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Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada

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Abstract Twenty-eight strains of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada were characterized on the basis of morphology, bioluminescence capacity, mating compatibility, and toxin composition. The distributions of these characters were evaluated in the context of regional patterns of paralytic shellfish poisoning (PSP) and coastal hydrography. Two morphospecies were identified – *A. tamarense* Lebour and *A. fundyense* Balech. The two are interspersed geographically though there are areas, such as the Gulf of Maine, where apparently only *A. fundyense* occurs. Southern waters (Cape Cod, Connecticut, and Long Island) have especially diverse populations. The two species are sexually compatible. Virtually all northern isolates are bioluminescent, whereas southern isolates include bioluminescent and non-bioluminescent strains. Cluster analyses, based on high performance liquid chromatography (HPLC) determinations of the suite of toxins produced by each isolate, revealed two and perhaps three distinct groups. One is comprised almost exclusively of northern strains, and the other of southern strains. A Cape Cod cluster may be separable from the southern group. These analyses explain a previously reported north-to-south trend of decreasing toxicity, as the northern isolates produce greater proportions of the more potent toxins than do southern forms. The overall perspective is that the biogeography of toxic *Alexandrium spp.* in the study region is not that of a single, widespread, homogeneous population, but rather is comprised of several sub-populations, each with its own physiological characteristics and history. Two scenarios are considered with respect to this regional biogeography. The first invokes recent and continuing dispersal of isolates to the south from a center of origin in the north, followed by recombination and strong selection. The second holds that the northern and southern populations diverged from a common ancestor (vicariance), but now represent localized populations with little mixing of genotypes. Neither hypothesis can be completely refuted by the data presented here, though the weight of the evidence favors the latter. The correct scenario may be a combination of both, with recent and continuous spreading occuring within the Gulf of Maine and perphaps the Gulf of St. Lawrence, but with endemic localized populations persisting without genetic exchange in most southern locations. These data also indicate that although morphological criteria separate toxic *Alexandrium* isolates from the study region into two morphospecies, these assignments do not coincide with clusterings based on toxin composition or allozyme electrophoresis, and they are further violated by mating results. A revision of taxon designations to the varietal level could be justified.

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Introduction

Some species in the dinoflagellate genus *Alexandrium* produce potent neurotoxins which are well known because they cause paralytic shellfish poisoning (PSP), a syndrome of human illness and death following the consumption of contaminated shellfish. *Alexandrium spp.* toxins also move to other levels of the food chain, impacting zooplankton, fish, and even marine mammals. PSP is a global phenomenon that affects many coastal countries. Along the western Atlantic, it represents a real or potential threat from New Jersey in the United States north to the St. Lawrence estuary and Newfoundland in Canada (Fig. 1 A). Shellfish toxicity has been a problem for hundreds of years in east-

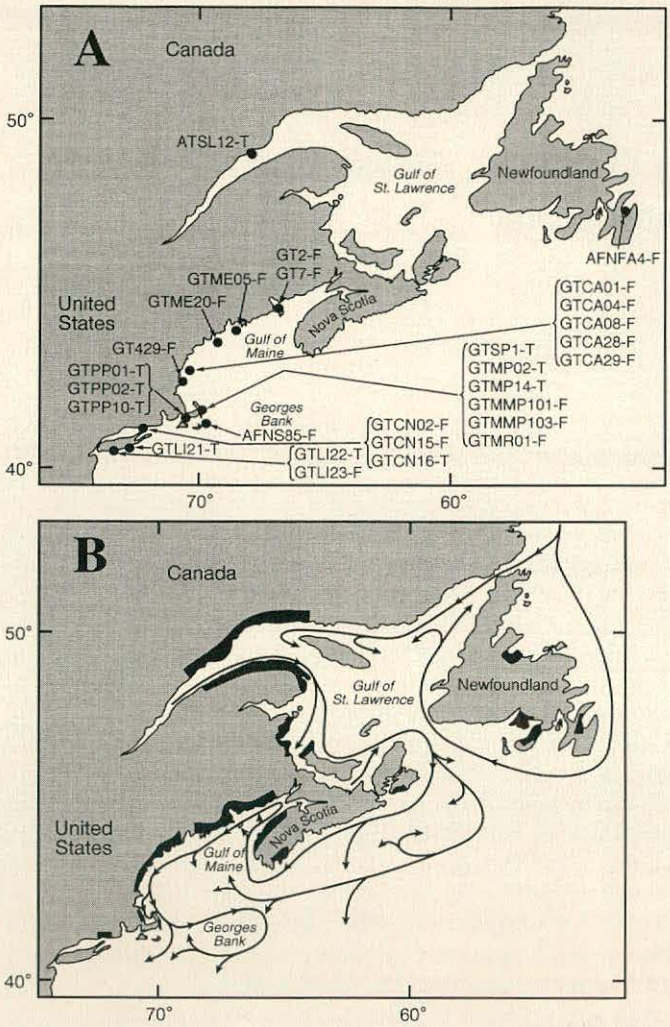


Fig. 1 The study region. **A** Origins of the isolates examined in the present study. Strain names followed by "F" or "T", designating *Alexandrium fundyense* and *A. tamarense*, respectively. **B** General pattern of non-tidal surface circulation in the study region (*arrows*). Coastline areas affected by paralytic shellfish poisoning (PSP) are blackened. Not shown: PSP toxicity also occurs in offshore shellfish within the central Gulf of Maine, on Georges Bank, and Nantucket Shoals (located just to the west of Georges Bank). Qualitative flow patterns derived in part from Smith and Schwing (1991) and references therein

ern Canada, but the western Gulf of Maine and areas further south have only been affected during the last two decades (Prakash et al. 1971; Anderson et al. 1982). A common belief is that the recent history of PSP in southern waters reflects the dispersal of *Alexandrium spp.* from endemic populations in the north during a massive 1972 red tide, an event which caused PSP toxicity for the first time in southern New England (Hartwell 1975). Shellfish toxicity is now a recurrent annual problem in these previously unaffected areas.

PSP is not evenly distributed throughout the region (Fig. 1 B). At the northern extreme, it is associated with shellfish along the lower estuary of the St. Lawrence River, including the Gaspé peninsula and the western Gulf of St. Lawrence (Cembella et al. 1988), as well as in scattered locations on Newfoundland (White and White 1985). Far to the south, toxic *Alexandrium spp.* blooms occur in isolated embayments and estuaries in Cape Cod, Connecticut, Long Island, and New Jersey (Anderson et al. 1982; Schrey et al. 1984; Cohn et al. 1988) and PSP has recently been detected at high levels in offshore shellfish on Georges Bank (White et al. 1993). Between these southern and northern extremes, the Gulf of Maine (including the middle and lower reaches of the Bay of Fundy) experiences PSP along virtually its entire coastline. Toxicity is localized and infrequent along the eastern coast of Nova Scotia.

There is considerable potential for genetic exchange among *Alexandrium spp.* populations for two reasons. First, the organisms undergo a sexual phase resulting in the production of resistant, overwintering cysts during the late stages of blooms (Anderson and Wall 1978). Furthermore, the areas affected by PSP are linked hydrographically (Smith and Schwing 1991) and there is a general north-to-south flow in the region (Fig. 1 B). North of Cape Cod, *Alexandrium spp.* blooms are geographically widespread phenomena, affecting hundreds of kilometers of coastline (Cembella et al. 1988; Martin and White 1988; Franks and Anderson 1992), facilitating population dispersal over large distances. In contrast, southern blooms are localized within shallow embayments and estuaries (Anderson et al. 1982; Schrey et al. 1984), with little or no exchange with local coastal waters.

Little is known of the genetic and ecological relationships that link different populations of *Alexandrium spp.* within this large region. Whether the 1972 outbreak was large enough to disperse these species as far south as Connecticut or New Jersey in one spreading event is uncertain, nor is it known whether toxic *Alexandrium* species were present in southern waters prior to that date, or whether there have been spreading events on a smaller scale since 1972. The first comparison of regional *Alexandrium spp.* populations was a survey of the toxin content of isolates originating between Nova Scotia and New York (Maranda et al. 1985). That study revealed a systematic decrease in toxicity from north to south, an observation that appears inconsistent with the hypothesized recent dispersal of toxic cells from a common source during the 1972 bloom. A research program was established to compare isolates from throughout the region using five different characters: morphology, mating compatibility, bioluminescence capacity, toxin composition, and allozyme electrophoresis. Electrophoretic results are given by Hayhome et al. (1989). Here we present information on the other characters and provide an overview of the entire study.

Materials and methods

Isolation and culture

Twenty-eight clones of *Alexandrium tamarense* and *A. fundyense* from waters between the Gulf of St. Lawrence, Canada and Long Island, NY were established in culture (Table 1). GT2 and GT7 were

Table 1 *Alexandrium tamarense* and *A. fundyense*. List of isolates and their characteristics, ordered from north-to-south. (*a* archived sample. Due to evaporation and chemical conversions during long-term storage, toxin contents not reported)

Clone	Species	Location	Toxin		Biolum
			(fmol cell)[1]	(fgSTX cell)	
ATSL12	*A. tamarense*	Baie Comeau, St. Lawerance Estuary (49°03′N, 68°20′W)	187.9	58695	Yes
AFNFA4	*A. fundyense*	Harbour Grace, New Foundland, (47°30′N, 53°10′W)	88.8	38075	Yes
GT2	*A. fundyense*	Campobello Island, Bay of Fundy (44° 46′N, 67° 11′W	a	a	Yes
GT7	*A. fundyense*	Campobello Island, Bay of Fundy (44°46′N, 67° 11′W)	31.9	10927	Yes
GTME05	*A. fundyense*	Greenlaw Neck, Deer Isle, Maine (44° 13′N, 68° 40′W)	a	a	Yes
GTME20	*A. fundyense*	Monhegan Island, Maine (43° 45′N, 69° 19′W)	50.7	17507	Yes
GTCA29	*A. fundyense*	Gulf of Maine, NH (43° 00′N, 70° 19′W)	69.8	25627	Yes
GTCA28	*A. fundyense*	Gulf of Maine, NH (43° 00′N, 70° 19′W)	61.5	21372	Yes
GTCA08	*A. fundyense*	Gulf of Maine, NH (43° 00′N, 70° 19′W)	53.8	19195	Yes
GTCA04	*A. fundyense*	Gulf of Maine, NH (43° 00′N, 70° 19′W)	67.3	26894	Yes
GTCA01	*A. fundyense*	Gulf of Maine, NH (43° 00′N, 70° 19′W)	a	a	No
GT429	*A. fundyense*	Ipswich Bay, MA (42° 40′N, 70° 45′W)	34.5	11435	Yes
GTSP1	*A. tamarense*	Salt Pond, Eastham, MA (41° 50′N, 69° 58′W)	a	a	Yes
GTMP14	*A. tamarense*	Mill Pond, Orleans, MA (41° 47′N, 69° 57′W)	74.0	19071	No
GTMP02	*A. tamarense*	Mill Pond, Orleans, MA (41° 47′N, 69° 57′W)	a	a	No
GTMMP101	*A. fundyense*	Mill Pond, Orleans, MA (41° 47′N, 69° 57′W)	a	a	No
GTMMP103	*A. fundyense*	Mill Pond, Orleans, MA (41° 47′N, 69° 57′W)	a	a	Yes
GTMR01	*A. fundyense*	Mitchell River, Chatham, MA (41° 40′N, 69° 58′W)	118.3	29859	Yes
AFNS85	*A. fundyense*	Nantucket Shoals, MA (41° 36′N, 69° 45′W)	131.5	53698	Yes
GTPP01	*A. tamarense*	Perch Pond, Falmouth, MA (41° 30′N, 70° 35′W)	29.3	7814	No
GTPP02	*A. tamarense*	Perch Pond, Falmouth, MA (41° 30′N, 70° 35′W)	a	a	N/A
GTPP10	*A. tamarense*	Perch Pond, Falmouth, MA (41° 30′N, 70° 35′W)	72.3	15894	No
GTCN02	*A. fundyense*	Palmer Cover, Groton, CN (41° 20′N, 72° 00′W)	127.6	46553	No
GTCN15	*A. fundyense*	Mumford Cove, Groton, CN (41° 20′N, 72° 01′W)	48.0	11621	No
GTCN16	*A. tamarense*	Mumford Cove, Groton, CN (41° 20′N, 72° 01′W)	51.3	7772	No
GTLI21	*A. tamarense*	Mud Creek, Moriches Bay, NY (40° 45′N, 72° 49′W)	25.5	6199	Yes
GTLI22	*A. tamarense*	Mud Creek, Babylon, NY (40° 40′N, 73° 20′W)	a	a	No
GTLI23	*A. fundyense*	Mud Creek, Babylon, NY (40° 40′N, 73° 20′W)	a	a	N/A

obtained from A. W. White, and GT429 was isolated in 1972 by C. Martin. All others were isolated between 1978–1991 by Anderson and co-workers. Most cultures (those termed "non-archived") were grown in duplicate 25-ml volumes of f/2 medium (Guillard and Ryther 1962) made with 0.2-μm filtered seawater [31 psu (practical salinity units)]. The f/2 medium was modified by adding H_2SeO_3 and reducing $CuSO_4$, both to a final concentration of 10^{-8} *M*. Cultures were incubated at 20 °C on a 14 h light:10 h dark cycle (ca. 250 $\mu E\, m^{-2}\, s^{-1}$ irradiance, cool white fluorescent bulbs). Growth was monitored on a Turner model 10 fluorometer. When cultures reached mid-exponential stage, duplicate samples were removed for cell counts and the remaining cells harvested by centrifugation at $1700 \times g$ for 3 min at 23 °C. The supernatant was aspirated and the cell pellet containing approximately 1.5×10^5 cells was resuspended in 0.5 ml 0.05 *M* acetic acid and sonified with a Branson sonifier for 3 min at a setting of 6 A in an ice water bath. Samples were frozen at −20 °C until analysis, then thawed, mixed, recentrifuged as above and filtered through 0.45-μm syringe filters. Some isolates were lost from the culture collection and could only be analyzed using stored samples prepared for a previous study. These archived samples had been grown and extracted as described by Hayhome et al. (1989). A subsample was processed for toxin analysis and the extracts frozen and thawed three times prior to analysis to release the toxins.

Pore morphology

Cultures grown under standard conditions were fixed in 4% formalin and used for taxonomic analyses. Species designations were assigned according to the criteria of Balech (1985). The morphology of cells from selected cultures produced by mating *Alexandrium tamarense* and *A. fundyense* were also examined to determine if a ventral pore was present in the progeny. Cysts were isolated and placed individually into tissue culture wells containing medium and incubated under normal culture conditions. The germinated planomeiocytes were allowed to divide twice in the wells, and the resulting four cells were individually isolated and placed into four separate culture tubes. In some cases, all four cultures survived, representing a tetrad. In other cases when one or two cultures did not survive, incomplete tetrads were obtained. In one instance, division proceeded too quickly and six cultures were established from one cyst. Clearly, two of these cultures are identical to two others. The presence or absence of the ventral pore in cells from each culture was ascertained using Calcofluor staining (Fritz and Triemer 1985) and microscopic observation of at least 40 thecae. Mating type was determined by backcrossing these first filial generation (F_1) cultures with the parents. Several F_1 cultures were also crossed with each other and induced to form cysts, which were then induced to germinate, and the progeny cultured to demonstrate the viability of the second filial (F_2) generation.

Mating compatibility

Mating compatibility was assessed by inoculating individual isolates alone, or in combination with another isolate, into f/2 medium with nitrogen supplied only as NH_4^+ at 25 μ*M*. Initial concentrations were 100 cells ml^{-1} of each strain. Crosses were incubated under the temperature and lighting conditions described above and checked for resting cyst formation after 1 mo.

Bioluminescence

Cultures were grown under standard conditions and tested for bioluminescence in a darkened room by the addition of several mls of 0.5 *M* acetic acid to a 25-ml culture in mid-exponential growth. Negative observations were verified at a later date.

Toxin analysis

Non-archived and archived samples were analyzed by the three-step isocratic elution method of Oshima et al. (1989) for high perfomance liquid chromatography (HPLC) determination of the saxitoxins, with some modifications. For the C1-C4 toxins, column temperature was 30 °C and the post column reaction temperature 45 °C to enhance separation and sensitivity. The mobile phase was adjusted to pH 5.8 with 0.05 *M* acetic acid. Gonyautoxins were analyzed with a column temperature of 23 °C and a post column reaction temperature of 35 °C to enhance fluorescence. Column and post column reaction temperatures were 24 and 50 °C, respectively, for the saxitoxin group in order to increase neosaxitoxin flurorescence. The column eluate was mixed with 7 m*M* periodic acid in 80 m*M* sodium phosphate buffer. The HPLC system consisted of a Waters 600E multi-solvent delivery system connected to a Waters Wisp 700 autosampler and a Shimadzu RF-535 fluorescence detector. A Beckmann C8 Ultrasphere Octyl 55m analytical column (25×46 mm) and a Brownlee MPLC New Guard Column were used for the separation of the toxins. External standard solutions were run prior to sample analysis and after every fourth sample. Toxin composition profiles were determined from replicate analyses (two injections) from each of two separate samples. Abbreviations used throughout this text are: STX – saxitoxin; NEO – neosaxitoxin; GTX 1,4 – gonyautoxins 1 and 4; GTX 2,3 – gonyautoxins 2 and 3; GTX 5 – gonyautoxin 5 (or B1; Hall et al. 1990); GTX 6 – gonyautoxin 6 (or B2); C1,2 – toxins C1 and C2; C3, C4 – toxins C3 and C4; dc – decarbamoyl. The terms GTX1,4, GTX2,3, C1,C2 and C3,C4 are used to represent the pooled concentrations of two toxins to account for possible epimerization. Toxicities (in STX equivalents cell^{-1}) were calculated from the molar composition data using individual potencies (Oshima et al. 1992). Specific toxicities of the individual toxins (pg STX eq.) are as follows: C1 = 15; C2 = 239; C3 = 32; C4 = 143; GTX1 = 2468; GTX2 = 896; GTX3 = 1286; GTX4 = 1803; GTX5 = 160; dcGTX2 = 1617; dcGTX3 = 1872; NEO = 2295; dcSTX = 1274; STX = 2483.

Non-archived toxin extracts were analyzed within 3 mo of extraction, whereas archived materials had been stored for several years at 4 °C in 0.05 *N* acetic acid. One concern was that prolonged storage could alter the toxin composition; another was that evaporation could cause the calculated total toxin concentrations to increase. In some analyses described below [e.g. clustering and principal component analyses (PCA)], archived and non-archived data were combined. For others, only non-archived samples were used. Justification for these decisions is given in the text.

Data analysis

Toxin composition data were examined using multivariate matrix cluster analysis employing Pearson correlation coefficients and the average linkage method (program CLUSTER, option JOIN MATRIX, in the SYSTAT package, Systat, Inc., Evanston, IL). This is a hierarchial clustering procedure in which rows and columns are permuted to maximize indentification of clusters, and the cutoff points between ranges of the correlation coefficients are determined by Tukey's gapping method. This multivariate clustering method is more appropriate than the more common univariate procedures in that it simultaneously yields information on the relationships among the isolates and among the toxins. This method was applied to toxin data transformed with the weighted-ratio method of Cembella et al. (1987), where the maximum mole percent (mol %) of a given toxin across isolates was assigned a value of 1, and the relative mol % of the toxin in other isolates was scaled relative to this maximum value. This treatment weights each toxin component equally, avoiding bias against toxins present in low concentrations in all isolates. In order to correct for the role of evaporation and possible epimerization in archived samples, the values for the non-archived preparations were scaled against themselves whereas the archived samples were scaled against the highest archived samples.

Toxin composition was also examined using PCA on the correlation matrix (program FACTOR, options VARIMAX, EIGEN = 0, in the SYSAT package). This method was used with toxicity expressed as total molar concentrations and as STX equivalents cell^{-1}. Observed differences in toxin composition of non-archived and archived samples of the same isolates led to a concern that inclusion of the archived samples might systematically bias the analyses. Therefore, each analysis was performed using the combined (archived plus non-archived) data, and the non-archived data alone, to determine if cluster patterns would be affected. The loading of each variable on the principal components is available from the authors on request.

Results

Morphology

All clones examined belong to the "tamarensis/catenella" group, with two species represented, i.e. *Alexandrium tamarense* Lebour and *A. fundyense* Balech. There were ten of the former species and 16 of the latter (Table 1). The distribution of the two species across the study region was not uniform (Fig. 1A), as 11 of the 12 northernmost isolates were *A. fundyense*, the one exception being an *A. tamarense* isolate from the St. Lawrence estuary. All northern Gulf of Maine isolates were *A. fundyense*. In the south, the two species were interspersed.

Mating compatibility

None of the seven *Alexandrium fundyense* and three *A. tamarense* isolates cultured by themselves in low nutrient medium formed resting cysts (hypnozygotes), but numerous cysts were formed from both inter and intra-species crosses. Of the 16 crosses of *A. fundyense* isolates with other *A. fundyense* (not including self-crosses), nine produced cysts (data not shown). Of 17 crosses of *A. fundyense* with *A. tamarense*, eight produced cysts. After a suitable maturation interval, cysts obtained from one *A. fundyense/A. tamarense* cross (GTSP1 and GT7) were germinated to produce typical planomeiocytes (von Stosch 1973; Anderson and Wall 1978) which divided to produce vegetative cells that were isolated and cultured as tetrads. Crosses of these tetrad cultures yielded cysts 50% of the time in a pattern consistent with the presence of two mating types in this heterothallic organism.

Complete and incomplete tetrad cultures produced by this mating between *Alexandrium tamarense* and *A. fundyense* were examined to determine mating type, whether the cells were bioluminescent, and if a ventral pore was present on the theca (Table 2). Both parents were bioluminescent, and one (GTSP1, *A. tamarense*) had a prominent ventral pore but the other (GT7, *A. fundyense*) did not. Of the 14 tetrad progeny assayed for bioluminescence, 13 were positive and one was negative. Mating types were equally divided and showed no relationship to bioluminescence or to the presence of a pore. Only one of the 21 progeny cultures contained cells with a prominent ventral pore. All others either had no pore whatsoever or had a very small, generally indistinct pore on a few (maximum 8%) of the cells. [Close examination of the clonal GT7 parent

Table 2 *Alexandrium* spp. Characteristics of progeny from mating between *A. tamarense* (*GTSP1*) and *A. fundyense* (*GT7*). Isolates grouped together are from the same cyst (*Biolum.* bioluminescent; *N/A* data not available)

Isolate	Biolum.	Mating type	Ventral pore	Comments
Parents				
GT7	Yes	+	–	3 of 59 cells, small pore
GTSP1	Yes	–	+	40 of 40 cells, normal pore
Progeny				
17C8A	Yes	+	+	38 of 40 cells, normal pore
17C8B	N/A	–	–	3 of 40 cells, small pore
17C8C	Yes	–	–	
17C8D	Yes	+	–	1 of 40 cells, small pore
17D5A[a]	Yes	–	–	
17D5B[a]	N/A	–	–	
17D5C[a]	Yes	+	–	1 of 40 cells, small pore
17D5D[a]	Yes	+	–	1 of 40 cells, small pore
17D5E[a]	Yes	+	–	3 of 40 cells, small pore
17D5F[a]	Yes	+	–	2 of 40 cells, small pore
17C3A	Yes	N/A	–	
17C3B	N/A	N/A	–	
17C3C	Yes	N/A	–	
17C4A	Yes	N/A	–	
17C4B	N/A	N/A	–	
17C4C	Yes	N/A	–	
17C5A	N/A	N/A	–	
17C5B	N/A	N/A	–	
17C5C	N/A	N/A	–	
17C1A	No	N/A	N/A	
17C1C	Yes	N/A	N/A	

[a] These cultures represent the first six cells formed after a single cyst germination. Ordinarily, four are isolated, but an additional division occurred. Therefore, two of the isolates are identical to two others (probably C,D = E,F)

culture revealed that 3 of 59 thecae examined (5%) had a small ventral pore.]

Bioluminescence

Of 26 cultures from the regional survey examined for bioluminescence capacity, 18 were positive (Table 1). Four were *Alexandrium tamarense* and 14 were *A. fundyense*. Five of the eight non-bioluminescent isolates were *A. tamarense*. All but one of the northern isolates were bioluminescent, but those from Cape Cod and areas to the south were mixed.

Toxin composition

Individual *Alexandrium* isolates can produce a suite of neurotoxins in the family of compounds collectively called the saxitoxins. They include the parent compound, saxitoxin, and 17 derivatives (Hall et al. 1990) whose potency can vary by nearly two orders of magnitude. The term "toxin content" refers to the overall toxicity of a cell, the integrated potency of all toxins present (expressed as μg STX equivalents $cell^{-1}$). "Toxin composition" refers to the number and quantity of individual toxins that are present in an extract, typically determined using HPLC.

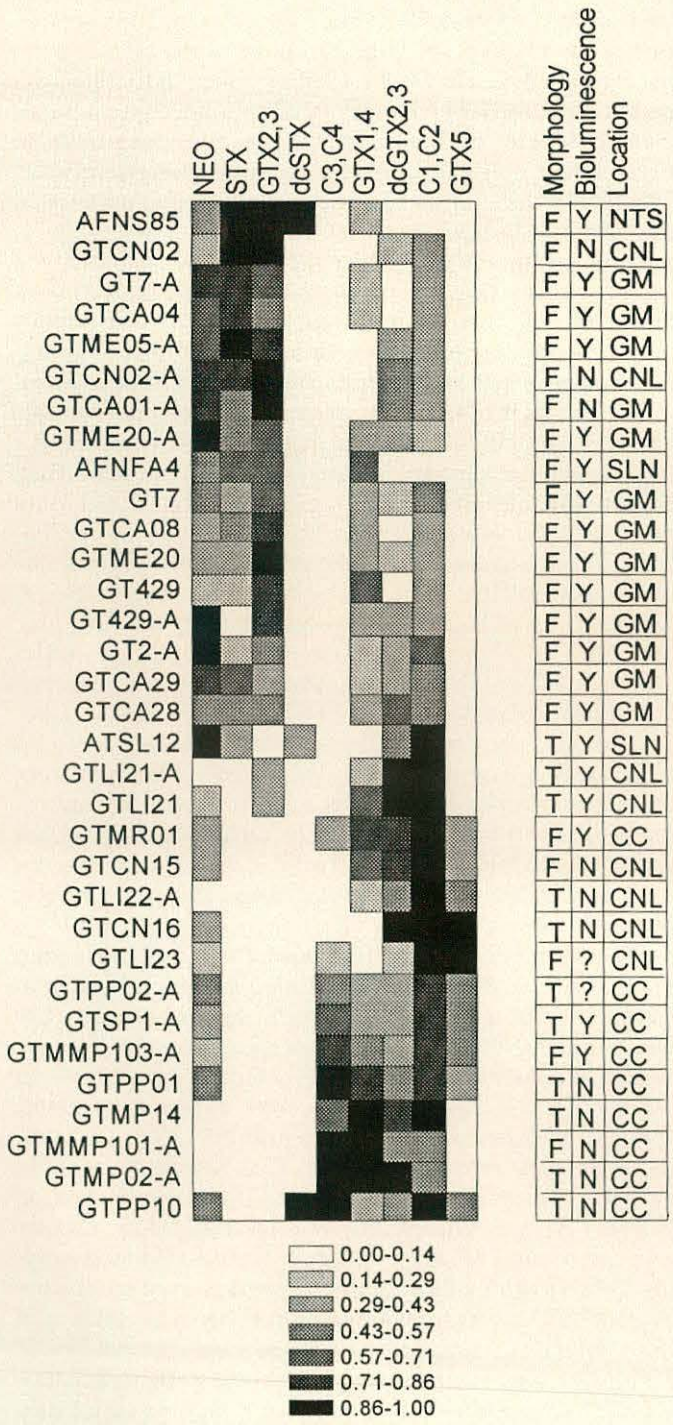
During this extended study, a number of *Alexandrium* isolates were lost. For many, preserved samples and toxin extracts were available, but it was not clear whether epimerization and evaporation during storage would introduce errors into their analyses. HPLC analysis of isolates which were still living, but also represented in the archived samples, demonstrated that storage of extracts in 0.05 *N* acetic acid at −20 °C caused changes in the relative abundance of each component of the epimer pairs GTX1,4, GTX2,3, C1,C2, and C3,C4. This effect, previously noted by Hall et al. (1990), does not alter the percent composition of the toxins if the concentrations of the epimer pairs are combined (i.e., as GTX1,4 rather than as individual toxins GTX1 and GTX4). However, when toxin content is calculated from the concentrations of the individual toxins in each isolate, epimeric conversions during long-term storage do introduce errors because of the different potency of the epimers (Oshima et al. 1989). Furthermore, evaporation during storage can reduce the volume of the archived samples, increasing the calculated molar concentration of the toxins and their overall toxicity. Analyses of non-archived and archived extracts demonstrated that evaporation had occurred in the stored samples (data not shown).

When five isolates were examined using both non-archived and archived samples in the cluster analysis, the two "duplicate" samples always grouped in the same cluster with the same major toxin signatures (Figs. 2 and 3). In two cases, the duplicates were each other's closest neighbors. The relative distances between archived and non-archived duplicates is indicated by the dashed lines in Fig. 3. Clustering analyses run on the non-archived samples alone revealed two main clusters with ATSL12 being an outlier (data not shown), just as was the case when non-archived and archived data were pooled for the analyses. PCA analyses of only non-archived samples also produced similar patterns to the combined analysis. Archived samples were therefore included in analyses that are based on relative toxin composition (i.e., clustering and PCA analyses), but were excluded from all that rely on absolute toxin concentrations (e.g. toxin content comparisons) due to the effects of evaporation.

The toxin composition of *Alexandrium* isolates varied dramatically over the region. Detailed, tabular information for all isolates is available from the senior author on request. Toxins C1 and C2 were present in most isolates, though proportions ranged broadly from 0.1 to 67.5% of the total toxin concentrations. With a few exceptions, southern isolates had high concentrations of C1,C2 compared to northern isolates. Toxins C3,C4 were detected in only ten isolates, all of which were either from Cape Cod or Long Island; concentrations were always low, usually < 1% of the total. GTX1,4 were present in all isolates, ranging from 5 to 46% of the total; no geographic patterns were evident. GTX5 was found in highest relative abundance in Long Island, Connecticut and Cape Cod isolates (10 to 30%), and in trace amounts (1%) in three nothern isolates. GTX2,3 varied from 0.5 to 49%, with highest concentra-

tions typically in northern isolates. The patterns for NEO and for STX were similar, with proportions ranging from 0 to 40 and 0 to 29%, respectively, and the highest concentrations in northern isolates. The decarbamoyl toxins dcGTX2,3 were present in very low concentrations in most of the isolates; dcSTX was found in three – one from the St. Lawrence, and two from Cape Cod.

The multivariate clustering matrix derived from these toxin composition data reveals two main clusters (Fig. 2).



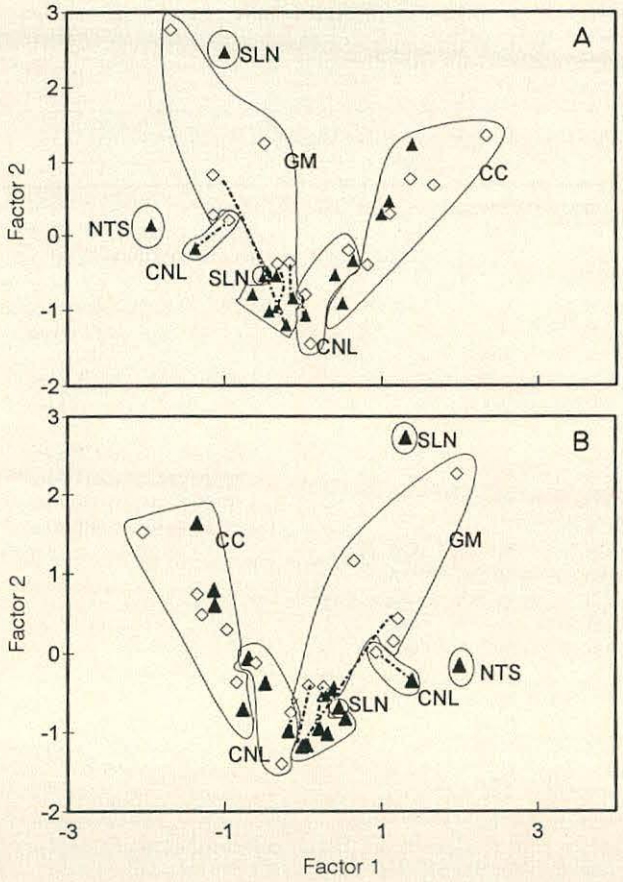


Fig. 3 *Alexandrium* spp. Principal components analysis for first two components for: **A** fmol of each toxin cell^{-1} and **B** fg saxitoxin equivalents cell^{-1}. Filled triangles represent values for fresh samples and open diamonds represent archived samples. Fresh and archived samples from same isolate connected by a dashed line. Groups of isolates from the same geographic location are surrounded by a continuous line. Abbreviations as in Fig. 2

Fig. 2 *Alexandrium* spp. Multivariate clustering matrix for toxin composition standarized using weighted ratio method (see "Materials and methods–Data analysis"). This multivariate clustering method simultaneously reveals the relationships of the isolates and the various toxins to each other and differs from more commonly used univariate clustering methods that only reveal the relationships of the strains. The figure is a three dimensional representation in which the most closely related isolates (on the basis of toxin composition) are nearest each other (rows) and the most closely associated toxins (columns) are nearest each other. Relative importance of a particular toxin in an isolate is indicated by shaded areas, with darkest shading representing the highest relative abundance. Clusters of isolates with similar toxin composition thus stand out visually. Right panel provides additional information on the isolates. (*F*–*A. fundyense*; *T*–*A. tamarense*; *Y*–Yes; *N*–No; ?–not known; *CC*–Cape Cod; *CNL*–Connecticut or Long Island; *GM*–Gulf of Maine; *NTS*–Nantucket Shoals; *SLN*–St. Lawrence Estuary.) Toxin abbreviations given in the "Materials and methods–Toxin analysis". Isolates with the suffix "A" analyzed using archived toxin extracts. Several isolates represented by both archived and fresh samples

The first is comprised of isolates with strong signatures for NEO, STX, and GTX2,3. With one exception (GTCN02), the isolates in this cluster originate in the Gulf of Maine or further north. All 17 members of the first cluster are *Alexandrium fundyense* and all but three are bioluminescent. Those three [AFNS85, GTCA01, and GTCN02, isolated from Nantucket Shoals (offshore of Cape Cod), southwestern Gulf of Maine, and Palmer Cove, Connecticut, respectively] share the same toxin profiles as the northern group and also have *A. fundyense* morphology.

The second main cluster is comprised of isolates with strong signatures for C1,C2, dcGTX2,3, and GTX1,4. These isolates all originated in Connecticut, Long Island, and Cape Cod. Both *Alexandrium fundyense* and *A. tamarense* morphologies are present, with *A. tamarense* being more common. Most, but not all, are bioluminescent. Cape Cod isolates also have strong signatures for C3,C4, and it is possible that these might belong to a third cluster that could be better resolved if larger sample sizes were available.

One isolate (ATSL12) had a unique profile containing both NEO and C1,C2 in relatively high proportions; it consequently did not fall into either main cluster. This isolate had *Alexandrium tamarense* morphology, was bioluminescent, and originated in the St. Lawrence estuary.

PCA was performed on the toxin composition data expressed as the molar content of each toxin and as STX equivalents (Figs. 3A,B). In the former analysis, the first two principal components account for 62.3% of the variance, and the first three account for 75.3%. With toxicity expressed as STX equivalents, the first two components account for 66% of the variance, with the third accounting for an additional 13%. For both analyses, the highest correlations (positive or negative) for the first principal component were STX, C3,C4, GTX2,3, and GTX5. For the second axis, they were C1,C2, and dcGTX2,3. The plots for the scores on the first two axes are very similar to the results of the clustering analysis (Fig. 2) and do not support an hypothesis of a simple north-to-south progression of traits. One isolate from Connecticut (GTCN02) and one from Newfoundland (AFNFA4) appear to be very similar to the Gulf of Maine isolates. The isolates from the St. Lawrence (ATSL12) and from Nantucket Shoals (AFNS85) are outliers. The isolates from Connecticut and Long Island have toxin signatures that are intermediate between the northern group and those from Cape Cod.

Toxin Content

When the molar concentrations of all toxins in an isolate are totaled, or if they are converted to STX equivalents, estimates of the total toxicity or toxin content are obtained. Among isolates, toxin content varied by nearly an order of magnitude (Table 1). The range was 25.5 to 187.9 fmol cell^{-1} (or 6199 to 58 695 fg STX cell^{-1}) in the non-archived toxin extracts. There was no apparent relationship between toxin content and species designation, as *Alexandrium fundyense* and *A. tamarense* were similar in toxicity, averaging 69.6 and 74.2 fmol cell^{-1} (or 25 718 and 20 758 fg STX cell^{-1}), respectively. When toxin content is plotted versus the latitude of the isolates' origins, a slight increase in toxicity from south-to-north is suggested (Fig. 4). A linear regression of toxin content (expressed as molar concentration) versus latitude was not significant at the 95% confidence level (data not shown). A regression expressing toxin content as STX equivalents cell^{-1} (Fig. 4) was significant ($P<0.05$).

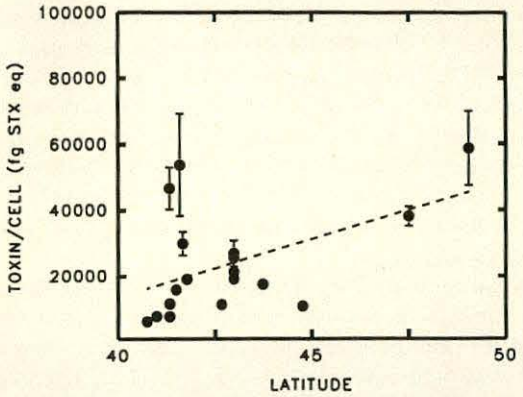


Fig. 4 *Alexandrium* spp. Toxin content of *Alexandrium* isolates as a function of latitude of origin. Toxicity determined from high performance liquid chromatography toxin composition analysis, with each individual toxin concentration converted to saxitoxin equivalents (STX eq) using the conversions listed in the text. Dashed line represents linear regression, correlation coefficient $r=0.49$. All samples analyzed in replicate; error bars denote 1 SD

Discussion

Genetic linkages between populations of closely related toxic dinoflagellates can be evaluated using several different characters. In this, and in a previous study (Hayhome et al. 1989), toxic dinoflagellates of the genus *Alexandrium* from the northeastern United States and Canada have been characterized on the basis of morphology, bioluminescence capacity, mating compatibility, allozyme banding patterns, and toxin composition. Of these, toxin composition provides the most useful insights into the genetic relationships between geographically separated populations. The other characters either provide no resolution at the regional scale under study here or add complementary information of use in interpreting the toxin composition results. The perspective on the PSP phenomenon that emerges is that the biogeography of toxic *Alexandrium spp.* in the study region is not that of a single, widespread, homogeneous population, but rather is comprised of several sub-populations, each with its own physiological characteristics and history. Mixing and genetic exchange among these populations may be infrequent.

Four discrete toxic *Alexandrium spp.* populations can be proposed on the basis of the timing of outbreaks, the

geographic separation between episodes of PSP, and an understanding of regional hydrography (Fig. 1B). These represent: (1) the lower St. Lawrence estuary, including the Gaspé peninsula, the western Gulf of St. Lawrence, and Newfoundland; (2) the Gulf of Maine, including the Bay of Fundy; (3) Cape Cod; and (4) Connecticut and Long Island. Further subdivisions are perhaps possible (e.g. Georges Bank), but these four provide a useful framework for analyses.

Sexual compatibility between *Alexandrium tamarense* and *A. fundyense*, demonstrated here for the first time, allows gene flow between these populations as long as hydrographic pathways facilitate intermixing. The extent to which this has occurred is difficult to ascertain, however. Our data suggest that exchange has been limited and reproductive isolation common. Below, we summarize the distributions of several morphological and physiological characters and use these to evaluate the validity of the proposed population separations and the extent of interchange. We also consider our data in light of the species designations assigned to our strains on morphological grounds and argue that *A. tamarense* and *A. fundyense* could be varieties of a single species and not separate species.

Morphotaxonomy

The taxonomy of the armoured, STX-producing dinoflagellates called the "tamarensis/catenella group" has long been contentious. There is agreement that these dinoflagellates should be in the genus *Alexandrium* and not *Gonyaulax* or *Protogonyaulax* (Steidinger and Moestrup 1990), but concerns about species assignments remain. Some believe the "tamarensis/catenella" group represents a single species complex comprised of numerous biochemically distinct varieties whose allozyme and toxin composition patterns do not demonstrate clear differentiation (Taylor 1985; Cembella and Taylor 1986; Cembella et al. 1987). An opposing view is held by those who differentiate approximately 30 species on the basis of small morphological features such as the shapes of thecal plates or the presence or absence of pores (Balech 1985). The isolates investigated in the present study were examined and initially categorized using these characters.

Some authors (e.g. Turgeon et al. 1990) have argued that the St. Lawrence populations might be a separate species called *Alexandrium excavatum*. Recently, however, one of us (E.B.) examined many natural and cultured specimens and concluded that it is possible to find examples of all possible transitions between *A. tamarense* and *A. excavatum*. On morphological grounds, *A. excavatum* cannot be distinguished from *A. tamarense* and therefore we use the latter, older name throughout this text.

There are only two morphospecies responsible for the production of PSP toxins within the region, i.e., *Alexandrium tamarense* and *A. fundyense*. The critical feature distinguishing the two taxa is the ventral pore on the margin between plates 1′ and 4′ in *A tamarense* that is absent in *A. fundyense* (Balech 1985). *A. tamarense* and *A. fundyense* are interspersed geographically though there are areas, such as the Gulf of Maine, where only one (*A. fundyense*) occurs. Southern waters are especially heterogeneous. Far to the north where our data are limited to two isolates from the St. Lawrence estuary and Newfoundland, both species are represented. Turgeon et al. (1990) and Cembella et al. (1988) report that *A. tamarense* (=*A. excavatum*) is the dominant morphotype in the lower St. Lawrence, but *A. fundyense* also occurs. The regional taxonomic distribution is thus a mixture of *A. tamarense* and *A. fundyense* in the St. Lawrence, Newfoundland area (apparently dominated by *A. tamarense*), a pure population of *A. fundyense* in the Gulf of Maine, and a mixture of the two species from Cape Cod southwards.

Mating compatibility

Crosses between isolates and within species produced hypnozygotic cysts approximately 50% of the time, whereas self-crosses never yielded cysts. This is the expected result for a heterothallic species when crosses are made of isolates with unknown mating types. The same rate of success was obtained with crosses between *Alexandrium tamarense* and *A. fundyense*, and the resulting cysts germinated to produce viable F_1 progeny. Additional crosses of F_1 cultures yielded cysts which were germinated to produce normal vegetative cells and viable cultures representing the F_2 generation. It is thus established that *A. tamarense* and *A. fundyense* are sexually compatible.

Mating experiments also provided information on the manner in which characters such as bioluminescence, the ventral pore, and toxin composition are inherited. Vegetative cells and gametes of most dinoflagellates are haploid (Pfiester and Anderson 1987), and thus the presence or absence of any trait in the progeny cannot be attributed to dominance. The most likely explanation for the absence of a pore in almost all of the progeny of a cross between *Alexandrium fundyense* and *A. tamarense* (Table 2) is that this apparently simple trait is a quantitative character whose expression is affected by more than one gene. This conclusion is also supported by the observation that the presence or absence of a pore was not always clear. In several cultures, a few cells could be found that had a "vestigial" pore while the vast majority of cells had no pore. Since the precise pattern of inheritance and numbers of genes involved is not known at present, it would be unwise to conclude that all matings between *A. tamarense* and *A. fundyense* will yield predominantly *A. fundyense* cells.

Bioluminescence

Loeblich and Loeblich (1975) used bioluminescence to separate species within the "*tamarensis*" group of *Alexandrium*, but this distinction was subsequently rejected (Schmidt and Loeblich 1979). Our results confirm this latter conclusion, since bioluminescent and non-bioluminescent strains of both *A. fundyense* and *A. tamarense* occur

(Table 1). Nevertheless, there is a geographic pattern to bioluminescent capacity within the region; virtually all of the northern isolates were bioluminescent, whereas southern isolates were mixed, with a slight bias towards non-bioluminescence. The underlying reasons for this distribution are unclear. Mating between two bioluminescent parents produced at least one daughter cell that was non-bioluminescent (Table 2). Crosses between bioluminescent and non-bioluminescent strains have not been attempted.

Toxin composition and content

A north-to-south trend of declining toxin content in *Alexandrium* species within our study region was first reported by Maranda et al. (1985) based on mouse bioassay results, and later augmented by Cembella et al. (1988) who provided HPLC toxin analysis of St. Lawrence isolates not represented in the former study. Our HPLC analyses further support the existence of a geographic trend in toxicity, though the relationship is weak. A regression between toxin content (expressed in total moles of all toxins per cell) and latitude was not significant ($P > 0.05$; data not shown), but when molar concentrations were converted to STX equivalents (i.e., potency), the regression against latitude was significant at the 95% level (Fig. 4). The correlation coefficient was only 0.49, whereas a similar regression by Maranda et al. (1985) of many of the same strains had a correlation coefficient of 0.82. A partial explanation for the difference is that five high toxicity, northern strains that Maranda et al. analyzed had died and could not be included in our study. We also included a new isolate from Nantucket Shoals, offshore of Cape Cod, which was very toxic relative to southern isolates which were predominantly of estuarine origin. If our study had included more strains from the northern Gulf of Maine or from the St. Lawrence, representatives of which have consistently been highly toxic (Cembella et al. 1988), the north-to-south trend in toxicity would likely have been stronger. Further resolution of this issue awaits toxin composition studies currently in progress on *Alexandrium* isolates from the St. Lawrence region (Cembella personal communication).

This north-to-south trend of declining toxicity, though only weakly evident in our data, is probably a characteristic of the regional population. It is not due to a higher intrinsic toxicity of *Alexandrium fundyense*, the predominant morphotype of northern isolates, since that species is, on average, similar in toxicity to *A. tamarense* (Table 1). Nor is it because northern isolates produce more moles of toxin, since a regression between latitude and toxin content expressed in moles was not significant. Rather, the trend arises because southern isolates produce proportionally more of the less-potent toxins than do northern strains (Fig. 2). The carbamate toxins that predominate within the northern isolates are much more potent (Oshima et al. 1989) than the sulfamate toxins that are in highest proportions in the southern strains. Given this distribution of toxin compositions, it follows that toxin content would decrease from north-to-south when it is determined by mouse bioassay or by HPLC and expressed in STX equivalents cell^{-1}, since both are measures of potency.

Cluster analysis and PCA of the toxin composition data both confirm the identification of at least two distinct groups of isolates. One is comprised almost exclusively of northern isolates (the exception being the Connecticut strain GTCN02), all of which are *Alexandrium fundyense*. The second cluster consists of southern isolates, which are a mixture of *A. tamarense* and *A. fundyense*. PCA analysis (Fig. 3) identifies two clear outliers: ATSL12 from the St. Lawrence Estuary and AFNS85 from Nantucket Shoals. The former strain has a toxin profile that overlaps with both the northerly and southerly groups, while the latter has a stronger signal for dcSTX than the rest of the isolates in the northern group (Fig. 2). PCA shows that isolates from Cape Cod are quite different from Connecticut and Long Island strains, which more closely resemble the northern group. A larger sample size would likely further delineate this Cape Cod cluster from the other two.

Biogeographic scenarios

Two alternate hypotheses can account for the distribution of characters among toxic isolates of *Alexandrium* in our study region. The first is a scenario of recent and continuing dispersal to the south from a center of origin in the north, followed by recombination and strong selection. The second hypothesis is that northern and southern populations diverged from a common ancestor (vicariance) but now represent localized populations, with relatively little mixing of genotypes among the regions delineated earlier. Neither hypothesis can be completely refuted by the data presented in the present study, but the weight of evidence leans towards the "local population/vicariance" hypothesis.

The "recent and continuing dispersal" hypothesis is consistent with: (1) retention of sexual compatibility among strains regardless of origin or morphospecies designation; (2) the north-to-south flow of coastal waters in this region; (3) the existence of high toxicity forms in the south (e.g. strains GTCN02 and AFNS85); and (4) the relative homogeneity of characteristics such as bioluminescence and morphology in the north compared to the heterogeneity in the south.

Under this scenario, the heterogeneity in southern populations could be accounted for by genetic recombination followed by selection and/or drift. The observed patterns would have evolved very rapidly, since 1972 was presumably the first major introduction event. However, selective forces capable of generating and maintaining this gradient in characters have neither been identified nor hypothesized. Another unappealing aspect of this hypothesis is that recombination and selection would have to maintain morphological and bioluminescent homogeneity in the northern populations but be relaxed in some way to allow heterogeneity in southern forms. Furthermore, ongoing mating studies between northern and southern isolates have yielded progeny with toxin composition patterns that are

generally equivalent to the parents' (D.M.A. unpublished data). Similar results were reported for *Alexandrium catenella* and for *A. tamarense* by Sako et al. (1992) and Ishida et al. (1993). Those workers demonstrated that simple Mendelian inheritance predominated and that on only one occasion, out of several dozen cyst germinations, did the progeny of a cross between strains with two different toxin compositions produce a suite of toxins different from either of the parents. If recent and continuing dispersal were occurring in our study region, matings between northern and southern populations should be yielding both low and high toxicity progeny in the south, yet the latter are very rare. The validity of this hypothesis therefore depends upon the existence of an additional type of environmental selection in southern waters – one that favors strains producing sulfamate toxins and is ultimately lethal to strains producing mostly carbamates. This is again a theoretical possibility, but its likelihood is questionable.

In the alternative "local population/vicariance" scenario, northern and southern populations would have been established from at least one common ancestor in the distant past and would have evolved more or less independently since their initial separation. Under this model, occasional dispersal from one area to the other could occur, but the populations would be generally isolated from each other. The high toxicity strain AFNS85 could be an example of such an occasional introduction. It was found in southern waters off Nantucket Shoals, an area to the east of Cape Cod influenced by flow from the Gulf of Maine, and possibly from the Scotian Shelf as well (Fig. 1 B).

The evidence in favor of this alternative hypothesis includes: (1) Cape Cod and Georges Bank act as hydrographic barriers to force the predominant north-south flow offshore, away from the estuaries and embayments where southern *Alexandrium* spp. cells and cysts are localized; (2) the toxin composition profiles of most Cape Cod isolates cluster together and are generally distinct from those of the Gulf of Maine and Connecticut/Long Island; (3) germination of Cape Cod *Alexandrium* spp. cysts is apparently regulated by a different mechanism than the internal clock that controls cysts in the Gulf of Maine (Anderson and Keafer 1987); (4) the geographic distribution of *Alexandrium* spp. populations has several large gaps, such as the area along the Scotian shelf between the St. Lawrence and the Gulf of Maine, or between Cape Cod and Connecticut (Fig. 1 B); (5) there was a report of *A. tamarense* (=*Gonyaulax tamarensis*) in Vineyard Sound, southern Cape Cod long before the massive 1972 New England red tide (Lillick 1937); (6) mating of parents with northern and southern toxin composition genotypes yields progeny resembling the parents (D.M.A. unpublished data), and strains with "northern" toxin composition are rarely observed in the south; and (7) localized southern populations of toxic *Alexandrium* spp. could have persisted for many years without detection because of their low toxicity.

Neither of these two biogeographic scenarios can be completely discounted, but we favor the "local population/vicariance" hypothesis since it does not require highly directional, unknown selective mechanisms and is consistent with all available data. Nevertheless, the correct story may well be a combination of both, with recent and continuing spreading from the north occurring within the Gulf of Maine (as clearly occurred during the 1972 New England red tide; Hartwell 1975) and possibly within the Gulf of St. Lawrence, but endemic, localized populations persisting without genetic exchange in most southern locations.

The species concept

The cumulative data on *Alexandrium* spp. from the present study and others illustrate the conflict between traditional classification and the needs of ecologists. Traditional taxonomy is based primarily on morphological traits and biogeographic distributions, and for phytoplankton, the most commonly used definition of a species is that group of individuals which maintains discrete, morphological features that are genetically fixed. There is no requirement that this be a physically large feature, only that it be discrete and constant. In this context, the stability of the ventral pore in culture (Balech 1985) argues that *A. fundyense* and *A. tamarense* are "good" species. Here we demonstrate that these two forms are sexually compatible, which might be interpreted to mean just the opposite, i.e., that they are not good "biological" species as originally defined by Mayr (1942). The retention of sexual compatibility in culture does not necessarily imply that genetic exchange is a regular event in nature, however, as compatibility can be a primitive trait retained by species which are not closely related, (e.g. hybridization; Costas 1986). Therefore, our mating results do not by themselves refute the classification of the two species of *Alexandrium* studied here as distinct taxa. A more serious challenge to their taxonomic separation as species comes from the analyses reported here and by Hayhome et al. (1989), all of which indicate that in the toxic *Alexandrium* spp., morphology is a poor predictor of the pattern of differences in biochemical or physiological traits such as allozyme banding, toxin composition, or bioluminescence. Even stronger evidence challenging the utility of morphospecies designations for ecological purposes in toxic *Alexandrium* spp. comes from the recent work of Scholin and Anderson (1994) on the ribosomal RNA genes in the "tamarense/catenella" group, which clearly demonstrates that isolates cluster together more logically on the basis of geographic origin that morphotaxonomy.

This discussion is not simply an academic exercise. Paradoxically, the impact of species designations falls most heavily on ecologists, who commonly assume that a species definition predicts a pattern of physiological differences (in our case, toxicity or bioluminescence) that can be used to define a niche. Surprisingly, this important assumption has rarely been tested quantitatively, as it has been here. Other quantitative studies of the issue for marine phytoplankton support our conclusion that species designations can sometimes be of little use for ecological purposes (Beam and Himes 1982; Gallagher 1986; Wood 1987; Brand 1991).

We recognize that the science of taxonomy seeks only to delimit stable groups of individuals that share common features (Manhart and McCourt 1992; Wood and Leatham 1992) and that there need not be other declared purposes, such as predictability or utility to other fields. To some extent, the problems of classification discussed here are artifacts of what ecologists and evolutionary biologists want species to "do" rather than they often "are". A reasonable solution is to reserve species-level designations for taxa where there is consistency among morphological, biochemical, molecular, and physiological data. Using this approach, the morphological variants identifiable as *Alexandrium tamarense*, *A. catenella* and *A. fundyense* might best be described as "varieties" in the botanical sense (Taylor 1976; Steidinger et al. 1980).

Designation of *Alexandrium tamarense* and *A. fundyense* as varieties would not solve the many problems inherent in their biology; it simply displaces them to a different level. It informs users of this classification that these entities are not completely isolated from each other and may not be stable. In taking this position, we recognize that recommending the use of trinomials is not risk-free. Many ecologists and systematists are reluctant to use infraspecific names and they are often dropped. In the case of the *Alexandrium* species, dropping the varietal designation completely would result in a genuine loss of information.

The studies on *Alexandrium* spp. represent one of the most complete data sets on the patterns of species and population-level genetic diversity in any protist. When these data are combined with more fragmentary compilations for other species, they indicate that many assumptions inherent in field and laboratory investigations of "species" by phytoplankton ecologists may be incorrect. The problems of speciation and genetic diversity at lower taxonomic levels in protists need further study in order to determine if this conclusion is correct.

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