenatum*. Cysts exposed to light exhibited germination rates four times higher than those maintained in darkness. Nevertheless, light is apparently not essential for germination, as shown by the excystment of 80% of the cysts kept for 3 months in darkness. In four of five dinoflagellates studied by Anderson *et al.* (1987), darkness delayed germination, but did not prevent it. According to these studies and earlier ones in which germination was not appreciably different in darkness and light (Huber and Nipkow, 1923; Anderson and Wall, 1978; Krupa, 1981; Hall, 1982), the results of experiments studying the effect of light on germination seem to depend not only on the species of dinoflagellate, but also on the experimental methods used. Binder and Anderson (1986) demonstrate that brief exposure to low-intensity light before and during the experiment may be sufficient to act as a photomorphogenic trigger to initiate dinoflagellate cyst germination. Since brief exposure to light is almost unavoidable in experimental cyst studies, it is difficult to say with absolute certainty that germination of *G.catenatum* will occur in complete darkness. In the present study, it is very possible that at the beginning of the experiment, the cysts received sufficient light during the net tow incubations or during isolation to initiate excystment, which could then proceed slowly, but progressively, once darkness was imposed.

Temperature effects

Temperature is the environmental variable most frequently mentioned in the control of dinoflagellate cyst germination (Anderson and Morel, 1979; Dale 1983; Pfiester and Anderson, 1987). Low temperatures maintain quiescence in the cysts of many species, and transfer to higher temperatures is sufficient to initiate germination. The present study shows that there is an optimal temperature range in which germination rates are maximal (Figure 5B). Germination was not observed at 6°C in the experimental incubation period and was very low (6%) at 11°C. According to the experiments described here, there is an optimal temperature range between ~17 and 29°c, in which germination exceeds 50%. Outside this range, germination rates diminish significantly, although the possibility of excystment at temperatures <11°C cannot be excluded if incubation time is lengthened.

The optimal temperature range for vegetative growth was similar to that found for cyst germination (Figure 5A). This result differs from those for *Ceratium hirundinella* (Huber and Nipkow, 1923) and *S.trochoidea* (Binder and Anderson, 1987), in which the optimal temperature for excystment was higher than that for growth. In the present study, a threshold around 16°C was observed, below which the growth rate diminished drastically, even though germination was still possible below 16°C. At high temperatures, both growth rate and excystment success decreased sharply around 30°C. This reflects a clear physiological limitation for growth of this species.

Growth medium effects

Nutrient concentrations in growth media did not appreciably influence cyst germination (Figure 4). Binder and Anderson (1987) noted a marked delay in cyst germination with *S.trochoidea* maintained in medium without nutrients. However, when those cysts were individually isolated into tissue culture chambers, germination rates in enriched and unenriched media were similar. The authors suggested that environmental perturbations caused by the cyst isolation technique stimulated excystment. In the present study, such manipulations were not performed and a similar effect of nutrient concentration on germination was not observed.

Ecological implications

Dinoflagellate species with a short mandatory dormancy period have the potential to cycle rapidly between a resting state and a motile, vegetative existence. Under such circumstances, at the time of excystment the cysts will still contain most of the energy and metabolites present in the gametes. This has been demonstrated in *S.trochoidea*, which has a dormancy period of 25 days (Binder and Anderson, 1987), and is likely in *G.catenatum* which has a dormancy period of ≤2 weeks. In nature, *G.catenatum* cysts may thus reach the sediment already in the process of germination, or in a condition to do so rapidly. These two factors suggest that *G.catenatum* could be a motile resident of

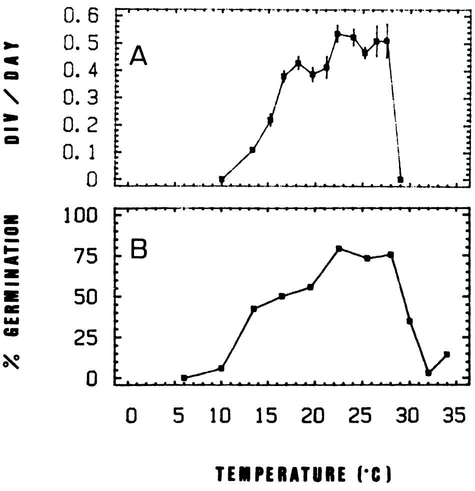

Fig. 5. Temperature effects on *G.catenatum* growth rate (**A**) and germination rate (**B**).

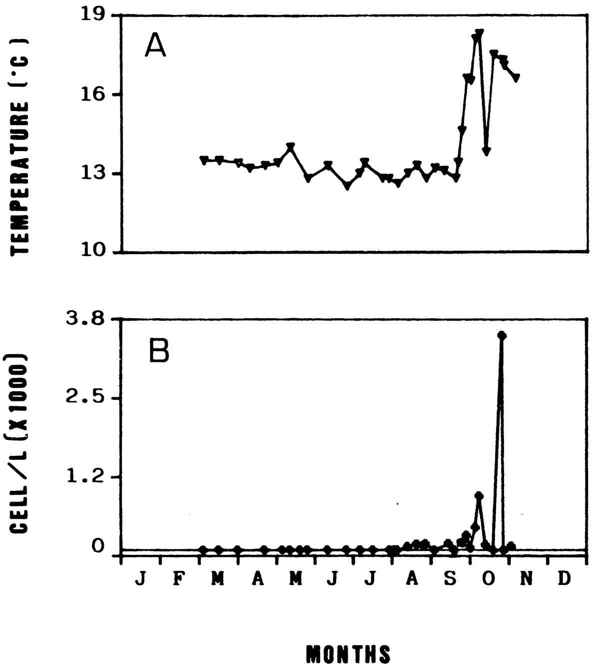

Fig. 6. (**A**) Bottom temperature (30 m depth) and (**B**) concentration of *G.catenatum* cells at Station 5 during 1987.

the water column throughout the year, yet blooms of this species are highly seasonal (Fraga *et al.*, 1988; Figueiras and Pazos, 1991). To determine the extent to which life cycle transformations play an important role in bloom dynamics in this species, factors which prevent the appearance of blooms in some seasons must be identified.

The normal pattern of blooms of *G.catenatum* in Galician coastal waters consists of small, intermittent populations in summer and large, sometimes massive, blooms in autumn. Throughout the year, the amount of light which reaches the depths where *G.catenatum* cysts are found in the rias is generally undetectable by the sensors normally used, and only in August and September does it reach maximum values around 1 μE m^{-2} s^{-1} (F.G.Figueiras, personal communication). In the deeper continental shelf areas with fine sediments where cysts could accumulate, light levels are even lower. Since darkness retards *G.catenatum* germination (Figure 4), low levels of light in bottom sediments might be a limiting factor in the development of early vegetative populations. Light could thus play a role by retarding germination throughout much of the year, except during specific intervals in August and September.

Upwelling occurs in spring and summer on the west and northwest coasts of the Iberian Peninsula between Lisbon and Finisterre. This causes bottom temperatures on the shelf and in the rias to fall to 12–13°C. This could influence *G.catenatum* blooms in two ways. On the one hand, more intense upwelling in summer could resuspend cysts to levels with more light. This might explain the intermittent populations of *G.catenatum* found in summer. In addition, since upwelling is associated with temperatures around 13°C (which do not favor rapid cyst germination or vegetative growth), the upwelling would not necessarily lead to large blooms but, more likely, small intermittent populations. This is in fact what is observed in summer months. In our cultures, division rates at 13°C do not exceed 0.2 divisions day^{-1}, which is probably not sufficiently rapid in ecological terms to produce sizable blooms. Under this scenario, temperature may be more important in limiting vegetative growth then in restricting germination. Our data indicate that ~40% excystment can occur at 14°C, about half the maximal rate. Growth would be about one-fifth of the highest rates observed at that temperature.

Moita (1993) has described the appearance of *G.catenatum* blooms in relation to seasonal upwelling on the north Portuguese coast. This coast and the Galician coast as far north as Cape Finisterre are subject to the same upwelling system, and information obtained in recent years shows similar patterns of phytoplankton bloom development in both areas. According to Fraga *et al.* (1988, 1993), autumn blooms on the Galician coast are related to the intrusion of warmer surface water into the rias from offshore as upwelling relaxes. Studies of bottom temperatures on the shelf and in the rias show that it increases by at least 2–3°C in autumn every year. The bottom temperature in Ria de Vigo reached 19°C in 1987 during the autumn (Figure 6), and generally exceeds 16°C. These temperatures are suitable for *G.catenatum* bloom development, since they allow high germination and growth rates. Autumn thus seems to be the most propitious season for the development of large *G.catenatum* blooms, based on the temperature and light environment, and this is in fact what is generally observed.

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