Journal of Plankton Research Vol.20 no.9 pp.1743–1755, 1998

Cysts of Danish *Gymnodinium nolleri* Ellegaard et Moestrup sp. ined. (Dinophyceae): studies on encystment, excystment and toxicity

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Abstract. Life cycle dynamics of *Gymnodinium nolleri* Ellegaard et Moestrup sp. ined. were studied under different temperature and nutrient conditions. Five culture strains originating from cysts found in Danish marine sediments were used for the experiments. Both encystment and excystment were found to vary with temperature. Maximal encystment occurred at 22–28°C, with no cysts formed below 13°C or above 33°C. Cyst production was slightly higher under phosphorus limitation than under combined nitrogen and phosphorus limitation. Maximal excystment occurred at 26.5°C with negligible excystment under 11°C and over 35°C. The resting period for cyst maturation was typically 3–4 weeks. Cysts produced under both phosphorus and nitrogen limitation were tested for paralytic shellfish poisoning (PSP) toxins by HPLC, and none were detected. It remains unclear why vegetative cells of this species have not yet been recorded in plankton samples from Scandinavia, despite the widespread distribution of the cysts.

Introduction

Resting stages (cysts) of *Gymnodinium nolleri* Ellegaard et Moestrup sp. ined. were found in Northern Europe for the first time in 1987 (Ellegaard *et al.*, 1993, as *Gymnodinium catenatum*) and have subsequently been found to be widespread in the region (Nehring, 1995). Because of this discovery, it has been suggested that blooms in the region might be expected (Nehring, 1995). Initially, they were thought to be cysts of *G.catenatum* because of a great morphological similarity with cysts of this species. However, cultures of *G.nolleri* from Denmark revealed several differences compared with strains of *G.catenatum* Graham 1943 from Spain, Japan and Australia (Ellegaard *et al.*, 1993; Ellegaard and Oshima, submitted): the Danish strains do not form chains longer than two cells, the cells are slightly smaller, and there are differences in rRNA sequence and isoenzymes. *Gymnodinium catenatum* has been intensively studied as it has caused PSP (paralytic shellfish poisoning) worldwide, with the first confirmed report in 1976 (Estrada *et al.*, 1984; Anderson *et al.*, 1989). As only the cyst stage of *G.nolleri* has been identified in Scandinavia, it is of interest to determine whether the cysts are toxic and also to understand the dynamics of cyst formation. These data, coupled to life cycle studies on *G.catenatum* from Australia (Blackburn *et al.*, 1989) and Spain (Anderson *et al.*, 1988; Bravo and Anderson, 1994), are shedding light on the relationship between Danish *G.nolleri* and toxic populations of *G.catenatum*. In this study, the effect of environmental factors on the life cycle of Danish strains of *G.nolleri* is examined and Danish cysts are analysed for PSP toxins.

The name *Gymnodinium nolleri* Ellegaard et Moestrup will be validly published shortly.

Method

Strains

Five strains of Danish *G.nolleri* (Table I), established from cysts collected from two locations in Danish waters (Figure 1), were used in this study. The cysts were isolated from sediment samples collected using a HAPS corer (Kanneworff and Nicolaisen, 1973). The top few centimetres of sediment were transferred to sample jars and kept cold (4°C) and dark until processing. Subsamples were rinsed with filtered sea water through a 25 µm Retsch sieve, sonicated for 6 min in a Branson 3200 Sonicator, rinsed again, and examined for cysts under the microscope. Individual cysts were isolated with a Pasteur micropipette, rinsed in 2–3 drops of culture medium, and transferred into individual wells of a polystyrene Microwell plate containing culture medium. To induce excystment, the wells were kept at ~20°C [12/12 h light/dark (L/D) and 60 µmol photons m^{-2} s^{-1})]. For experimental purposes, the strains were grown at 250 µmol photons m^{-2} s^{-1} at 20°C and 30 p.s.u. in f/2-Si (Guillard, 1975). Since each strain originated from a cyst, they are not necessarily clonal.

Table I. Strain designation and source of strain

Strain	Source
DK5	Cyst from Øresund (Figure 1, 1)
DK6	Cyst from Øresund (Figure 1, 1)
922I	Cyst from Kattegat (Figure 1, 2)
922II	Cyst from Kattegat (Figure 1, 2)
922III	Cyst from Kattegat (Figure 1, 2)

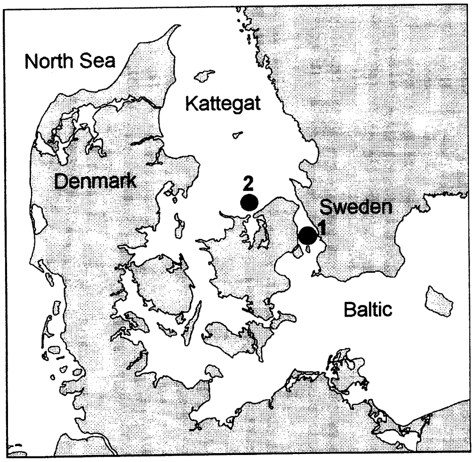

Fig. 1. Sampling stations in Danish waters. 1: North of the island of Hven in Øresund (The Sound), a flat area at 30 m depth, containing fine-grained sediment. 2: Sampling station 922 of the Danish National Environmental Research Institute.

Cyst production and harvesting

Cysts used for excystment and toxicity studies were produced in culture tubes containing 25 ml of f/2 medium without added NO_3^- and/or PO_4^{3-} (hereafter termed 'encystment medium'), inoculated with 2 ml of each of two different exponentially growing cultures of *G.nolleri* (cell density in the inoculum: 3000–9000 cells ml^{-1}). Cell densities were determined by counting a subsample, fixed with Utermöhl's fixative, in Sedgwick–Rafter counting chambers. Cyst production was induced by the same method in larger volumes in 100–500 ml flasks. Two millilitres of single strains were also introduced into encystment medium, but no cyst production was seen. Cysts were harvested 3–4 weeks after incubation and isolated from vegetative stages by removing as much medium and swimming cells as possible above the cyst pellet by aspiration. The removed supernatant was examined for cysts under the microscope and contained <1% of the cysts. For toxin analysis, the pellet was transferred to a pre-weighed centrifuge tube and the culture vessel was scraped with a Teflon policeman and rinsed with filtered sea water. When cysts from several culture vessels had to be combined, the pellets were pooled, allowed to settle overnight and the medium again removed by aspiration. The remaining vegetative cells in the cyst slurry were broken by probe ultrasonication (Braun-Sonic 200 with micro tip attachment) for 50 s. A fixed subsample was enumerated for remaining motile cells, broken cysts and intact cysts. If >5% motile cells remained, the samples were resonicated.

Temperature effect on encystment

The effect of temperature on encystment was determined using a lighted (400 µmol photons m^{-2} s^{-1}, 14/10 h L/D) aluminium temperature gradient bar (Watras *et al.*, 1982), allowing up to 80 50-ml culture tubes. A temperature gradient was established by heating one end of the bar and cooling the other.

In the first experiment, duplicate tubes containing 25 ml each of two types of encystment medium, f/2 without added PO_4^{3-}, and f/2 without added NO_3^- and PO_4^{3-}, were inoculated with 1–2 ml of each of cultures DK5 and 922I to give an initial density of 800 cells ml^{-1}. The cultures were incubated at nine different temperatures ranging from 15 to 30°C.

In the second experiment, duplicate tubes with 25 ml of f/2 medium without added PO_4^{3-} were inoculated with 0.3 ml of each of DK5 and DK6 to give an initial density of ~300 cells ml^{-1}, and placed at 6–35.5°C (16 temperature levels). To minimize shock caused by sudden temperature changes, inoculum cultures were pre-conditioned for 2 days at 15, 20 or 25°C (to within 10°C of the encystment temperature they were to be exposed to).

Cyst production was monitored by viewing the culture tube under a dissecting microscope every few days. The tubes were sampled for total cyst yield after 3–4 weeks of incubation by scraping the sides with a Teflon policeman and vigorously mixing the tube before sampling. All samples were counted in triplicate.

Excystment

Excystment as a function of temperature was studied at 16 temperature levels from 6 to 36°C using the same temperature gradient bar as described above. Thirty-two days after DK5 and DK6 were inoculated into f/2 medium with no added PO_4^{3-}, the resulting cysts were harvested from a flask as described above. About 5000 cysts (0.3 ml of cysts + medium) were added to duplicate tubes containing 25 ml f/2 medium. The tubes were monitored every 4–7 days for empty cysts and harvested after 32 days, when empty and intact cysts were counted. To ensure that maximal germination had been achieved, the tubes at 25–29.5°C were left in the temperature gradient bar for one additional week. As a control for possible production of new cysts during the experiment, 2 ml each of DK5 and DK6 were inoculated at the beginning of the experiment into tubes containing 25 ml f/2. Two tubes were placed at each of 15, 20 and 25°C.

Maturation

Fifteen days after inoculation of DK5 + DK6 into f/2 without added NO_3^-, 18 newly formed cysts were isolated and individually transferred into microtitre plate compartments containing f/2 medium. The plate was kept at 20°C and 300 µmol photons m^{-2} s^{-1}, and monitored every few days for excystment.

Toxin analysis

A known number of harvested cysts were pelleted by centrifugation at moderate speed for 3 min, and 100–500 µl of 0.05 M acetic acid (depending on the number of cysts in the sample) were added. Total cyst counts ranged from a few thousand to 300 000 (9–376 million cysts l^{-1} acid). The cyst samples were broken by probe

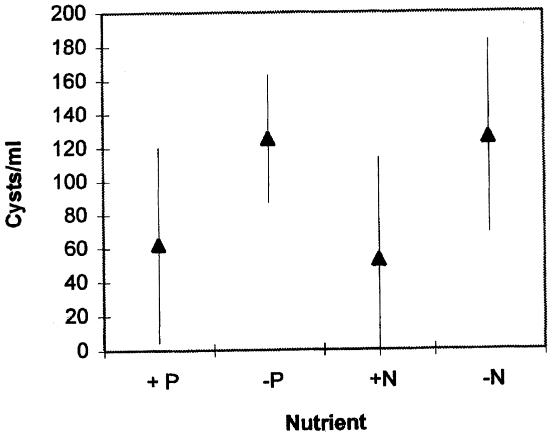

Fig. 2. Effects of adding the limiting nutrient 2 weeks after inoculation of two strains into nutrient-deplete medium. Average ± 95% confidence interval. +P = crosses inoculated in f/2 – PO_4^{3-}, with PO_4^{3-} added later. Sample size = 3. – P = f/2 minus PO_4^{3-}. Sample size = 6. + N = f/2 minus NO_3^-, with NO_3^- added later. Sample size = 2. – N = f/2 minus NO_3^-. Sample size = 3. ▲ = average.

ultrasonication for 1.5–3 min while on ice and a 10 μl subsample was examined for remaining intact cysts. The extract was stored frozen at –20°C. Before injection into the HPLC, the thawed samples were mixed and centrifuged to remove debris from the injection sample. The samples were tested for STX, GTX and C toxins according to Oshima (1995) with the modifications described by Anderson *et al.* (1994). Samples with possible trace amounts of C toxins were hydrolysed by mixing the extract with 1 M HCl (11:1, final concentration = 0.1 M HCl) and heating for 7 min in boiling water to convert C toxins into GTX toxins.

Photography

Cysts were photographed using brightfield and Nomarski differential interference contrast with a Zeiss MC100 35 mm camera mounted on a Zeiss axioscope.

Data analysis

The data were tested statistically by fitting to linear models using PROC GLM (general linear models) in the statistics software Statistics Analysis Systems (Anonymous, 1990).

Results

Cyst production

Cyst production occurred in all attempted crosses between the five strains (one cross was not attempted) and no cyst production occurred when any single strain was transferred to encystment medium (Table II). The implications of these results are further discussed in Ellegaard and Oshima (submitted), where more strains, including some from Spain and Australia, were crossed.

Cyst production was slightly higher under phosphorus (P) limitation than under combined nitrogen (N) and P limitation (Figure 3). Cyst production was induced in both tubes (25 ml) and flasks (200–500 ml) with comparable yields. Crosses with DK5 + DK6 (cysts shown in Figure 4) and DK5 + 922I produced useful quantities of cysts, and these strains were used in further experiments to study the effect of temperature on encystment and excystment, as well as the toxin content of the cysts.

Table II. Crossing matrix for the five Danish strains. DK5 and DK6 are from sampling site 1; 922I, II and III are from sampling site 2

	DK5	DK6	922I	922II	922III
DK5	–	+	+	+	+
DK6		–	+	+	/
922I			–	+	+
922II				–	+
922III					–

–, no cysts produced; +, cysts produced; /, cross not attempted.

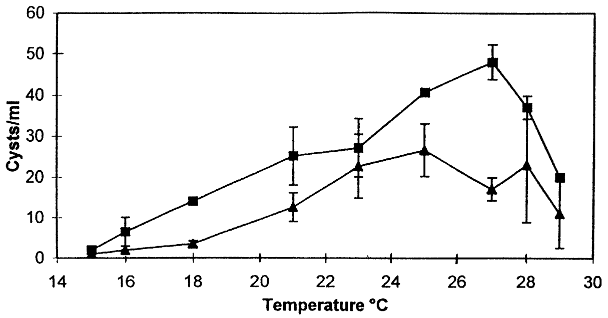

Fig. 3. Cyst production (cysts ml^{-1}) as a function of temperature, first experiment. Strains DK5 and 9221 were inoculated into nutrient-deplete f/2 medium. Average ± SD. ■ = f/2 without added PO_4^{3-}. ▲ = f/2 without added PO_4^{3-} and NO_3^-.

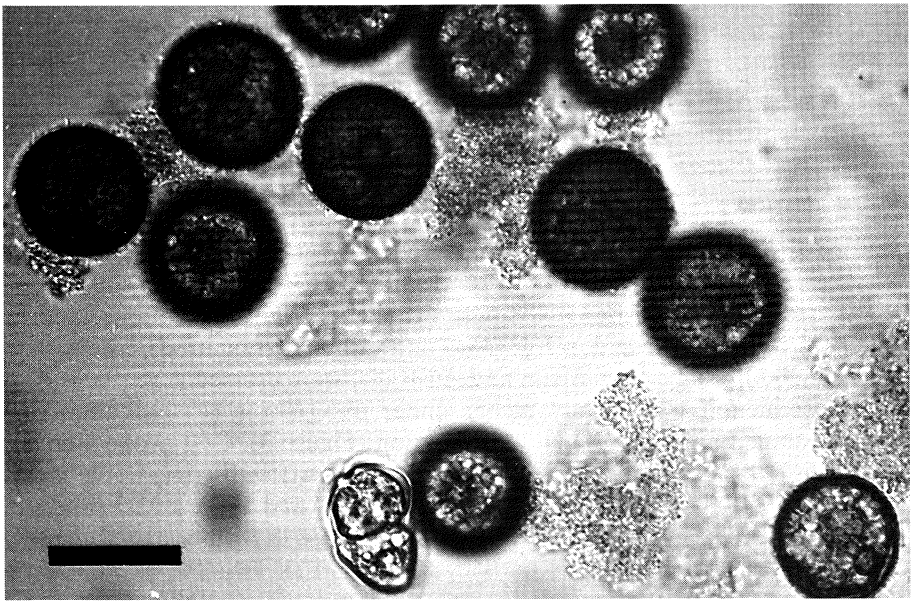

Fig. 4. Light micrographs (Nomarski differential interference contrast) of freshly produced cysts of DK5 + DK6. Scale bar = 35 μm.

When studying cyst production in *Gyrodinium uncatenum*, Anderson *et al.* (1985) showed that a large proportion of the vegetative population initiate sexuality and become planozygotes, but never complete the transition to cysts. The authors suggested that the low nutrient concentration required to initiate sexuality might become too low to allow cyst formation. In this study, however, adding the limiting nutrient (N or P), in full f/2 strength, to the culturing vessel ~2 weeks after inoculation did not improve cyst yield (Figure 2).

Temperature effect on encystment

In the first experiment (strains DK5 and 922I; 15–30°C), the first cysts were observed 7 days after inoculation at all temperatures between 16 and 28°C. After 13 days, cysts were present across the whole range of temperatures, and after 26 days the tubes were harvested and the cysts counted. Cyst production was maximal (~50 cysts ml^{-1}) at 27°C, and was slightly higher in tubes without PO_4^{3-} than in tubes lacking both NO_3^- and PO_4^{3-} (Figure 3).

In the second experiment (strains DK5 and DK6; 6–36°C), the first cysts were observed after 7 days at 22–29.5°C. After 15 days, cysts had been seen at 11–33°C and no cysts were seen outside this temperature range. At temperatures above 33°C, the vegetative cells died very quickly, while there was survival and limited growth at 6–11°C. Maximum cyst production (~1000 cysts ml^{-1}) was seen at 22–28°C (Figure 5). The discrepancy in cyst yield between duplicates in experiment 2 was probably caused by a difference in sampling intensity of the two rows as the tubes in row 1 were monitored to assess cyst production. Disturbance during sampling may have enhanced cyst production. The diameter of the cysts was 35–40 mm (29.5°C, 20 cysts measured), which is comparable to natural cysts of Danish *G.nolleri* and slightly smaller than natural *G.catenatum* cysts (Ellegaard *et al.*, 1993).

Overall, cyst production varied with temperature; no cysts were produced at temperatures below 11°C or above 33°C. Maximum cyst production (~1000 cysts ml^{-1}) occurred at 22–28°C, but there was a relatively high production in the range 15–30°C.

The variation in cyst production is the result of both the effect of temperature on cyst production as such, and the result of the effect of temperature on vegetative growth. A model for the effect of temperature (T) and salinity (S) on vegetative growth has previously been developed (Ellegaard *et al.*, 1993): maximal growth rate (divisions per day) = $-1.294 + 0.1601T - 0.004018T^2 - 0.006806S - 0.0002739S^2 + 0.0008017TS$. From this model (growth rate at 30 p.s.u. = $-1.74469 + 0.18415T + 0.004018T^2$), the growth rate at each temperature was calculated. The number of cells at a given temperature = $N_t = N_0 \times e^{r(T) \times t}$, where N_0 is the

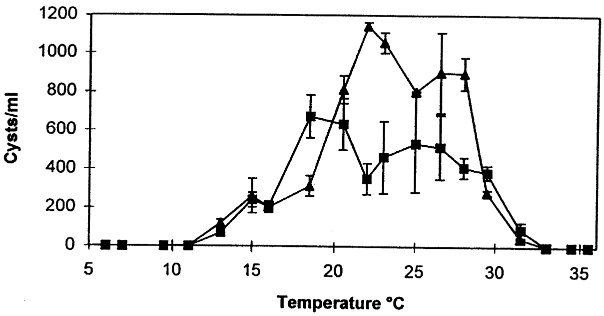

Fig. 5. Cyst production (cysts ml^{-1}) as a function of temperature, second experiment. Strains DK5 and DK6 were inoculated into f/2 medium without PO_4^{3-}. Average ± SD. ▲ = row 1 (more disturbance). ■ = row 2.

start population (300 cells ml^{-1}), r is the maximal growth rate × ln2 and t = days = 10 (cell density, measured as fluorescence, was maximal after 10 days). The curve of the variation in cyst production with temperature and the theoretical growth curve derived from the model indicate that cyst production, to a large extent, varies synchronously with the variation in cell density with temperature. The direct effect of temperature on cyst production was found as Φ where C (number of cysts) = $\Phi \times N$ (number of cells). Therefore, $C(T) = \Phi(T) \times N_0 \times e^{r(T) \times t} \Rightarrow \ln\Phi = \ln C - \ln N_0 - r \times t$.

The variation of $\ln\Phi$ as a function of temperature for each of the four datasets was tested by PROC GLM. In experiment 1, the curve for variation in cyst production with temperature was delayed relative to cell density and $\ln\Phi$ varied with temperature in the form $\ln\Phi = A + BT$ (Table III). For experiment 2, there was no significant relationship between temperature and Φ, and the variation in cyst production can be explained by the variation in cell density.

The maximal number of cysts formed depended greatly on the strains used. The cross between DK5 and 922I (experiment 1) resulted in a maximal cyst production of 50 ml^{-1}, while DK5 + DK6 (experiment 2) resulted in a maximal cyst production of >1000 ml^{-1}.

Excystment

The effect of temperature on excystment was tested at 6–36°C on cysts produced from DK5 + DK6. There was no excystment at 6°C, and the excystment percentage rose gradually with increasing temperature to a maximum of ~70% at 26.5°C, decreasing thereafter to 5–10% at 35.5°C (Figure 6). No cyst production occurred in the control tubes in the course of the experiment and no further excystment took place in tubes left an additional week in the temperature gradient bar. The excystment percentage as a function of temperature was fitted by PROC GLM to the model: excystment % = $27.97 - 8.11T + 0.73T^2 - 0.015T^3$ [d.f. = 15; F = 69.5*** (significant at the 0.001 level); $r^2 = 0.93$].

Maturation

Of the 18 individually isolated cysts, all were still intact 7 days after isolation. After 15 days, one cyst had germinated and after 19 days five cysts were empty. After 22 days, eight cysts had germinated (44%) and the experiment was terminated. From the encystment experiments, cyst formation is known to begin 7 days

Table III. Statistical testing. Student's t-test for the null hypothesis of parameter = 0 for the three parameters, A and B. Correlation coefficient (r^2), degrees of freedom (d.f.) and F statistic for the models

Data	Intercept (A)	B	r^2	d.f.	F
Without P and N	–9.462***	0.138***	0.927	8	89.5***
Without P	–8.661***	0.126***	0.843	8	37.5***

***Significant at the 0.001 level.

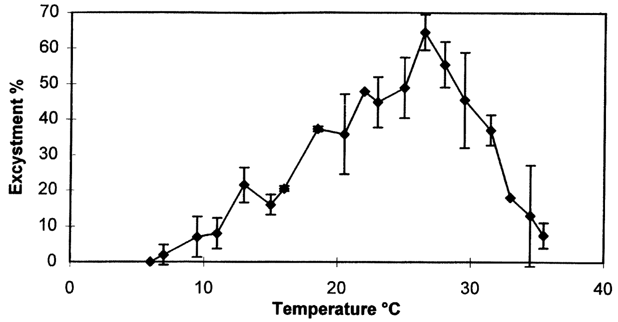

Fig. 6. Excystment percentage (empty cysts as a percentage of total cysts) as a function of temperature. Cysts of DK5 + DK6. ◆ = average (± SD).

after inoculation into encystment medium; thus, as the cysts were harvested from a culture inoculated 15 days earlier, the age of the cysts can be assumed to have been between 1 and 8 days at isolation. Most cysts germinated ~20 days after isolation, i.e. 21–28 days after formation. This indicates that the mandatory dormancy period (Pfiester and Anderson, 1987) is of the order of 3–4 weeks.

Toxin analysis

Cysts of DK5 + DK6 and DK5 + 922I were produced in tubes or flasks with N or P as the limiting nutrient. Analysis for STX toxins (STX, dcSTX, neoSTX) and GTX toxins (GTX 1, GTX 2, GTX 3, GTX 4, GTX 5) showed no detectable toxin in any of the samples. Analysis for C toxins (C1, C2, C3, C4) showed the possible presence of trace amounts of C2 and/or C4 in some samples. However, hydrolysis of these samples yielded no detectable amounts of GTX toxins. Extracts of the vegetative cells of the three strains also showed no detectable toxins.

Additionally, two samples (DK5 + DK6 with 400 million cells l^{-1} acid produced in f/2-P and DK5 + 922I produced in f/2-P) were screened for the presence of PSP toxins by receptor binding assay (Doucette *et al.*, 1997). This method is comparable in sensitivity to HPLC for the detection of PSP toxins (G.J.Doucette, personal communication). No toxins were detected.

Discussion

Encystment

Nutrient limitation, either N or P or both, is commonly used to induce sexuality and cyst formation in dinoflagellate cultures (Pfiester, 1976; Anderson *et al.*, 1984, 1985; Blackburn *et al.*, 1989). In Danish strains of *G.nolleri*, P limitation was most effective in cyst induction, although good cyst yields were also achieved with N limitation or combined N and P limitation.

Cyst production varied with temperature; no cysts were produced at temperatures below 13°C or above 33°C and maximal cyst production (~1000 cysts ml^{-1}) occurred at 22–28°C.

The effect of temperature on encystment of *G.catenatum* has not been reported, but it has been studied in other dinoflagellates. *Alexandrium tamarense* from eastern North America was found to have a maximum cyst production of 500 ml^{-1} with a sharp optimum at 21°C, no cyst production above 25°C and low production at 10–18°C (Anderson *et al.*, 1984). *Gyrodinium uncatenum* showed a maximum of 250 cysts ml^{-1} at 23°C and some cyst production at 12–32°C (Anderson *et al.*, 1985). Maximum numbers of cysts formed by the Danish strains of *G.nolleri* are higher than the numbers reported for those two species. *Alexandrium tamarense* has a very sharply defined temperature maximum, *Gyrodinium uncatenum* a somewhat wider maximum, and *G.nolleri* a plateau maximum, producing cysts over a large range of temperatures.

Cysts of *A.tamarense* have a mandatory resting period of 1–4 months (Anderson, 1980), while Danish strains of *G.nolleri* have a resting period of ~3 weeks. The short dormancy period and the lack of a sharply defined temperature effect on encystment and excystment indicate that the life cycle of Danish *G.nolleri* is less coupled to changes in environmental conditions than that of *A.tamarense*. This is also true of Australian *G.catenatum* (Blackburn *et al.*, 1989).

Excystment

Excystment of Danish *G.nolleri* was minimal at 7°C and below, increasing steadily with temperature to a maximum of 70% at 27°C, and decreasing sharply until few cysts germinated at 36°C. Spanish *G.catenatum* shows a similar pattern with maximal excystment of 75% at 22–27°C and some excystment between 5 and 35°C (the entire range tested) (Bravo and Anderson, 1994). Individually isolated cysts from the German Bight and the Kiel Bight showed comparable excystment percentages (40–80% excystment at 18°C, depending on growth medium and salinity; Nehring, 1996).

What is presumably the vegetative stage of *G.nolleri* has only been recorded twice (as *G.* cf. *catenatum*) from northern European waters [the Dutch North Sea (Peperzak *et al.*, 1996) and the French North Sea (Paulmier, 1992)], yet *G.nolleri* cysts are widespread and common in the sediments of that region (Ellegaard *et al.*, 1993; Nehring, 1995). This might be explained by the vegetative population being small and intermittent, and possibly overlooked because the vegetative stage is short lived and not easily identified. Perhaps the vegetative stage is only present briefly during periods of warmer waters, with encystment occurring relatively soon after excystment. The water temperature in Danish waters rarely reaches the temperatures at which *G.nolleri* has optimal growth (~25°C; Ellegaard *et al.*, 1993), encystment and excystment. However, other dinoflagellates are known to bloom at conditions found to be suboptimal for growth in culture studies. A strain of *A.tamarense*, for example, showed optimum growth and encystment at 21°C (Anderson *et al.*, 1984), yet blooms where that strain originated are typically over when the water temperature reaches 12–15°C (Anderson *et al.*, 1983).

The population in Danish waters may be a remnant of larger populations in the past. In a survey of sediment cores from Skagerrak, Dale and Nordberg (1992) found *G.* cf. *catenatum* cysts in high numbers in sediments from a period where the climate was warmer than now.

Toxin analysis

Compared with the motile cells, cysts of *Alexandrium* spp. have been reported to contain toxins in different relative amounts, sometimes at much higher levels (Dale *et al.*, 1978; Lirdwitayaprasit *et al.*, 1990; Oshima *et al.*, 1992), comparable levels (White and Lewis, 1982) or even lower levels (Cembella *et al.*, 1990). In laboratory-produced cysts of two different crosses of Danish cultures, no toxins were detected even in extremely concentrated samples, regardless of the nutrient limitation used to induce cyst production. Also, no PSP toxins were detected in the vegetative stage of 11 Danish strains of *G.nolleri* (Ellegaard and Oshima, submitted). Trace amounts of putative C toxins were detected in very concentrated samples of vegetative cells (Ellegaard and Oshima, submitted), but hydrolysis of cyst samples in the present study, containing similar tentative C toxins, did not result in the formation of the corresponding gonyautoxins. We therefore conclude that the putative C toxin peaks witnessed in the HPLC chromatograms do not represent toxins, but rather spurious peaks that elute at or near standard solution peaks. Since no toxins have been detected in either vegetative or cyst stage of any strain and under any of the varying environmental factors, the Danish strains of *G.nolleri* can be considered non-toxic. A non-toxic strain of *G.catenatum* has been reported from Australia (Oshima *et al.*, 1993) and non-toxic strains are also known from other toxin-producing species [i.e. *A.tamarense* (Prakash, 1967)]. Lack of toxin production is not in itself considered an adequate criterion for separating species (Taylor, 1985).

The lack of toxins in all Danish strains tested is significant, as the presence of cysts of toxic species is often seen as a warning of potential bloom and toxicity problems. We believe that the presence of *G.catenatum*-like cysts in northern Europe does not indicate a risk of PSP.

When such cysts are reported in northern Europe, material should be analysed carefully before warnings about shellfish toxicity are issued.

Acknowledgements

This work was supported in part by grants from the Danish Botanical Association and the American Women's Club in Denmark to M.E., and from the US National Science Foundation (grant # OCE-9415536) to D.M.A. Contribution no. 9704 from the Woods Hole Oceanographic Institution.

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Received on April 2, 1997; accepted on May 6, 1998