J. Phycol., 14(2), 1978, pp. 224–234

POTENTIAL IMPORTANCE OF BENTHIC CYSTS OF *GONYAULAX TAMARENSIS* AND *G. EXCAVATA* IN INITIATING TOXIC DINOFLAGELLATE BLOOMS[1,2,3]

Donald Mark Anderson[4]

Department of Civil Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

and

David Wall[5]

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

ABSTRACT

Thick-walled, nonmotile cysts (termed hypnocysts) of two dinoflagellates were isolated from estuarine sediments in Cape Cod, Massachusetts, and germinated to produce their respective motile, thecate stages. Hypnocysts from Orleans district were identified as Gonyaulax excavata *(Braarud) Balech sensu Loeblich & Loeblich. Visually identical hypnocysts from Falmouth district were provisionally identified as* Gonyaulax tamarensis *Lebour. Both species were toxic. A geographic survey in September detected hypnocysts in only the sediments of locations where toxic blooms developed the preceding and following Spring. Laboratory incubation (16 C) of hypnocysts from sediment samples stored in the dark (5 C) for 6 mo initiated excystment by the temperature increase, with no appreciable effect from light regime, nutrient, or chelator concentrations. Motility of excysted germlings was optimum in highly chelated medium and in the presence of light. We conclude that hypnocysts of both taxa are important in seeding recurrent annual blooms, synchronizing early bloom development with vernal warming of seawater and increasing the geographic range of the species. We suggest that many red tides in New England and eastern Canadian waters are initiated through the displacement of motile estuarine populations into nearshore areas by tidal advection and surface runoff, although the potential existence and importance of offshore cyst reservoirs cannot be discounted. Evidence is presented that hypnocysts are probably sexual zygotes whereas the thin-walled cysts readily formed in laboratory cultures (pellicle cysts) are asexual. Pellicle cysts are of limited durability, do not overwinter in nature, and therefore do not play a significant role in initiating toxic blooms.*

Key index words: Cape Cod (Massachusetts); cysts; dinoflagellates; Gonyaulax; *hypnocysts; pellicle cysts; red tide; spores, resting; toxic blooms, dinoflagellate*

The possibility that toxic dinoflagellate blooms are initiated from benthic resting cysts was suggested by Prakash (16) during his early investigations into the relationship between *Gonyaulax tamarensis* Lebour

[1] *Accepted: 10 January 1978.*

[2] Dedicated to Luigi Provasoli on the occasion of his official academic retirement with sincere wishes for continued scholarship and productivity.

[3] Contribution No. 3989 from the Woods Hole Oceanographic Institution.

[4] Address for reprint requests after 1 August 1978: Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543.

[5] Present address: Amoco Production Company, Security Life Building, Denver, Colorado 80202.

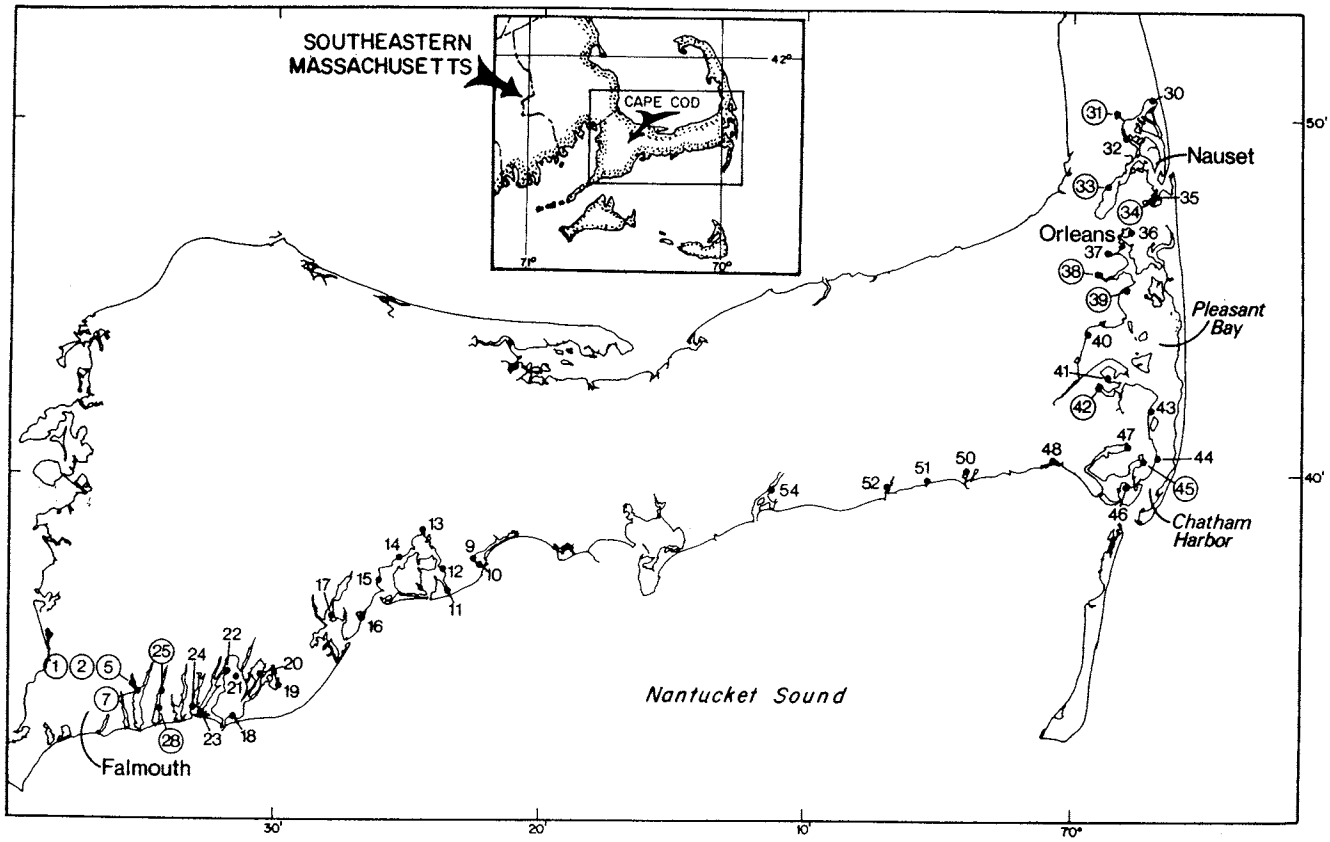


FIG. 1. Geographical distribution of hypnocysts in the Cape Cod region, Massachusetts. Circled numbers denote the presence of benthic cysts of *Gonyaulax excavata* or *G. tamarensis*. Samples 1, 2, 5, 7, Perch Pond; 9, 10, East Bay; 11, Eel River; 12, West Bay; 13, Prince Cove; 14, 15, Cotuit Bay; 16, Popponesset Beach; 17, Popponesset Bay, Ryefield Point; 18, 21, Waquoit Bay; 19, Jehu Pond; 20, Hamblin Pond; 22, Seapit River; 23, 24 Eel Pond; 25, 28, Green Pond; 30, Nauset Bay, North Beach; 31, Salt Pond; 32, Salt Pond Bay, Hemenway Road; 33, Town Cove; 34, Mill Pond, Orleans south of Mill Pond Road; 35, Mill Pond, north of Mill Pond Road; 38, Areys Pond; 39, Paw Wah Pond; 40, Pleasant Bay, near Tar Kiln Road; 41, Crows Pond; 42, Ryders Cove; 43, Chatham Harbor, near Cow Yard Lane; 44, Chatham Harbor, near Holway Road; 45, Mill Pond, Chatham; 46, Stage Harbor; 47, Oyster Pond, Chatham; 48, Ridgevale Beach; 50, Wychmere Harbor; 51, Allens Harbor; 52, Herring River; 54, Bass River, near Uncle Freemans Road.

and paralytic shellfish poisoning along the eastern coast of Canada. The seeding function of such dormant populations was also linked to toxic blooms along the coasts of New England (3,9,15,20) and Florida. Wall (25) itemized several functions that resting cysts potentially fulfill in the ecology of estuarine-neritic marine dinoflagellates, including i) locating annually recurrent blooms in certain sites where cysts overwinter in the sediments; ii) synchronizing early bloom initiation with seasonal changes in the physical environment; and, iii) enabling toxic species to extend their geographical distribution to areas without previous histories of shellfish toxicity. Collectively, these three roles will be referred to as the "cyst hypothesis" for toxic dinoflagellate bloom initiation and geographical spreading of shellfish toxicity.

In September 1972, Cape Cod, Massachusetts, was the southern limit of a massive red tide outbreak extending up the New England coast to southern Maine. The causative dinoflagellate, originally identified as *G. tamarensis* Lebour, was later classified as *G. excavata* (Braarud) Balech *sensu* Loeblich & Loeblich (13) (synonym: *G. tamarensis* var. *excavata* Braarud). This study represents an attempt to examine the validity of the cyst hypothesis with respect to Cape Cod, where the recent history of shellfish toxicity includes two noteworthy features. First, shellfish toxicity has been a recurrent annual event since the initial outbreak in 1972 (Table 1). Second, the toxicity spread locally to a previously unaffected district (Falmouth) after four years of negative test results.

Two indirect methods were employed to evaluate the cyst hypothesis. The sediments of salt ponds and estuaries along the southern coast of Cape Cod were surveyed to establish a relationship between detectable benthic cyst populations and historical shellfish toxicity sites. Also, laboratory experiments were conducted to identify the most important external "triggering mechanism" for excystment.

The latter experiments utilized natural cysts from the sediment samples and cysts produced in laboratory cultures. These two cyst types possess differ-

ent morphological and physiological characteristics which are detailed in our results as a secondary objective of this study. We thus refer to the former as hypnocysts (overwintering, thick-walled cysts) and to the latter as pellicle cysts, a term chosen because the cyst wall corresponds to the pellicle of other dinoflagellates as defined by Loeblich (12). Their respective roles in toxic bloom initiation are discussed later.

An unexpected discovery was that the toxic dinoflagellate in the Falmouth district (Perch Pond, see Fig. 1) possessed an antero-ventral pore on the right margin of the first apical thecal plate, a characteristic which was not present in the organism from the Orleans district (Mill Pond). The latter organism was identical with *G. excavata* from the 1972 New England red tide, and we have provisionally identified the former as *G. tamarensis*. A taxonomic discussion follows our main conclusions.

MATERIALS AND METHODS

Forty-five sediment samples were taken from 13 salt ponds and estuaries along the south and east shores of Cape Cod (Fig. 1) in September 1976. Sediments were collected in a 35 μm pore size plankton net towed across the water-sediment interface, and stored in the dark at 5 C in seawater from the original site. Hypnocysts were isolated by micropipette after the sediment slurry was sieved to retain only the 20–75 μm size fraction (Nitex mesh or Micro-mesh sieve material, Buckbee Mears, St. Paul, Minnesota). Organic detritus in the slurry was disaggregated by sonification (4 min at 1.4 A with a Branson S-75 sonifier). Hypnocysts were washed repeatedly in seawater medium and sealed in Palmer-Maloney nannoplankton counting slides (26) either in f/2 medium (8) (using filtered Vineyard Sound seawater), or a modification of the artificial seawater Aquil (hereafter Aquil A) with 10 times normal trace metals and chelator and three times normal nitrate (1,14). Excystment responses were generally tested by 16 C incubation under cool white fluorescent light (50 $\mu E \cdot m^{-2} \cdot s^{-1}$ measured with a quantum sensor) on a 14:10 LD cycle with daily observations for 5–11 days. Variations included incubation in constant dark, no nutrients (silicate, phosphate, nitrate, vitamins), chelator concentrations ranging from 0 to $10^{-4.3}$ M EDTA in Aquil and f/50 medium with and without soil extract. Excystment was defined as the complete emergence of the protoplast from the cyst even if the germling remained nonmotile. The number of motile germling cells was tabulated prior to the first division.

One sediment sample from Green Pond, Falmouth (collected 29 May 1976, 10 days after toxic shellfish were discovered) was stored at room temperature (18–22 C) in diffuse daylight to test excystment response without cold conditioning at 5 C.

Pellicle cysts were obtained from laboratory cultures of Isolate 429 of *G. excavata* obtained from A. R. Loeblich III (hereafter *G. excavata* 429) and new isolates from germinated hypnocysts of *G. tamarensis* (Perch Pond) and *G. excavata* (Mill Pond). Two modifications of Aquil medium were used—Aquil A and Aquil B (normal trace metals, $10^{-6.3}$ M EDTA and 10^{-3} M Tris, 1). Culture conditions were as described previously for hypnocysts.

Formation of pellicle cysts was induced by subjecting cultures of motile cells to cold, nutrient starvation and toxic copper concentrations. Exposure to cold involved removal of exponential-phase cultures from 16 C to 0 C. Nutrient depletion was produced by a 1:10 dilution of early stationary phase cultures into Aquil without nitrate, phosphate silicate and vitamins. Copper toxicity was imposed by the addition of a solution of cupric sulfate and EDTA to an exponential phase culture in Aquil B with final concentrations of $10^{-6.3}$ M EDTA, $10^{-5.3}$ M copper, 10^{-3} M Tris and a calculated cupric ion activity of $10^{-9.7}$ M (the lowest level at which all cells remain nonmotile for an extended period (1)). Pellicle cysts produced by these methods settled rapidly and were collected within 24 h using separatory funnels, with the procedure repeated twice to insure 100% nonmotile cells. Cysts formed from temperature stress were stored at 0 C, all others at 5 C. Excystment of pellicle cysts was induced by incubation in Aquil A with the percentage of motile cells determined after 48 h by the method of Anderson and Morel (1).

Microchemical tests on the cell walls of hypnocysts and pellicle cysts followed the acetolysis technique described in Gray (7) and the iodine-hydriodic acid staining technique for thecae described by von Stosch (23).

RESULTS

Hypnocyst distribution and identification. A survey along the east and south shores of Cape Cod revealed a localization in two main areas—an eastern embayment complex near Orleans (Nauset Bay, Pleasant Bay) and a southwestern complex near Falmouth (Great Pond, Perch Pond, Green Pond), 48 km away (Fig. 1). These two complexes were also the only districts on Cape Cod where shellfish became toxic in 1976 (in the spring, 5 mo before the sediments were collected). Further, the following spring the same two areas experienced toxic blooms: Mill Pond was again closed for shellfish harvesting whereas Perch Pond had measured *G. tamarensis* cell densities of 60,000 cells · $liter^{-1}$ (Anderson unpubl.).

Incubation of Mill Pond hypnocysts produced >100 motile cells identified as *G. excavata* according to thecal morphology. They were identical with *G. excavata* as described by Loeblich and Loeblich (13). Perch Pond hypnocysts produced motile cells identified provisionally as *G. tamarensis* due to the presence of a pore on the first apical plate. Thecae in plankton hauls from Mill Pond and Perch Pond during the spring 1977 confirmed the consistent difference between these two organisms.

Hypnocysts of both species were identical under the light microscope. They were also indistinguishable from hypnocysts in sediments from Gloucester, Massachusetts collected by C. Martin in September 1972 and from hypnocysts isolated from Oslofjord by Dale (3) identified as *G. excavata.*

The hypnocysts of *G. excavata* (Figs. 22–26) and *G. tamarensis* (Figs. 27–32, 48, 50) are elongate-cylindrical cells with rounded ends. Length varied from 43–72 μm and width from 26–39 μm, with a mean L:B ratio of 1.8. The wall is thick (2–5 μm) and multilayered. There is an outer covering of mucilage to which particles such as sand grains and small diatoms adhere, plus two cyst wall layers—the exospore (24) and endospore. The former is very thin and resists both acetolysis and concentrated sulphuric acid. It thus may contain a sporopollenin-like polymer (2) in common with other marine *Gonyaulax* cysts (25). The endospore is thicker and stained red with iodine-hydriodic acid mixture. It is destroyed by acetolysis and sulfuric acid, after first swelling to distort the cyst. It probably consists of cellulose.

TABLE 1. *Monthly records of shellfish toxicity in Cape Cod inlets 1972–1976.*[a]

Month	J	F	M	A	M	J	J	A	S	O	N	D
1972 Positive									6	17	6	5
Total tested									30	63	11	12
1973 Positive	4	1	0	0	0	0	0	0	0	0	0	0
Total tested	9	5	10	0	22	35	25	34	23	8	0	2
1974 Postive	0	0	0	0	1	18	6	0	0	0	0	0
Total tested	2	2	2	2	23	84	45	43	42	27	4	6
1975 Positive	0	0	0	0	4	8	0	0	0	0	0	0
Total tested	8	8	7	4	41	74	38	28	32	3	3	4
1976 Postive	0	0	1	2	22	2	0	0	0	0	0	0
Total tested	8	7	6	13	75	53	35	38	28	7	7	0

[a] Numbers indicate separate shellfish toxicity tests using a mouse bioassay: positive tests had toxin levels > 80 μg/100 g meat. (Figures courtesy of Commonwealth of Massachusetts, Department of Environmental Quality Engineering.)

TABLE 2. *Excystment results for* Gonyaulax tamarensis.[a]

Medium	Chelator conc.	No. of hypnocysts	Excystment (%)	Motility (%)
Aquil A	$10^{-4.3}$ M EDTA	32	100	97
Aquil A (no nutrients)	$10^{-4.3}$ M EDTA	22	100	100
f/50	$10^{-6.3}$ M EDTA	256	47	2
f/50 (soil extract)	$10^{-6.3}$ M EDTA, soil extract	35	97	60
f/2	$10^{-4.9}$ M EDTA	30	93	70

[a] Incubation at 16 C for 5 days: all hypnocysts from sediment sample collected from Perch Pond, Falmouth on 14 September 1976 and stored at 5 C in dark for 155–170 days (most probable time of cyst formation was May 1976).

Cell contents determined the characteristic appearance of hypnocysts. Distinctive features include microgranules in Brownian motion, starch grains, lipid globules, one or more orange-red pigmented accumulation bodies and the nucleus. Typically the starch grains surround the nucleus in the central zone of the hypnocyst (Fig. 28).

Excystment of hypnocysts. Excystment was preceded by a transformation of the protoplast towards that normally seen in the motile stage, including chloroplast development and intensification of the pigmentation, the disappearance of microgranular cytoplasm and starch accumulations, and the appearance of a pale zone (the nucleus) lying obliquely across the girdle region (Fig. 33).

During excystment, the protoplast emerged by an amoeboid motion with cytoplasmic streaming through a small breach at one end of the hypnocyst (Figs. 33–36). The emergent germlings were unusually large (40 × 50 μm) with a shallow longitudinal sulcus (Figs. 38–40). Posterior biflagellation in germlings was observed several times and by both authors independently (Fig. 37). The transverse sulcus developed several minutes after emergence with an oblique orientation not typical of mature thecate cells. Cell volumes decreased with successive cell divisions (beginning within 24 h of excystment, Fig. 41), such that after two divisions the cells were typically 35 μm diam. (13).

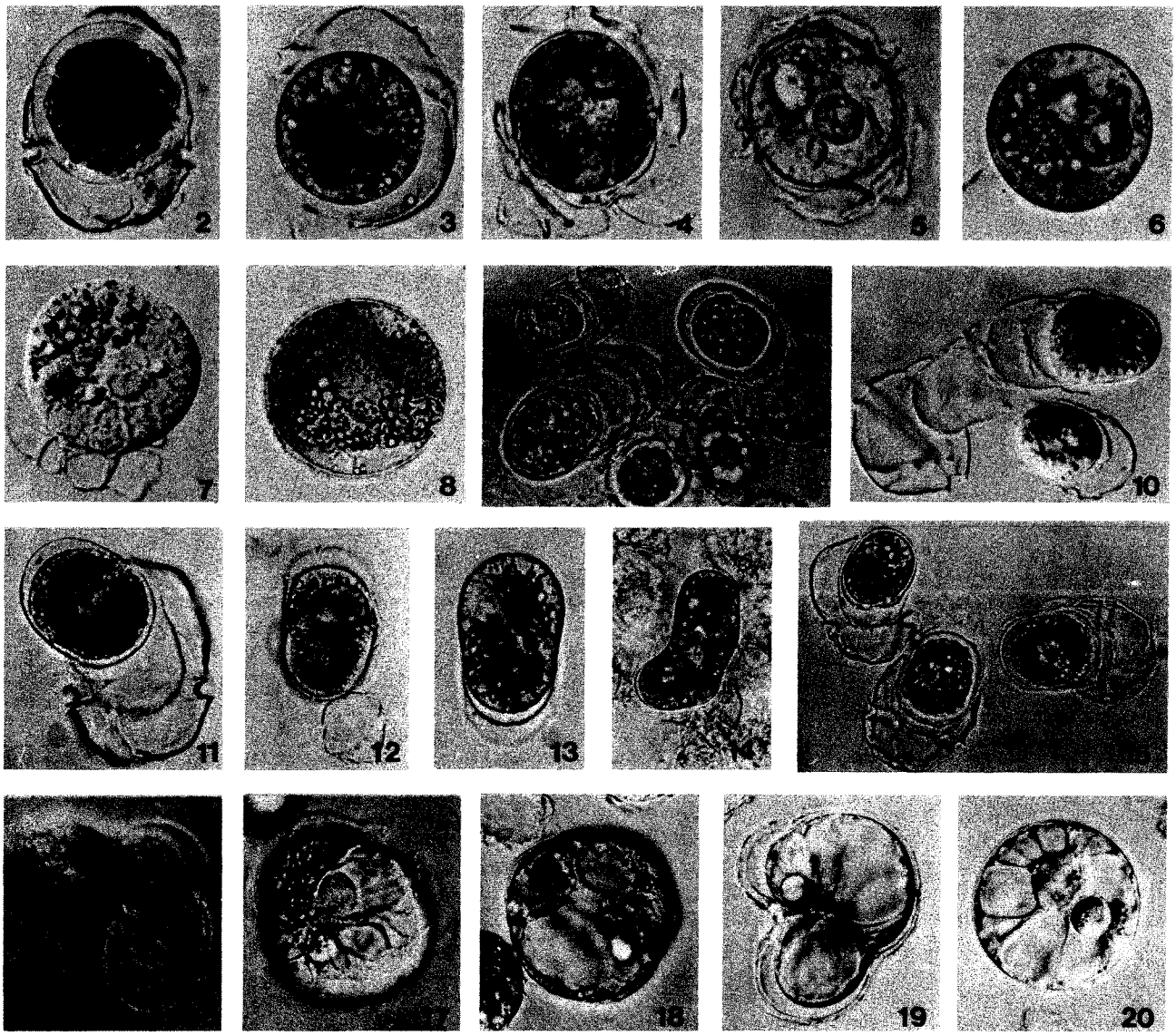
Experiments to test the viability of hypnocysts stored at 5 C since September 1976 were conducted during February and March 1977, several weeks before toxic blooms typically occur in Cape Cod salt ponds (early May). Results of the incubations under different light/dark conditions and media composition are summarized for each species in Tables 2 and 3.

Over a thousand hypnocysts from the two ponds excysted in laboratory tests. In individual experiments under widely varying conditions, 80–100% excystment was generally observed, although on one occasion only 47% were successful. Disregarding this one unusually low value, excystment did not vary appreciably with nutrient concentration, light regime, or chelator concentration. In contrast, the success of the excysted cells in achieving motility was much higher when the media (both artificial and natural seawater) were more heavily chelated and when light was available. A lack of nitrate, phosphate, silicate and vitamins did not affect either excystment success or motility among the germlings, although it was not possible to completely eliminate detritus (or bacteria) during cyst isolation, and the seawater salts of Aquil undoubtedly contained nutrients as contaminants.

Hypnocysts did not germinate when stored at 5 C for 6 mo, though many excysted within several days when incubated at 16 C. Low storage temperature was thus effective in suppressing germination. However, in one experiment, a 1 h exposure to room temperature followed by a return to 5 C resulted in 17% germination after 5 days, an indication that the onset of excystment may have been triggered, despite the subsequent return to 5 C.

Hypnocysts stored at room temperature since May 1976, and never exposed to cold conditioning also had low germination success. When placed in fresh medium at 16 C 9 mo later, only one of 14 excysted and became motile, whereas after 12 mo, 26 of 49 excysted and 11 became motile. Hypnocysts can thus germinate without "cold conditioning" and thermal activation, albeit less successfully than those exposed to cold for a prolonged period.

Pellicle cysts. Pellicle cysts formed in laboratory cultures were simple, nonmotile, athecate cells of highly variable shape. In *G. excavata* 429 there were three subtypes: coccoid, hypnoid, vacuoloid. The coccoid subtype was spherical (24–42 μm diam.) and found either as a free cell or partly or completely enclosed within its parental theca (Figs. 2–8). The hypnoid subtype was ovoid with maximum and minimum dimensions of 43 and 22 μm, respectively. It was characterized by chains of cast-off pellicles in many instances (Figs. 9–16), hence the term "hypnoid" (5,27). Vacuoloid cysts (Figs. 17–20) were spherical, ovoid or trilobate and distinguished mainly by their cell contents and greater size (40–70 μm).



FIGS. 2–20. Pellicle cysts of *G. excavata* 429 and *G. tamarensis* (FIG. 16 only) from laboratory cultures stored at 5 C. FIGS. 2–8, coccoid subtype; FIGS. 9–16, hypnoid subtype; FIGS. 17–20, vacuoloid subtype. The cysts were formed under different stresses: temperature-induced cysts shown in FIGS. 3, 8, 14, 17–19 (light storage) and 2, 7 (dark storage): Cysts induced by nutrient starvation shown in FIGS. 20 (light storage) and 4, 5 (dark storage): Cysts induced by copper toxicity shown in FIGS. 9, 10, 15 (light storage) and 11, 13 (dark storage). FIG. 2. Young coccoid cyst (36 × 34 μm) enclosed by theca in early apical ecdysis. FIG. 3. Immature coccoid cyst (32 × 31 μm) with chloroplasts; also enclosed by dehisced theca. FIG. 4. Mature coccoid cyst (34 × 31 μm) with areas of microgranular cytoplasm, two large pigment bodies and thecal remains. FIG. 5. Mature coccoid cyst (31 × 29 μm) with remains of chloroplasts, two large pigmented bodies, enclosed by thecal remains. FIG. 6. Typical mature coccoid cyst with microgranular cytoplasm, pale nucleus, single large pigment body and refractive starch grains; 31 μm diam. FIG. 7. Free mature coccoid cyst; 42 μm diam. (Normarski phase-interference). FIG. 8. Unusually large coccoid cyst; 57 μm diam., probably developed from vacuoloid subtype. FIG. 9. Immature hypnoid cysts emerging from thecae; largest cyst 29 × 24 μm. FIG. 10. Immature hypnoid cyst, with train of abandoned pellicles; largest 34 × 27 μm. FIG. 11. Immature hypnoid cyst; protoplast 34 × 26 μm. FIG. 12. Mature free hypnoid cysts; protoplast 43 × 29 μm. FIG. 13. Immature free hypnoid cyst; protoplast 34 × 26 μm. FIG. 14. Mature elongate hypnoid cysts; length 50 μm; among mass of decayed thecal remains. FIG. 15. Immature hypnoid cysts emerging from thecae; largest protoplast 34 × 26 μm. FIG. 16. Hypnoid cyst releasing its protoplast; 53 × 51 μm. FIG. 17. Vacuoloid cyst; 55 × 50 μm. FIG. 18. Vacuoloid cyst; 55 μm diam. FIG. 19. Vacuoloid lobed cyst with concentric pellicles; maximum size 70 μm. FIG. 20. Vacuoloid cyst showing radial strands of cytoplasm; nucleus (left) and oil globule (right); 53 μm diam.

The wall of pellicle cysts was thin, smooth, single-layered and unornamented; it did not form an operculum or archeopyle (6) on excystment. Chemically its composition is unknown but it is not cellulosic or lipid since it was not stained by iodine-hydriodic acid (23) or Sudan III. It was more resistant to concentrated sulphuric acid than the theca, remaining intact after a week of immersion. Acetolysis destroyed pellicle material fixed in a 3:1 alcohol-acetic acid solution for 1 wk.

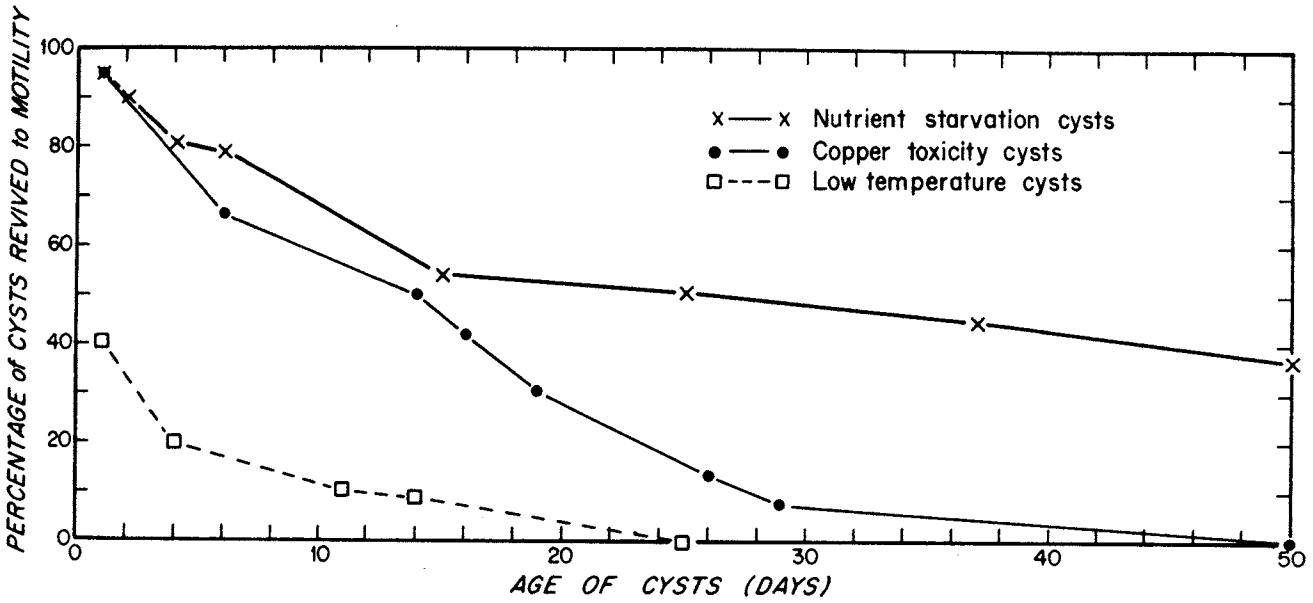


FIG. 21. Frequency of revival to motility (excystment) for pellicle cysts as a function of time elapsed since initial loss of motility (encystment) in laboratory cultures: cysts collected within 24 h of encystment.

Pellicle cyst cell contents varied between two extremes. At one extreme, the contents were indistinguishable from the protoplasts of vegetative cells, with chloroplasts, organelles near the cell wall and a pale equatorial nucleus. At the other extreme, contents resembled the protoplasts of hypnocysts (cf. Figs, 8, 22) with large areas of streaming microgranular cytoplasm, pigmented accumulation bodies, starch grains and a nucleus. Some vacuoloid subtypes contained radial strands of cytoplasm (Figs. 19, 20).

The relative frequencies of occurrence of the three subtypes differed according to the imposed stress. Coccoid cysts were predominant in subcultures exposed to cold temperatures and to nutrient depletion, as well as in some old, neglected transfer cultures, whereas hypnoid cysts occurred only in subcultures exposed to copper. Vacuoloid cysts occurred only in subcultures where formation resulted from low temperatures or nutrient starvation. This subtype developed after 9 wk of 5 C storage in the light. Several weeks later they were transformed to large coccoid cells with hypnocyst-like cell contents (Fig. 8).

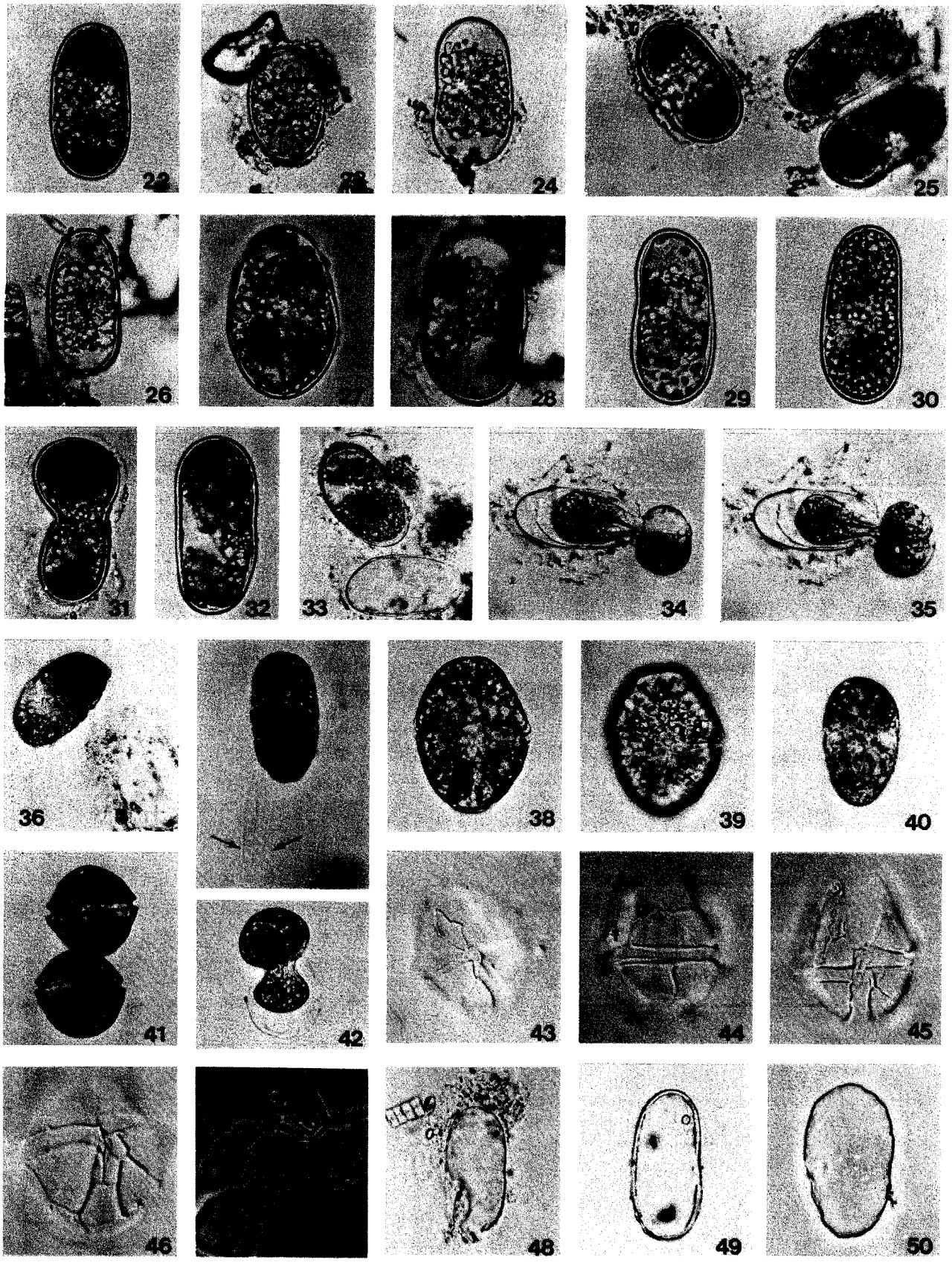
Excystment of pellicle cysts. Excystment involved either liberation of a flagellated stage with one transverse and one longitudinal flagellum or the formation of a train of cast-off pellicles in hypnoid fashion (Fig. 16). Figure 21 demonstrates the decrease in pellicle cyst viability with age. Where 90% of the cysts formed from nutrient and copper stresses revived after 2 days when returned to Aquil A medium, after 50 days dormancy, the best response was only 37% for cysts formed by nutrient depletion. Copper and temperature-induced cysts lost their viability much faster. Note, however, that these results are indicative of the chosen stress and storage conditions. Minor variations of the three stresses would probably change both the shape and relative positions of the curves in Fig. 21.

Storage of pellicle cysts at 5 C for longer time periods showed that the only subcultures with viable cysts were the two containing vacuoloid cysts. Less than 5% of these revived on 16 C incubation. In other subcultures, coccoid cysts with hypnocyst-like cell contents appeared to outlive other cyst types, but after 7 mo, even these more durable pellicle cysts were dead.

TABLE 3. *Excystment results for* Gonyaulax excavata.[a]

Medium	Conditions	No. of hypnocysts	Excystment (%)	Motility (%)
Aquil A	light	36	83	nd[b]
Aquil A	dark	38	79	nd
Aquil A	no nutrients	26	77	nd
Aquil A	light	18	100	83
Aquil A	light	32	88	63
Aquil A	dark	32	93	7
Aquil A	light	29	93	90
Aquil A	dark	42	90	12
Aquil	no EDTA	40	75	0
Aquil	$10^{-7.3}$ M EDTA	39	90	0
Aquil	$10^{-6.3}$ M EDTA	31	90	3
Aquil	$10^{-5.3}$ M EDTA	40	95	13
Aquil	$10^{-4.3}$ M EDTA	36	97	83
f/2	light	31	84	77

[a] Incubation at 16 C for 5 days: all hypnocysts isolated from sediments from Mill Pond, Orleans: cysts in Aquil or Aquil A collected 21 September 1976, stored at 5 C 179–187 days; cysts in f/2 collected 16 March 1977, stored 48 days at 5 C (most probable time of cyst formation was May 1976).
[b] Not determined.


22
24
25
26
28
29
30
31
32
33
34
35
36
38
39
40
41
42
43
44
45
46
48
49
50

DISCUSSION

The following conclusions are based on the preceding laboratory and field data. The rationale for each statement is discussed following.

i) Hypnocysts of *G. excavata* and *G. tamarensis* probably play a major role in initiating annually recurrent toxic blooms in the Cape Cod region. Pellicle cysts cannot perform this function.

ii) Hypnocyst germination begins primarily in response to vernal warming and thus determines the timing of spring bloom initiation each year.

iii) Two different dinoflagellates (*G. excavata, G. Tamarensis*) cause shellfish toxicity in the Cape Cod region. The latter was previously reported to be nontoxic (13).

iv) Hypnocysts of *G. tamarensis* are the probable mechanism for the introduction of this toxic species into the previously unaffected Falmouth district in 1976.

v) Many New England-eastern Canadian red tides can be conceptualized as estuarine-generated phenomena based on the physical and biological characteristics of hypnocyst accumulation and germination.

vi) Hypnocysts of *G. excavata* and *G. tamarensis* are probably sexual zygotes (hypnozygotes) whereas pellicle cysts are asexual.

Ecological roles. The functions of hypnocysts and pellicle cysts with respect to red tides may be sharply divided. Pellicle cysts were progressively less viable over time in laboratory cultures, and most were dead seven months after encystment. Moreover, pellicle cysts have not been found in sediments, which further suggests they are incapable of overwintering. The role of pellicle cysts in nature is thus limited to survival through short periods of nonmotility during vegetative growth—an adaptation to short-term perturbations in ambient conditions.

Our observations demonstrate that overwintering in a dormant condition and the subsequent re-establishment of a motile population through germination are the functions of the more durable hypnocysts. First, a geographical survey in the fall 1976 demonstrated the direct correlation between the presence of benthic hypnocysts and the only Cape Cod locations where toxic blooms occurred the preceding and following springs. Second, hypnocysts were shown to be physiologically capable of survival for more than 6 mo at 5 C (i.e., they can overwinter). Third, laboratory germination of cold conditioned hypnocysts was successful several weeks before blooms of the two species actually developed. We cannot, of course, eliminate the possibility that these blooms were seeded by some other mechanism (i.e., a small residual motile population surviving 0 C winter temperatures or a population introduced from coastal waters).

Seasonal timing, development. Our laboratory results indicate that a temperature increase is the main external stimulus for excystment. Thus hypnocyst germination in Cape Cod salt ponds would be initiated by vernal warming of the seawater and sediments. In Oyster Pond, for example, (5 km from Perch Pond) surface water temperature increases from 6 to 14 C in April (4). This conclusion is also consistent with the pattern of shellfish toxicity on Cape Cod (Table 1) which typically peaks in late spring.

Previous studies on freshwater dinoflagellate cysts such as *Ceratium hirundinella* (O.F.M.) Bergh by Huber and Nipkow (10,11), *C. cornutum* (Ehr.) Claparede & Lachmann, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* von Stosch (22–24) have demonstrated that temperature increases are of major importance in stimulating excystment. This is also true for *Gonyaulax* and *Pyrodinium* hypnocysts (26,28).

Our results indicate that once initiated, excystment generally proceeds through emergence regardless of the suitability of the ambient environ-

←

FIGS. 22–50. Hypnocysts and post-excystment developmental stages in *G. excavata* and *G. tamarensis*. FIGS. 22–26, 41, 42, *G. excavata* from Mill Pond; FIGS. 27–40, 43–48, 50, *G. tamarensis* from Perch Pond; FIG. 49, *G. tamarensis* from Green Pond. FIG. 22. Hypnocyst without mucilaginous outer coat; 62 × 31 μm. FIG. 23. Ovoid hypnocyst; 46 × 31 μm. FIG. 24. Weakly reniform hypnocyst; 58 × μm. FIG. 25. Three hypnocysts; left one 53 × 29 μm. FIGS. 26, 27. Weakly tapered hypnocyst; 60 × 39 μm. FIG. 28. Pale hypnocyst with starch grains localized in its median equatorial zone; 54 × 41 μm. FIG. 29. Hypnocyst; 62 × 29 μm. FIG. 30. Hypnocyst, completely filled with starch grains, extracted from hepatopancreas of *Mya*; 67 × 29 μm. FIG. 31. Dumbbell-shaped hypnocyst; 65 × 34 μm. FIG. 32. Hypnocyst with clear zone representing site of nucleus; 61 × 29 μm. FIG. 33. Ripe hypnocyst with transformed cell contents immediately prior to excystment; 54 × 30 μm: lower, abandoned exospore; 53 × 29 μm. FIGS. 34, 35. Protoplast emerging from hypnocyst (excystment), with 40 s of elapsed time; hypnocyst 60 × 31 μm. FIGS. 36, 37. Posteriorly biflagellate germling immediately after excystment; 53 × 31 μm: arrows point to two trailing, uncoiled flagella. FIGS. 38, 39. Post-excystment germling with focus on cell margin and ventral area, respectively; 55 × 43 μm. FIG. 40. Post-excystment germling; 50 × 31 μm. FIG. 41. Typical pair of motile cells (doublet), 7 days after excystment; transdiam. 38 μm. FIG. 42. Thecate cell releasing protoplast by apical ecdysis; transdiam. 31 μm. FIGS. 43, 46. Abandoned theca from a germling, no more than 2 days after excystment, antero-ventral and postero-ventral views showing typical plate pattern of *G. tamarensis*, transdiam. 43 μm (focus in antero-ventral view is on lower hemisphere so image is reversed left to right). FIGS. 44, 45. Second theca from another germling, no more than 3 days after excystment, in right lateral and mid-ventral views, showing tabulation pattern of theca; transdiam. 38 μm. FIG. 47. Epithecal plates of *G. tamarensis* from Perch Pond plankton (18 March 77) in internal view so left and right reversed: arrow points to ventral pore on plate 1' where it borders plate 4'. ×1,000 (Nomarski phase-interference). FIG. 48. Abandoned exospore of hypnocyst surrounded by mucilaginous layer; 60 × 29 μm. FIG. 49. Hypnocyst with endosporic layer intact; 62 × 29 μm. FIG. 50. Exospore of hypnocyst after soaking 5 days in concentrated sulfuric acid; endospore and external mucilage were destroyed. ×500.

ment, whereas motility and cell divisions are dependent on the presence of suitable light and chelator. Thus the successful development of blooms of *G. tamarensis* and *G. excavata* from germinated hypnocysts is clearly dependent on external factors that further constrain the timing and location of outbreaks.

Hypnocyst dispersal. The spread of shellfish toxicity to the Falmouth district in 1976 may have resulted from the unintentional introduction of *G. tamarensis* hypnocysts. In contrast with the first toxicity in Orleans in September 1972 during the massive outbreak along the New England coast, the first toxicity in Falmouth was in 1976, and it was caused by *G. tamarensis* rather than *G. excavata.* Both of these features are curious, as is the geographic discontinuity whereby shellfish were toxic in Orleans and in Falmouth but nowhere between. Taken together, these peculiarities suggest that toxicity in Falmouth was not initiated by dispersal of toxic dinoflagellates from elsewhere on Cape Cod, but from an external source. For example, transfer of sediments (e.g., dredging, dumping) or shellfish (e.g., transplantation) could introduce hypnocysts into new locations where indigenous populations could be sustained. Massachusetts has received numerous shellfish relays from Maine over the years, and Perch Pond was in fact seeded with quahogs in 1975, one year before the first toxic shellfish were detected.

New England red tides. Our investigations were limited to Cape Cod, but the results are applicable to the entire New England, eastern Canadian red tide phenomenon. Such red tides can be categorized as autochthonous or allocthonous. The former are those where dense motile populations develop in close proximity to benthic hypnocysts. As exemplified by Mill Pond and Perch Pond, such blooms would be annually recurrent and amenable to close monitoring and perhaps some degree of prediction. Some may be extremely localized and of brief duration but nonetheless highly toxic.

Allochthonous blooms would be those that occur beyond the confines of estuaries—far from their original seed beds of hypnocysts. The widespread 1972 New England red tide (which occurred after the passage of Hurricane Carrie) may be one example of this type. We theorize that such blooms develop when motile populations entrained in estuarine waters are flushed into adjacent coastal zones (by major storm or tidal events) where growth can continue until nutrients are depleted or physical mixing with coastal waters adversely changes the chemical environment. Certainly the importance of land runoff in supporting toxic blooms has been recognized (e.g., 1,9,16–18). We merely add evidence that emphasizes the role of runoff in creating a unique chemical and highly stratified environment, while coincidentally serving as a dispersal and seeding mechanism.

The essential difference between the two bloom types is their genesis. Open coastal environments are highly dynamic and probably prevent the dense accumulations of hypnocysts found in estuaries. Although the growth of coastal populations to toxic cell densities from an extremely low density excystment "inoculum" is possible, we suggest that large-scale blooms in this region do not generally begin in open coastal waters. However, we cannot rule out the possibility of non-seasonal cyst "reservoirs" at subthermoclinal depths (in near-shore topographic depressions, for example) where cysts could accumulate for years without annual germination before being recirculated to the euphotic zone by major storm activity or upwelling. Huber and Nipkow (11) found that constant exposure to low temperatures in deeper sections of Lake Zurich suppressed cyst germination of *Peridinium cinctum* (O.F.M.) Ehr. for as long as 16.5 yr.

The speculative nature of certain aspects of the foregoing discussion emphasizes the need for future research in several specific areas. First, direct evidence must be gathered that confirms the proposed role of germinating hypnocysts as the seed population for blooms. Second, a more detailed investigation of the encystment and excystment phenomena is needed to define threshold levels of the critical parameters that initiate these processes. Third, hydrodynamic, hydrologic, and vertical migration studies should define the conditions under which motile populations are flushed from estuarine locations into coastal waters. A parallel study should determine whether coastal accumulations of hypnocysts do exist and if so, what combination of physical factors is necessary for the direct initiation of a coastal bloom. A synthesis of these lines of research may then create a sound basis for the predictions of both local and regional red tides.

Sexuality of cysts. A secondary objective of this study was the documentation of the differences between hypnocysts and pellicle cysts. The former are larger, more elongate, have a non-renewable multilayered thick wall (i.e., no chains of discarded cell walls as in Fig. 16), greater longevity and do not form in culture in response to the chosen stimuli. Most importantly, hypnocysts release large posterior-biflagellated germlings upon excystment.

These characteristics suggest that hypnocysts are the sexual zygotes of *G. excavata* and *G. tamarensis,* whereas pellicle cysts are asexual. In particular, according to von Stosch (24), the unusually large single germling with posterior biflagellation indicates that the emergent cell is a planomeiocyte. Hence the hypnocyst is a zygote. However, we have not observed the fusion of gametes and cyst formation directly, so we use the term "hypnocyst" rather than the more specific "hypnozygote" which can be substituted once sexuality is proven.

The reason hypnocysts of *G. excavata* and *G. tamarensis* have not been observed to date in cultures is probably failure of the organisms to reproduce

sexually under the chosen culture conditions. This is commonplace among marine dinoflagellates where in the great majority of instances, only pellicle cysts occur in laboratory cultures.

Taxonomic considerations. Our discovery that the dinoflagellate from the Orleans complex lacks an antero-ventral pore (along the right margin of the first apical plate (1') where it joins plate 4') whereas the species from Falmouth possesses this pore, prompted us to identify them as *G. excavata* and *G. tamarensis,* respectively. This pore is the only morphological feature separating these two species according to Loeblich and Loeblich (13). However, we have not established that the taxon from Perch Pond is identical with *G. tamarensis* from the Tamar estuary in Britain or Isolate 173 of Loeblich and Loeblich (13) in all other respects. In particular, the last is reportedly nontoxic, whereas the Perch Pond organism has been tested and is definitely toxic (Y. Shimizu, pers. comm.). We do not consider toxicity to be a rigorous taxonomic criterion in species definition at this point and are willing to accept toxic and non-toxic strains of a single species.

Whether *G. excavata* should be considered a separate species or a variety (*G. tamarensis* var. *excavata*) is a separate issue, as is the contention that according to the rules of priority, the correct name for the species should be *G. phoneus* (Woloszynska & Conrad) nov. comb. (13,21). Steidinger (19), Taylor (21) and Wall (25) each emphasized that *G. tamarensis*-related dinoflagellates belong to a species complex whose taxonomy needs careful revision. The central problem is the differentiation between species and varieties, and until new methods are employed to rigorously address this question, further discussion seems futile. In this paper, we simply wish to emphasize that shellfish toxicity in New England is caused by two dinoflagellates instead of one as previously reported, with the only distinction between the two being the presence, or lack of, an antero-ventral pore in the theca.

This research was supported by the Office of Sea Grant in the National Oceanic and Atmospheric Administration, United States Department of Commerce, through grant numbers 04-6-158-44007 and 04-6-158-44016, by National Science Foundation Grant number DES75-15023, by International Copper Research Association Project Number 252, and by a grant to the Woods Hole Oceanographic Institution from the George Frederick Jewett Foundation. The authors thank the following individuals who assisted in this effort: F. M. M. Morel (Massachusetts Institute of Technology), J. Orphanos (Commonwealth of Massachusetts, Department of Environmental Quality Engineering), A. Russell Ceurvels, J. Hickey (Commonwealth of Massachusetts, Department of Fisheries, Wildlife and Recreational Vehicles), G. Souza (Shellfish Constable, Falmouth), G. Munsey (Shellfish Warden, Orleans), G. Russell and Y. Shimizu (University of Rhode Island).

1. Anderson, D. M. & Morel, F. M. M. 1978. Copper sensitivity of *Gonyaulax tamarensis. Limnol. Oceanogr.* 23:283–95.
2. Brooks, J. 1971. Some chemical and geochemical studies on sporopollenin. *In* Brooks, J., Grant, P. R., Muir, M., van Gijzel, P. & Shaw, G. [Eds.] *Sporopollenin,* Academic Press, London, 351–407.
3. Dale, B. 1977. Cysts of the toxic red-tide dinoflagellate *Gonyaulax excavata* (Braarud) Balech from Oslofjorden, Norway. *Sarsia* 63:29–34.
4. Emery, K. O. 1969. *A Coastal Pond.* American Elsevier Publishing Co., New York. 80 pp.
5. Entz, G. 1926. Beiträge zur Kenntnis Peridineen. I. Zur Morphologie und Biologie von *Peridinium borgei* Lemmermann. *Arch. Protistenk.* 56:397–446.
6. Evitt, W. R. 1967. Dinoflagellate studies II. The archeopyle. *Stanford Univ. Publ. Geol. Sci.* 10(3):1–83.
7. Gray, J. 1965. Palynological techniques. *In* Kummel, B. & Raup, D. [Eds.] *Handbook of Paleontological Techniques,* W. H. Freeman Co., San Francisco, 471–81.
8. Guillard, R. R. L. & Ryther, J. H. 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacae* (Cleve) Gran. *Can. J. Microbiol.* 8:229–39.
9. Hartwell, A. D. 1975. Hydrographic factors affecting the distribution and movement of toxic dinoflagellates in the western Gulf of Maine. *In* LoCicero, V. R. [Ed.] *Toxic Dinoflagellate Blooms,* Proceedings First International Conference, Massachusetts Science and Technology Foundation, Wakefield, 47–68.
10. Huber, G. & Nipkow, F. 1922. Experimentelle untersuchungen über die entwicklung von *Ceratium hirundinella* O.F.M. *Z. Bot.* 14:337–71.
11. ——— 1923. Experimentelle untersuchungen über die entwicklung und formbuildung von *Ceratium hirundinella* O.F.M., *Flora Jena.* 116:114–215.
12. Loeblich, A. R. III. 1970. The amphiesma or dinoflagellate cell covering. North American Paleontological Convention Proceedings G:867–929.
13. Loeblich, L. A. & Loeblich, A. R. III. 1975. The organism causing New England red tides: *Gonyaulax excavata. In* LoCicero, V. R. [Ed.] *Toxic Dinoflagellate Blooms.* Proceedings First International Conference, Massachusetts Science and Technology Foundation, Wakefield, 207–24.
14. Morel, F. M. M., Westall, J. C., Rueter, J. G., & Chaplick, J. P. 1975. Description of the algal growth median "Aquil" and "Fraquil." Technical Note 16, Massachusetts Institute of Technol., Department of Civil Engineering, Ralph M. Parsons Laboratory. 33 pp.
15. Mulligan, H. F. 1975. Oceanographic factors associated with New England red tide blooms. *In* LoCicero, V. R. [Ed.] *Toxic Dinoflagellate Blooms.* Proceedings First International Conference, Massachusetts Science and Technology Foundation, Wakefield, 23–40.
16. Prakash, A. 1967. Growth and toxicity of a marine dinoflagellate *Gonyaulax tamarensis. J. Fish. Res. Board Can.* 24:1589–606.
17. ——— 1975. Land drainage as a factor in "red tide" development. *Environ. Lett.* 9:121–8.
18. Ryther, J. H. 1955. Ecology of autotrophic marine dinoflagellates with reference to red water conditions. *In* Johnson, F. H. [Ed.], *The Luminescence of Biological Systems,* American Association for the Advancement of Science, Washington, D.C., 387–414.
19. Steidinger, K. A. 1971. *Gonyaulax balechii* sp. nov. (Dinophyceae) with a discussion of the *Gonyaulax* and *Heteraulacus. Phycologia.* 10:183–7.
20. ——— 1975. Basic factors influencing red tides. *In* LoCicero, V. R. [Ed.] *Toxic Dinoflagellate Blooms.* Proceedings First International Conference, Massachusetts Science and Technology Foundation, Wakefield, 153–62.
21. Taylor, F. J. R. 1975. Taxonomic difficulties in red tide and paralytic shellfish poison studies: the "*tamarensis* complex" of *Gonyaulax. Environ. Lett.* 9:103–19.
22. von Stosch, H. A. 1965. Sexualität bei *Ceratium cornutum* (Dinophyta). *Naturwissenschaften* 52:112–3.
23. ——— 1969. Dinoflagellaten aus der Nordsee. II. *Helgolandium subglobossum* gen. et. spec. nov. *Helgol. Wiss. Meeresunters.* 19:569–77.

24. ——— 1973. Observations on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. *Br. Phycol. J.* 8:105–45.
25. Wall, D. 1975. Taxonomy of cysts of red tide dinoflagellates. *In* LoCicero, V. R. [Ed.] *Toxic Dinoflagellate Blooms.* Proceedings First International Conference, Massachusetts Science and Technology Foundation, Wakefield, 249–55.
26. ——— & Dale, B. 1968a. Modern dinoflagellate cysts and evolution of the Peridiniales. *Micropaleontology* 14:265–304.
27. ——— 1968b. Quaternary calcareous fossil dinoflagellates (Calciodinellidae) and their natural affinities. *J. Paleont.* 42:1395–408.
28. ——— 1969. The "hystrichosphaerid" resting spore of the dinoflagellate *Pyrodinium bahamense* Plate 1906. *J. Phycol.* 5:140–9.