

Probable origin and toxin profile of *Alexandrium tamarense* (Lebour) Balech from southern Brazil

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Abstract

The distribution of the toxic dinoflagellate *Alexandrium tamarense* Lebour has apparently expanded within the southern hemisphere during the last 2 decades. Toxic blooms of *A. tamarense* were recorded in Argentinean coastal waters since 1980; however, the first documented bloom in southern Brazil was in 1996. In this study, 13 strains of *A. tamarense* from southern Brazil were isolated and kept in culture. Phylogenetic analysis using RFLP and DNA sequences of the D1–D2 region of large subunit ribosomal DNA (rDNA) clearly indicates that Brazilian strains are most closely related to other South American strains. The strains from South America are placed firmly within a phylogenetic clade which contains strains from North America, northern Europe and northern Asia, previously called the North American clade. Possible dispersal hypotheses are discussed. The cultures were also analyzed for saxitoxin and its derivatives by high performance liquid chromatography (HPLC). The main saxitoxin groups found were the low toxicity *N*-sulfocarbamoyl group, C1, 2 (30–84%), followed by the high potency carbamate toxins, gonyautoxins 1, 4 (6.6–55%), gonyautoxins 2, 3 (0.3–29%), neosaxitoxin (1.4–24%) and saxitoxin (0–4.4%). The toxin composition is similar to that of other strains from South America, supporting a close relationship between *A. tamarense* from southern Brazil and other areas of South America. Toxicity values were variable (7.07–65.92 pg STX cell⁻¹), with the higher range falling among the most toxic values recorded for cultures of *A. tamarense*, indicating the significant risk for shellfish contamination and human intoxication during blooms of this species along the southern Brazilian coast.

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1. Introduction

Some decades ago, the occurrence of PSP-producers (especially the genus *Alexandrium* Halim) were largely restricted to the northern hemisphere, mainly in North America and Asia (reviewed by Balech, 1995; Shumway, 1990; Taylor, 1985). These

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Fig. 1. Map of South America with the first record of *Alexandrium tamarense/catenella* at each location.

organisms have since been reported in a number of southern hemisphere locations, including Australia, New Zealand (Hallegraeff, 1993) and South America. Historical records for the South American *tamarense* complex species (*Alexandrium tamarense*/*A. catenella*/*A. fundyense*) and PSP occurrence are summarized in Fig. 1. The range of the *tamarense* complex appears recently to have spread around the southern tip and northward along the east coast of the continent. The earliest PSP outbreak recorded in 1886 in Chile was associated with *A. catenella* (Sengers, 1908), the next outbreak was reported in 1972 (Guzmán et al., 1975). In Argentina, the first toxic outbreak of *A. tamarense* occurred in Peninsula de Valdés in 1980 (Carreto et al., 1985) and since then this species has been periodically detected along the Argentinean coast. In Uruguay, the first PSP outbreak also was recorded in 1980 (Davison and Yentsch, 1985), but the conclusive identification of *A. tamarense* as the

causative species was not possible until the second toxic outbreak in 1991 (Brazeiro et al., 1997). Along the southern Brazilian coast, *A. tamarense* was recorded for the first time in 1996 (Odebrecht et al., 1997), at concentrations up to 2×10^5 cells l^{-1} . It was also observed at the same time in Uruguayan waters, 300 km south, at lower concentrations.

The continuing outbreaks of PSP in many areas and the appearance of toxicity in others motivated intensive studies of *A. tamarense* all over the world. However, some doubts still exist about the exact taxonomic position of the species within the *tamarense* complex. The distinctions between these species are mainly based on the presence or absence of a ventral pore on the 1' apical plate, overall cell shape and the ability to form chains (Balech, 1995). Besides morphological identification, several methods have been used to identify these species, such as sequencing of the ribosomal RNA gene and high performance liquid chromatography (HPLC) toxin analysis (Scholin et al., 1994; Cembella and Taylor, 1985; Anderson et al., 1994). But once again, results obtained using these methods are not always in agreement. An example of this is from the work of Scholin et al. (1994), who show that populations of different species from the same area are more similar genetically than geographically separated populations of the same morphologically defined species or morphotype.

To resolve geographical populations or ribotypes of the *tamarense* complex and provide fine scale population resolution, genetic analysis using restriction fragment length polymorphism (RFLP) of large subunit (LSU) ribosomal DNA (rDNA) sequences can be employed (Scholin and Anderson, 1996; Scholin et al., 1994). The isolates that appear to be "out of place", such as strains from the Thau Lagoon in France which show high affinity with strains from Japan and Asia (Lilly et al., 2002) suggest that recently human assisted dispersal may have occurred.

In this study, we explore possible origins of the *A. tamarense* population from southern Brazil through analysis of 13 *A. tamarense* strains (morphological classification) from the southern coast, using both genetic analysis and toxin profiles as well as historic accounts. We compare the RFLP patterns and DNA sequence data generated from our strains with those published by Scholin et al. (1994) and Scholin and Anderson (1996) and compare the toxin profiles with

other regional populations, including Uruguayan strains.

2. Methods

2.1. Origin of the strains

Sediment sampling was conducted with a gravity corer when possible or a Van-Veen dredge when conditions were turbulent. Sampling occurred from March to December 1997 and in March 1999. The sampling site was a muddy area close to Patos Lagoon Estuary mouth between 32°10' and 32°22'S and between 51°51' and 51°55'W, with water depth from 5 to 30 m (Fig. 2). Twelve clonal strains were produced by cyst isolation, germination and re-isolation of individual motile cells. Strain C6 was isolated directly from a vegetative cell collected in the water column on August 1997. All cultures were grown in 50 ml test tubes containing 25 ml of f/2 medium without silicate (Guillard and Ryther, 1962, as modified by Anderson et al., 1994) at 20 °C under a 14-h light:10-h dark cycle and ca. 250 $\mu\text{E m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent tubes. Cultures were harvested for toxin and genetic analyses during the mid-exponential growth phase.

The identification of motile cells was confirmed by detailed thecal plate observation (stained with calco-

fluor) under fluorescence microscopy on either live or recently fixed cultures. Cyst identification was performed using both morphological characteristics like shape, size and color and by observation of the germinated motile cells.

Cyst concentrations in the top 2–3 cm of sediment were estimated using aliquots of 1 cm^3 according to Yamaguchi et al. (1995). Cysts were stained with calcofluor white MR2 and counted in a Sedgwick-Rafter chamber under an epifluorescent Nikon Axioplan microscope at 100 $\times$ magnification.

2.2. Genetic analysis

2.2.1. DNA extraction

Samples (~150 ml) of each culture were harvested by gentle centrifugation and immediately frozen in liquid nitrogen until treatment according to modifications of the method developed by Adachi et al. (1994). The resulting pellets were resuspended in deionized water and the DNA concentration was measured using UV absorbance in a 1 cm path quartz cuvette and reported in $\mu\text{g l}^{-1}$.

2.2.2. Polymerase chain reaction amplification

The D1–D2 region of LSU rDNA was amplified using modifications of the method in Scholin and Anderson (1994). Primers D1R (5'ACCCGCTGAAT-ACCCGCTGAATTTAAGCATA3') and D2C (5'CC-CCTTGGTCCGTGTTTCAAGA3') were used and the PCR reaction was performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Each amplification reaction contained primers at a final concentration of 0.1 μM , 0.4 mM of dNTPs and 3.75 units of Taq polymerase from Perkin-Elmer, the PCR buffer supplied with the enzyme and 12 ng of each template. The success of each PCR reaction and the approximate product concentrations were verified using a 1.2% agarose gel and a low mass molecular weight ladder (Gibco BRI).

2.2.3. Digestion reaction with restriction enzymes

The restriction digests were performed according to modifications of the protocol used in Scholin and Anderson (1996), with the enzymes *Nsp* I, *Mse* I and *Apa* LI (from New England BioLabs). A master mix was prepared using the buffers supplied with each enzyme and BSA according to manufacturer's specifications. Approximately, 500 ng of PCR product was

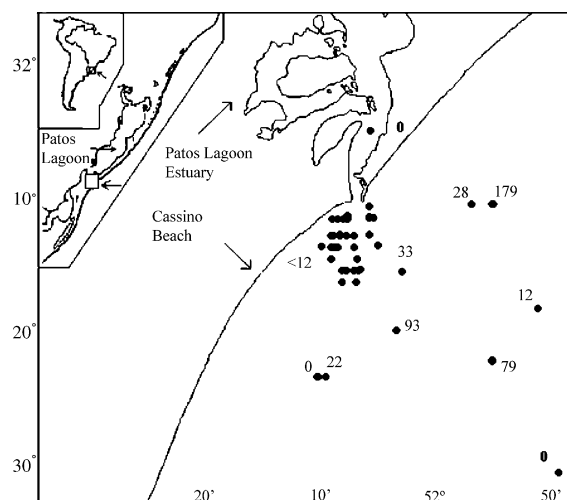


Fig. 2. Sampling area showing cyst concentration in the upper sediment layer (cyst cm^{-3}).

mixed with the master mix in a 50 μ l reaction and incubated for 2 h at 37 °C. Then, 40 μ l of each digestion for *Mse* I and 20 μ l for *Apa* LI and *Nsp* I was mixed with DNA loading dye at a 1:4 ratio. The digestions were run for approximately 1.25 h at 90 V on a 3% agarose gel (1 low EEO: 3 Nusieve, 1 $\times$ TAE buffer and ethidium bromide). Markers (BioMarker Low) were used to determine the size of the DNA fragments. Photographs of the gel runs were taken using an instantaneous Polaroid camera and ISSO 3000/36, 667 film in a UV system. RFLP patterns from our strains were compared to published data (Scholin and Anderson, 1996) and unpublished data generated by one of the authors (Lilly) for strains from Chile, Argentina and Uruguay.

2.2.4. Sequencing analysis

The entire D1–D2 region was sequenced for four strains, 2c–2f. To compare the sequences generated by these strains with others from South America, they were aligned with the sequences of two Uruguayan and two Chilean isolates (Lilly, 2003). Duplicate sequences from non-Brazilian cultures were removed and existing *Alexandrium* sequences from Scholin et al. (1994) were added to the alignment for phylogenetic analysis. The most appropriate substitution model was determined using ModelTest. Using Paup, 1000 replicates of maximum parsimony analysis were run to generate starting trees for maximum likelihood analysis. One hundred replicates of fast-step bootstrap were run using maximum likelihood.

2.3. Toxin analysis

Triplicate samples of each of the 13 strains were grown to mid-exponential phase. Three cell count samples were harvested and fixed with Utermöhl's solution for average cell concentration estimates. An aliquot (15 ml) of each culture was concentrated for toxin analysis via centrifugation (1700 $\times$ g for 5 min), the overlying seawater aspirated and 0.05 M acetic acid added to the cell pellet. Samples were sonicated with a Braun-Sonic 2000 sonifier at 10 W for 40 s while kept on ice and stored at –20 °C. Toxin analyses were carried out using a modification of the Oshima (1995) post-column derivatization HPLC method (Anderson et al., 1994). Unknowns were standardized with solutions containing toxins C1–C4, GTX1–5, NEO, dcSTX and STX provided by Oshima (Tohoku University, Sendi, Japan). Toxin content values were expressed as the molar concentration per cell and the toxicity values, in saxitoxin equivalents, were converted from the molar concentrations using the factors in Oshima (1995).

3. Results

The *A. tamarense* motile cells (Fig. 3A) showed an almost isodiametric shape, transdiameter from 30 to 50 μ m, total length from 40 to 50 μ m and usually were single cells or no more than two cells in chains.

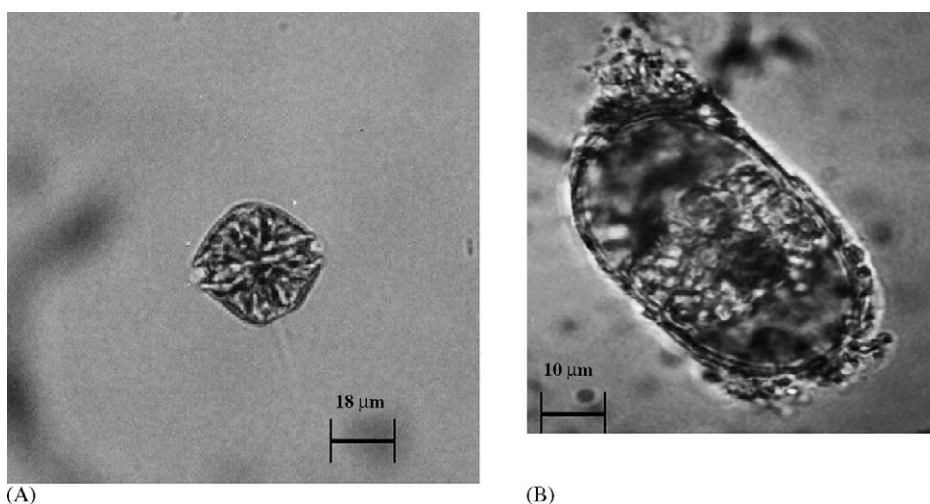


Fig. 3. *Alexandrium tamarense* from southern Brazil: (A) motile cell isolated from water column and (B) resting cyst isolated from sediment.

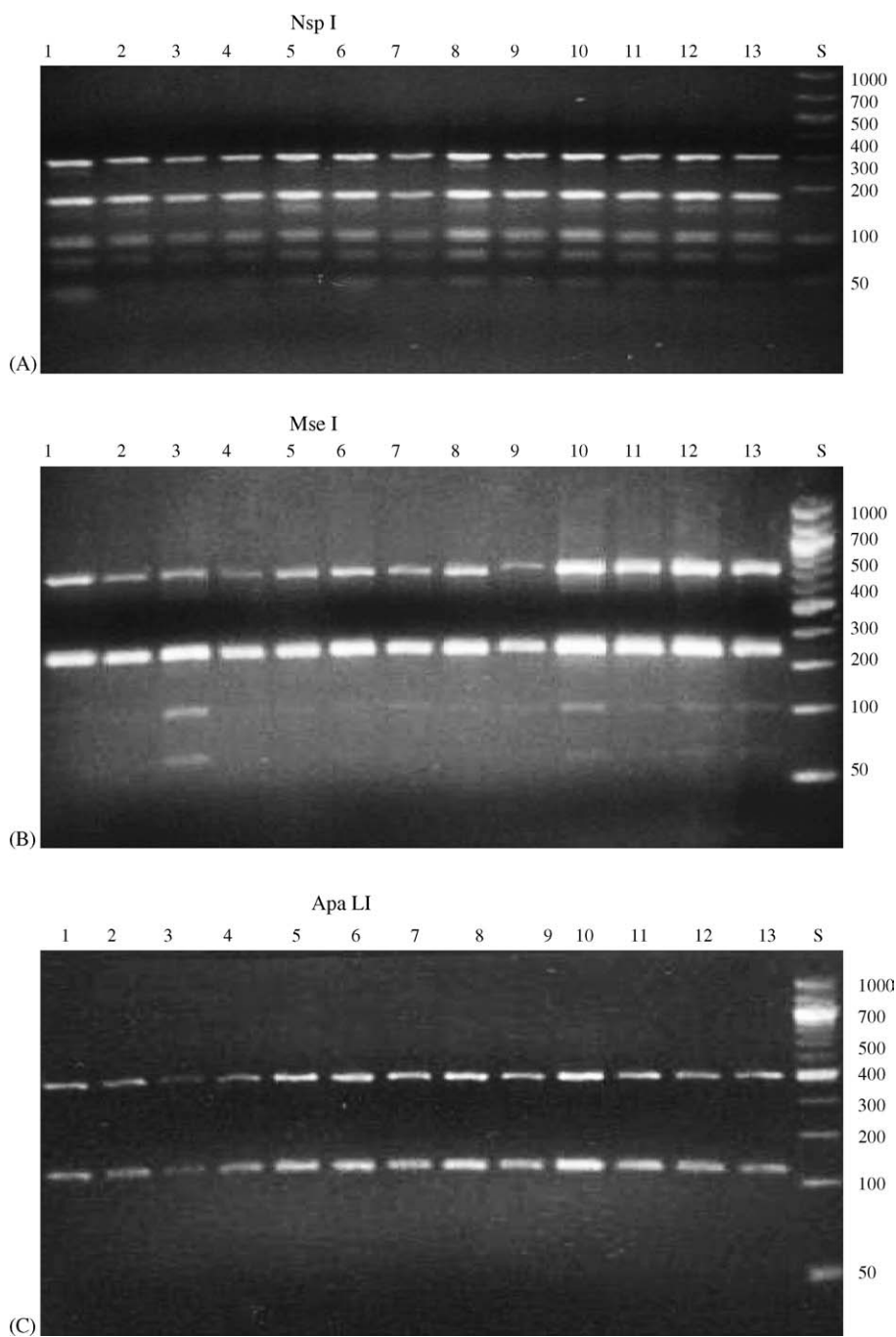


Fig. 4. Agarose gel with the restriction patterns obtained by digestion of D1–D2 LSU rDNA PCR products of 13 strains of *A. tamarensis* from southern Brazil, with the enzymes: (A) *Nsp* I, (B) *Mse* I and (C) *Apa* LI. 1, 2a; 2, 2b; 3, 2c; 4, 2d; 5, 2e; 6, 2f; 7, 2g; 8, 3a; 9, 3c; 10, 3d; 11, 1a7; 12, 1a10; 13, C6; S, molecular weight standard (bp).

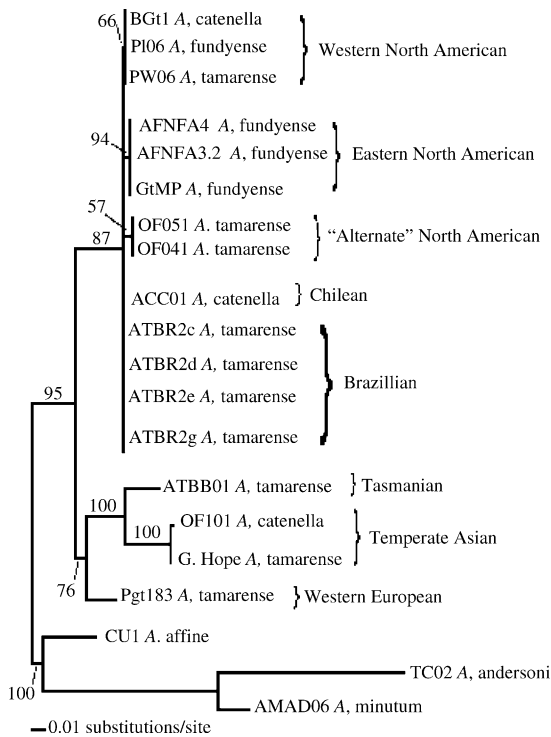


Fig. 5. Maximum likelihood tree showing the phylogenetic placement of the Brazilian strains within the *tamarensis* complex. Numbers represent 100 replicates of ML bootstrap.

The cysts from sediment samples were oval in shape, length from 32 to 52 μm and width from 20 to 32 μm (Fig. 3B). A mucilaginous cover was usually observed and the cytoplasm showed different degrees of vacuole development with one or two red bodies inside. Thecal plate analysis was consistent with the designation of *A. tamarensis* (Balech, 1995).

3.1. Cyst distribution

Cyst concentrations varied between undetectable values and 179 cysts cm^{-3} at the northernmost station. The higher values were usually found in deep areas, whereas the lower concentrations were associated with exposed areas and sites closer to the shore (Fig. 2). These concentrations are substantially lower than concentrations found in Argentina, which are up to 9000 cysts cm^{-3} (Orozco and Carreto, 1987).

3.2. Genetic analysis

RFLP analyses on the 13 strains were inconclusive. They indicated that the western North American and the eastern North American ribotypes, as defined by Scholin and Anderson (1996) might both be represented (Fig. 4A–C). Strains from Chile, Argentina and Uruguay displayed the western North American ribotype pattern (Lilly, 2003), therefore, sequence analyses were run on newly isolated material from four isolates that displayed the full range of patterns to clarify this issue. The sequences were identical with one another. The Brazilian sequences had several base changes in common with each of the western, eastern and alternate North American ribotypes published by Scholin et al. (1994), but did not possess all of the base changes common to any of the groups. The sequences from the Brazilian strains were identical to those from Uruguay while the sequences from two Chilean strains differed by only a single base change and the insertion of an extra “TA” repeat (data not shown). Phylogenetic analysis placed the Brazilian strains firmly within the “North American” clade, but not within any existing subclade (Fig. 5).

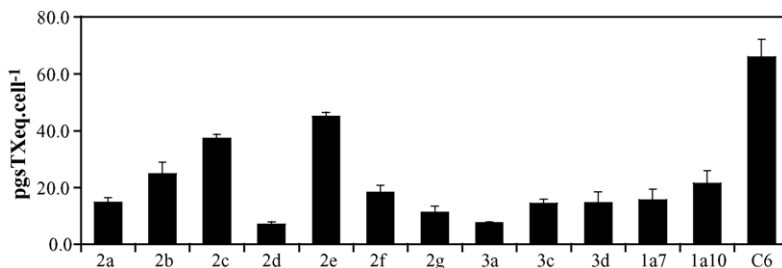


Fig. 6. Toxicity of *A. tamarensis* strains from southern Brazil.

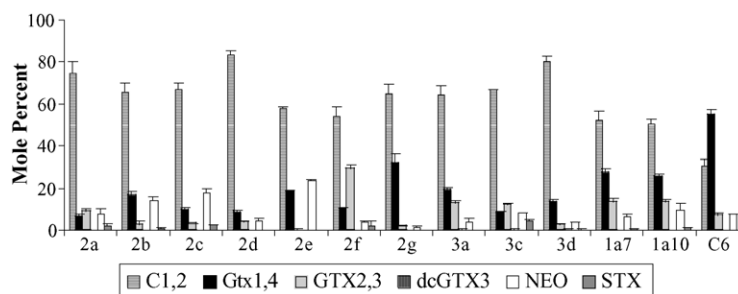


Fig. 7. Toxin profile of *A. tamarensis* from southern Brazil. C1, 2, toxins C1 and C2; GTX1, 4, gonyautoxins 1 and 4; GTX2, 3, gonyautoxins 2 and 3; dcGTX3, decarboxyl gonyautoxin 3; NEO, neosaxitoxin; STX, saxitoxin.

3.3. Toxin analysis

Total toxin content ranged from 42 to 199 fmol cell⁻¹ (data not shown) and toxicity from 7.07 to 65.92 pg STX eq cell⁻¹ (Fig. 6). Toxins C1, 2 were predominant in most of the strains, comprising up to 78.4% of the total toxin (Fig. 7). The only exception was strain C6, whose higher toxicity value was due to the high proportion of the potent GTX4 toxin (50% of the total).

4. Discussion

The occurrence of the “North American” RFLP pattern (Scholin and Anderson, 1996) in *A. tamarensis* from Brazil suggests a close relationship with strains from the northern hemisphere that also display this pattern, which include all *A. tamarensis*, *A. fundyense* and *A. catenella* from North America (Scholin et al., 1994), toxic *A. tamarensis* from northern Europe (Medlin et al., 1998) and some *A. tamarensis* and *A. catenella* from northern Asia (Scholin et al., 1994). The South American strains are not closely related to others of the *A. tamarensis* complex from the southern hemisphere, including *A. tamarensis* and *A. catenella* strains from Australia or South Africa which yield different RFLP patterns and DNA sequence data (Scholin et al., 1994; Scholin and Anderson, 1996; Lilly, 2003). It is interesting to note that these populations are discontinuous and that these temperate strains of *Alexandrium* would be unlikely to survive in the intervening tropical waters.

The origin of the “North American” type *Alexandrium* in South America is thus obscure, as

natural transport between the northern hemisphere and South America in modern times appears unlikely due to strong physical barriers including temperature. While the current data do not allow us to conclusively choose between them, we suggest two scenarios that could account for the current biogeographical patterns. One possible explanation could be that *Alexandrium* cells were transported to South American waters in the ballast water of cargo vessels from a region in which closely related populations occur, including the North American or Japanese coasts. Another possibility is that the *Alexandrium* populations in South America were established much earlier, during a period of cooler global oceans when natural transport across the hemispheres was possible, such as the last ice age. DNA sequence data support this hypothesis. The South American LSU rDNA sequences are distinct from sequences obtained from modern populations of the Asian, western North American and eastern North American populations of the North American ribotype, indicating that the populations have been separated long enough for evolution to have occurred.

By either means, it is hypothesized that *tamarensis* complex cells first arrived in South America along the western coast and then spread to eastern South America via oceanic current systems. This is supported by the historical precedence of PSP in Chile, but not in other South American countries (Sengers, 1908; Guzmán et al., 1975). PSP outbreaks subsequently occurred in Argentina, Uruguay and most recently, southern Brazil (Carreto et al., 1985; Davison and Yentsch, 1985; Brazeiro et al., 1997; Odebrecht et al., 1997). Experimental and field observations also support this hypothesis. First,

Table 1

Comparison of the toxicity and toxin profiles (mole %) of *Alexandrium tamarense* strains from southern Brazil and Uruguay

Location	Strain ID	Toxicity (pg STX eq cell)	Toxin profile (mole%)						
			C1,2	GTX1,4	GTX2,3	dcGTX3	NEO	dcSTX	STX
Brazil	ATBR2a, 2b, 2c, 2d, 2e, 2f, 2g, 3a, 3c, 3d, 1a7, 1a10, C6	7.07–65.92	30–85	7–55	0.3–29	0–0.3	1–24	0–1	0–4
Uruguay	ATUR01A, 01B, 02	10.00–26.00	48–67	2–46	3–24	0	1–11	0–1	1–4

Brazilian strain sequences are identical to those from Uruguay, indicating that these strains may represent a continuous population. The strains from Chile differ only slightly and are the closest relatives of the Brazilian/Uruguayan group. (this study; Lilly, 2003). There is also a correlation of *Alexandrium* populations and the presence of coastal fronts in both Argentina and Uruguay (Carreto et al., 1986 and Brazeiro et al., 1997). Satellite temperature data from 1996, when *A. tamarense* was first seen in Brazil, showed a cold-water front traveling northward from Uruguay to Brazil, which may have been a vector for *Alexandrium* transport. The high similarity found between Brazilian and Uruguayan *Alexandrium* toxin profiles (present study and Mendéz et al., 2001) (Table 1) supports a Uruguayan origin for the Brazilian *Alexandrium* strains. Both sets of strains presented a higher content of *N*-sulfocarbamate toxins C1, 2, followed by gonyautoxins 1, 4, gonyautoxins 2, 3 and neosaxitoxin, respectively. The toxicity values of the Brazilian strains were variable and some of the cultures were more potent than the Brazilian cultures but the magnitude of toxicity values were comparable.

The low cyst concentration in Brazilian sediments may reflect the very recent spreading of the cells from Uruguay in 1996. However, in this study, the sampling sites were limited to relatively shallow areas with high rates of sediment deposition from Patos Lagoon, which could dilute cyst concentrations. It should be noted that the use of calcofluor white MR2 versus the more effective primuline stain, may have underestimated the cyst density by a factor of two (Yamaguchi et al., 1995). It is probable that the deepest muddy sediments, mainly in the area close to Uruguay, have much higher cyst concentrations than the values found in the present study.

Although cyst concentrations were relatively low in the sediment samples analyzed, the cultures derived

from these cysts and vegetative cells collected in the environment can be quite toxic. The highly toxic culture (C6) assayed in this study (max. 65.92 pg STX eq cell⁻¹) is among the most toxic strains known for this species. Although toxin analysis was not performed when *A. tamarense* was first detected in southern Brazil in 1996, the toxicity levels presented here, coupled with and the high cell densities on that occasion (2×10^5 cell l⁻¹; Odebrecht et al., 1997), suggest that toxin concentration in the filter feeders was probably far over the safety limits. Additionally, in laboratory experiments, Brazilian *A. tamarense* strains exhibited high growth rates of up to 0.7 div day⁻¹ and a tolerance to a wide range of environmental conditions (Persich, in preparation) indicating the potential for *Alexandrium* blooms under a variety of circumstances. Therefore, it is possible that the southern Brazilian coastline may experience additional outbreaks of PSP, originating from localized cyst germination or from new populations that are transported from Uruguay via coastal currents.

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