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The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone

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Abstract

Toxin production has always been considered a constitutive characteristic of dinoflagellates in the genus *Alexandrium*. Here we demonstrate that saxitoxin production can be lost by an *Alexandrium* species during routine culture maintenance. This is the first report of any marine saxitoxin-producing alga ever to have completely lost the ability to produce toxins. A clonal toxic isolate of *Alexandrium lusitanicum* from Portugal has been maintained in culture since 1962. In 1992, a subculture was established and sent to a different laboratory. Recent comparisons of the parental strain and the subculture revealed that the former had lost its toxicity, whereas the latter still produces saxitoxins. This loss of toxicity was confirmed by three independent toxin detection methods: mouse bioassay, mouse neuroblastoma assay and HPLC. Sequence analyses of different rRNA domains demonstrated that the toxic and non-toxic cultures are genetically identical for those markers. Morphological analysis showed that both cultures have the same plate tabulation and are *A. lusitanicum*. These results strongly argue that the loss of toxicity is not a result of a culturing artifact or mistake, such as mislabeling or contamination. The clonal cultures also show a significant difference in growth. Possible explanations for the change include genetic mutations or the effects of prolonged treatment of the non-toxic culture with antibiotics.

Keywords: *Alexandrium*; Loss of toxin production; Paralytic shellfish poisoning; Saxitoxin

1. Introduction

Species within the dinoflagellate genus *Alexandrium* have been associated with paralytic shellfish poisoning (PSP) episodes in coastal waters throughout the world, from boreal to tropical latitudes. The saxitoxins, a family of potent neurotoxins, are produced by some of these species in varying amounts and combinations, leading to highly variable intrinsic potencies (Cembella, 1998). To date more than 20 saxitoxin derivatives that differ considerably in toxicity have been identified (Cembella, 1998; Gallacher and Smith, 1999), and numerous *Alexandrium* isolates have been characterized with respect to their toxin profile or composition (i.e. the suite of toxins produced).

Under normal growth conditions, a species or strain produces a consistent or characteristic toxin profile and toxin content (total toxicity) (Hall, 1982; Cembella et al., 1987; Anderson, 1990; Anderson et al., 1994). However, these and other studies have documented variation in toxin content (Anderson et al., 1990a; Anderson, 1990; Mascarenhas et al., 1995) and toxin composition (Anderson et al., 1990b; Franco et al., 1995), typically following sustained nutrient deprivation and adaptation. These variations reflect differences in a strain's growth conditions, such as nitrate and phosphate concentrations (Anderson et al., 1990c) or salinity (Flynn et al., 1996). Regardless of the variations in toxin content and composition in a strain, toxin production (or the lack thereof) is considered a highly stable

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characteristic of *Alexandrium* strains, i.e. isolates always produce some toxin, or none at all, but never switch between these two conditions. Although some strains kept in culture are reported to suffer reduction in toxin content (Hansen et al., 2003) the total loss of the ability to produce saxitoxins has never been observed to date.

The constitutive nature of toxin production seems to be true for other toxic marine algae, although there are a few exceptions. There are, for example, no reports of the total loss of brevetoxin toxicity in *Karenia brevis* cultures, but one strain apparently lost its ability to produce one of the brevetoxin derivatives while in culture (R. Pierce pers. comm.). For the diatom *Pseudo-nitzschia multiseries,* there are times during the growth cycle (exponential growth), when a strain will produce no domoic acid, but that same strain will produce toxin when growth slows or ceases in stationary phase (Bates, 1998). In this instance, the capability to produce toxin has not been permanently lost-toxin synthesis has simply been turned off during a particular growth phase.

One protist that can potentially alternate between toxic and non-toxic forms is *Pfiesteria piscicida*. For example, when toxic zoospores of *P. piscicida* are grown in culture with algae as food, they gradually lose their toxicity (Burkholder and Glasgow, 1997). Springer (2000) reports that some zoospores have residual levels of toxicity, and that full toxicity can be re-established by growing the now non-toxic zoospores on fish prey. Other workers report cases in which the non-toxic zoospores remain uninducible (Stoecker et al., 2002). These results suggest that toxin production can be lost in this species, but until the *Pfiesteria* toxin is chemically identified and analyzed, it is premature to state that toxin production is completely lost.

A culture of *Prorocentrum minimum* may represent another exception to the tendency towards constitutive toxin production. In a study by Sousa Silva (1990), bacteria previously isolated from a toxic *Gonyaulax tamarensis* (= *Alexandrium tamarense*) culture were added to a non-toxic clone of *P. minimum*. The inoculated clone showed some toxicity (as evidenced by mouse bioassay) while the controls remained non-toxic. Similar results were obtained for two clones of *Gyrodinium instriatum*, leading Sousa Silva to conclude that toxicity could be elicited in clones by adding the *A. tamarense* bacteria. Although this seems to be a case of de novo toxin production, the type of toxin produced was not defined and may not have even been saxitoxin. Furthermore, these results have not been successfully repeated by others.

Considering all available data, the ability to produce PSP toxins has heretofore been demonstrated to be a constitutive, highly stable characteristic in marine algae in general, and, with no exception, in the genus *Alexandrium*. For the first time, here we report the total loss of toxicity of a clone of *Alexandrium lusitanicum*.

2. Materials and methods

2.1. Culture history

The original culture of *A. lusitanicum* was isolated in 1962 from a bloom in Lagoa de Obidos, Portugal and given the strain designation 18-1. That strain has been maintained at the Laboratório de Microbiologia e Ecotoxicologia in Lisbon ever since, using Provasoli's ASP7 medium with bimonthly addition of AM9 antibiotic mixture every two new transfers (Silva and Sousa, 1981). AM9 is a mixture of polymixin B sulfate, dihydrostreptomycin, tetracycline, chloramphenicol, penicillin G and neomycin. In 1992, a subculture was established and sent to Dr Greg Doucette's Laboratory. The culture was kept in f/2 media (enriched Vero Beach, Florida seawater) with no further antibiotic treatment. Salinity was kept at 30 psu, similar to the ASP7 medium. We refer to this subculture as 18-1T since it retains all of the characteristics of the parent culture isolated in 1962. At some unknown point between 1995 (Franca et al., 1995; Mascarenhas et al., 1995) and 2000 (Pereira et al., 2000), the 18-1 culture maintained at the Laboratório de Microbiologia e Ecotoxicologia became non-toxic. For discussion purposes, this culture is now referred to as 18-1NT to distinguish it from the parent culture 18-1 and the other subculture 18-1T.

2.2. Growth

Both 18-1T and 18-1NT were grown in identical conditions for at least 2 years before performing the experiments reported here. Cultures were maintained in f/2 medium (Guillard and Ryther, 1962) at 20 ± 1 °C on a 16:8 h L:D cycle and transferred in mid-exponential phase. The growth of each culture was followed by periodic cell counts with a Sedgwick-Rafter counting chamber. All counts were made in triplicate. Additionally, a second set of cultures was established simultaneously in f/2 and in ASP7 media (Provasoli, 1963), which is an artificial seawater-based culture medium. Growth in ASP7 was monitored following the same conditions and quantification methods described for f/2. Separate cultures were grown for toxin analysis, morphology and sequencing (see below).

2.3. Morphology

The plate morphology of each of the two subcultures was studied by calcofluor staining (Fritz and Triemer, 1985). Three ml of mid-exponential phase cultures were fixed in 5% v/v formalin, treated with calcofluor and observed under an epifluorescence microscope.

2.4. Ribossomal gene sequencing

Cells were collected by filtration from mid-exponential culture and frozen in liquid nitrogen prior to DNA

extraction. A modified 3% hexadecyltrimethyl-ammonium bromide (CTAB) protocol (Doyle and Doyle, 1990) for DNA extraction was used following mechanical cell disruption in a bead beater (Biospect Products). Two regions of the large subunit (Lsu) ribosomal RNA (rRNA): D1/D2 and D9/D10 were amplified from the extracted genomic DNA. Primers used for D1/D2 fragment were those published by Scholin et al. (1994). The region including the D8, D9 and D10 hypervariable domain was amplified using the FD8 and RB primers (Chinain et al., 1998). In addition to the large subunit amplifications, the ITS1, 5.8S and ITS2 regions were also amplified by PCR using the ITS1 and ITS 4 universal primers described in D'Onofrio et al. (1999). The same PCR conditions were used for all amplifications, following the conditions described in Scholin and Anderson (1994).

The amplified regions were sequenced on an ABI automatic sequencer, following the reaction protocols described by the manufacturer. Reactions were completed with the PCR primers used. Sequences thus obtained were compared to each other and to a selected culture of *A. minutum* (AMD21), kindly provided by Santiago Fraga (Instituto Español de Oceanografia).

2.5. Toxin analysis

Mouse bioassay. Toxicity of cell extracts of both 18-1T and 18-1NT was first determined by mouse bioassay. Toxin extraction was performed from cell pellets from mid-exponential cultures, following the procedure as described in AOAC (1980). To ensure cell disruption, previous to boiling, samples in 10 ml of 0.1N HCl were sonicated. Extracts were maintained at pH3. Charles River test mice were injected intra-peritoneally with 1 ml of each acidic extract. All injections were performed in triplicate and time of death recorded. Two mice were also injected with 1 ml of 0.1N HCl as a control.

HPLC. Cultures grown in 25 ml f/2 medium were collected via centrifugation (5000*g*, 5 min, 23 °C) for toxin content and toxin profile measurement by HPLC. One millilitre of 0.05N acetic acid was added to the cell pellet and 3, 25 μl aliquots were removed from the mixed sample and preserved in separate 1 ml volumes of filtered seawater containing Utermöhl's solution (Utermöhl, 1958) for cell counts. The acetic acid cell slurry was then disrupted by sonication (10 W, 20 s.) in an ice bath. The samples were stored at −20 °C prior to analysis. A modification of the post-column oxidation method of Oshima (1995) was performed to quantify 14 saxitoxin derivatives as described in Anderson et al. (1994). Four mixtures of PSP toxin standards kindly provided by Prof. Oshima (Tohoku University, Sendai, Japan), were used for identification and quantification purposes. Toxicities of the derivatives (in fg STX equivalents $cell^{-1}$) were calculated from the molar composition data using individual potencies in mouse units per μmol^{-1} (Oshima, 1995): C1—15; C2—239; C3—32; C4—143; GTX1—2468; GTX2—892; GTX3—1584; GTX4—1803; GTX5—160; dcGTX2—382; dcGTX3—935; NEO—2295; dcSTX—1274; STX—2483.

Cells pellets were also collected from mid-exponential cultures grown in the two different growth media, f/2 and ASP7. To insure maximum cell number was not a factor in toxin detection, triplicates from each set of experiments were pooled together and processed as a single sample. Thus, four pellets were analyzed: the subculture grown in f/2 and ASP7 media and the parental culture grown in f/2 and ASP7 media. Toxin extraction and analysis were performed following the same experimental procedures described above.

Mouse neuroblastoma assay (MNA). For toxin content estimation by MNA, 250 ml of mid-exponential phase cultures were centrifuged. Toxin extraction was performed by mechanical disruption in a bead beater with glass beads in 0.05N acetic acid. A cell extract was also obtained from a culture of *Scrippsiella*, grown in the same conditions, for use as a negative control. All pellets were obtained in triplicate.

The MNA was carried out according to the method described by Gallacher and Birkbeck (1992). Cell extracts were assayed using the following dilutions in RPMI assay medium: 1/8, 1/16, 1/32, 1/64. Measured sodium channel blocking (SCB) activity was compared to the same dilutions of the *Scrippsiella* extract. This negative control was used to detect the effect of the cellular extract (no toxin) on neuroblastoma cells and its SCB activity per se. The combination of ouabain and veratridine that killed 80% of the neuroblastoma cells was previously determined by titration and chosen for routine detection of saxitoxin-like activity (typical values used were around 0.2 mM ouabain and 0.05 mM veratridine). SCB activity was compared to a STX dose-response curve using a certified reference standard (National Research Council, Halifax, Canada). Cell pellets from cultures grown simultaneously in f/2 and ASP7 media were also analyzed by MNA.

3. Results

3.1. Morphology

Examination of the thecal plates of the 18-1T and 18-1NT cells by calcofluor staining revealed no morphological differences between the two strains. Both possessed the plate formula characteristic of the Genus *Alexandrium* (Po, 4′, 6″, 5‴ 2⁗, 6c and 9–10 s, with the 1′ linked to the Po) (Fig. 1). The characteristics described by Balech (1995) that distinguish *A. lusitanicum* from the closely similar *A. minutum* (namely the narrow 6″ precingular plate and Type B form of the 2⁗ plate) were also observed in both strains.

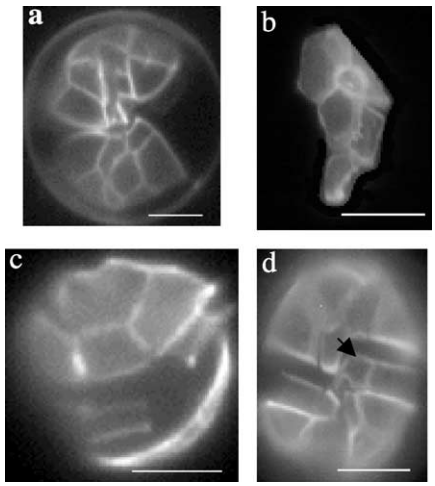

Fig. 1. Morphology of *Alexandrium lusitanicum* strains 18-1NT top, and 18-1T, bottom. Scale bar: approximately 12.5 μm. (a) General view of 18-1NT. (b) detail of $1'$, $4'$ plates and ventral pore. (c) Back, lower half view of 18-1T. Visible are the $2''''$, $2'''$, $3'''$, $4'''$ plates. (d) Details of the anterior sulcal plate (indicated by the arrow).

Table 1
GenBank accession numbers of strains sequenced

Strain/rDNA	D1/D2	D8/D10	ITS
18-1T	AY455827	AY455824	AY455826
18-1NT	AY455828	AY455823	AY455825

3.2. Ribosomal RNA gene sequencing

PCR amplification of the D1/D2 region of the Lsu rRNA gene produced fragments of approximately 665 bp. The 18-1T and 18-1NT strains had identical nucleotide sequences that differed from *A. minutum* by a single base (Table 1, Fig. 2). Similarly, no genetic variation between the two *A. lusitanicum* cultures was detected with the D8/D10 primers. In contrast to the results for the D1/D2 primers, the nucleotide sequences of the 720 bp fragment of all three cultures tested were identical, including that for *A. minutum*. Similar results were obtained for the whole ITS1, 5.8S and ITS2 region. Again, no nucleotide variations were detected between *A. lusitanicum* 18-1T, 18-1NT and *A. minutum*.

3.3. Growth

Significant differences in growth were observed between the two strains when grown in f/2 medium (Fig. 3A). 18-1NT was consistently slow growing and attained a lower maximum cell density. In f/2 medium, the maximum cell density reached was 20,124 ± 1356 cell ml^{-1} for 18-1NT versus 33,167 ± 2420 cell ml^{-1} for 18-1T, while calculated growth rates were 0.14 d^{-1} for 18-1NT and 0.19 d^{-1} for the toxic 18-1T.

Growth of the same two cultures was also measured in ASP7 media, with the same general results as for f/2 medium (Fig. 3B). The toxic 18-1T again showed a higher growth rate and attained a higher maximum cell yield. For both cultures, however, cell yield and growth rate were consistently lower than was the case in f/2 media. In ASP7, cell yield of 18-1T did not exceed 26,080 ± 4689 cell ml^{-1}. A similar pattern was observed for the 18-1NT (20, 130 ± 1356 cell ml^{-1} in f/2 vs. 13,900 ± 4291 cell ml^{-1} in ASP7).

3.4. Toxicity

Mouse bioassay. Cell pellets of at least 22×10^6 cells were used for this assay. The mice injected with 0.1N HCl or with the 18-1NT extract had a similar negative response. All mice survived the injection and did not show any unusual symptoms or behavior. Mice injected with the 18-1T extract died within 8 min and the median toxicity calculated was 4.1 MU or 352.8 fg STX.$cell^{-1}$.

HPLC. Analysis of the cell pellet extracts for the 18-1T and 18-1NT strains of *A. lusitanicum* using HPLC were remarkably different from each other. The chromatograms obtained for 18-1T showed a toxin profile consisting of mainly GTX 1, 4, with lower quantities of GTX 2, 3 (Fig. 4). No compounds with retention times comparable to the standards were detected in the STX and N-sulfocarbamoyl isocratic runs (Fig. 5). Total toxin content of the 18-1T extract was calculated to be 8.4 ± 0.4 fmol $cell^{-1}$ in f/2, or 3784.6 + 150.8 fg STX equivalents.$cell^{-1}$.

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18-1-NT  ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT
AMD21    ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT
18-1-T   ATATGGTTGATGTGGGTGCGATGGTTCTTACCTTGAATGTCAGCTTCTATTTCTGCAAAT

18-1-NT  CATTACCCTTGCACATGAATGGTAATTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA
AMD21    CATTACCCTTGCACATGAATGGTAACTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA
18-1-T   CATTACCCTTGCACATGAATGGTAATTTGCCTGCGGGTATTGGAATGCATGTGTTTGCAA

18-1-NT  TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
AMD21    TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
18-1-T   TGATTTGTGATTTGACGCATGTGTTTGGTGAAATTTGTATATGCTCTTTTGTCGAAGGGG
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Fig. 2. Partial sequence of isolates 18-1T, AMD21 and 18-1NT cultures for the D1/D2 region of the Lsu rRNA. Bases 420–600 are represented. The base pair difference between 18-1 and AMD21 is highlighted.

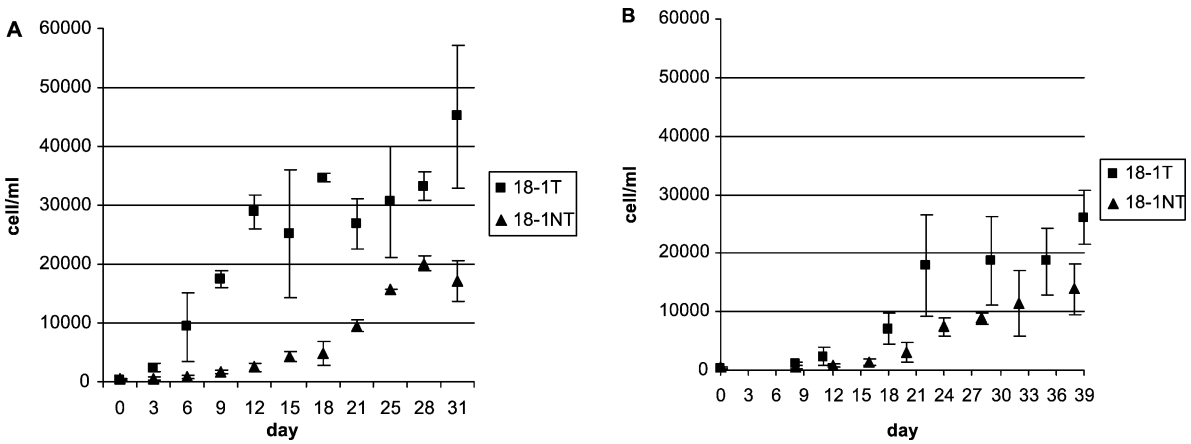

Fig. 3. Comparison of cell numbers of the non-toxic 18-1NT and toxic 18-1T isolates grown in f/2 (A) and ASP7 (B) media. Standard deviations are shown for each count ($n = 3$).

In contrast, no peaks with retention times similar to the toxin standards were observed for 18-1NT for GTX and C toxins (Fig. 4). A peak with a retention time fairly close to that of STX was detected for this strain (noted as peak X in Fig. 5). However, peak X was consistently offset from the standard, eluting slightly after it. The STX peak in the standard showed good reproducibility in its retention time and never coincided with peak X. To confirm that peak X

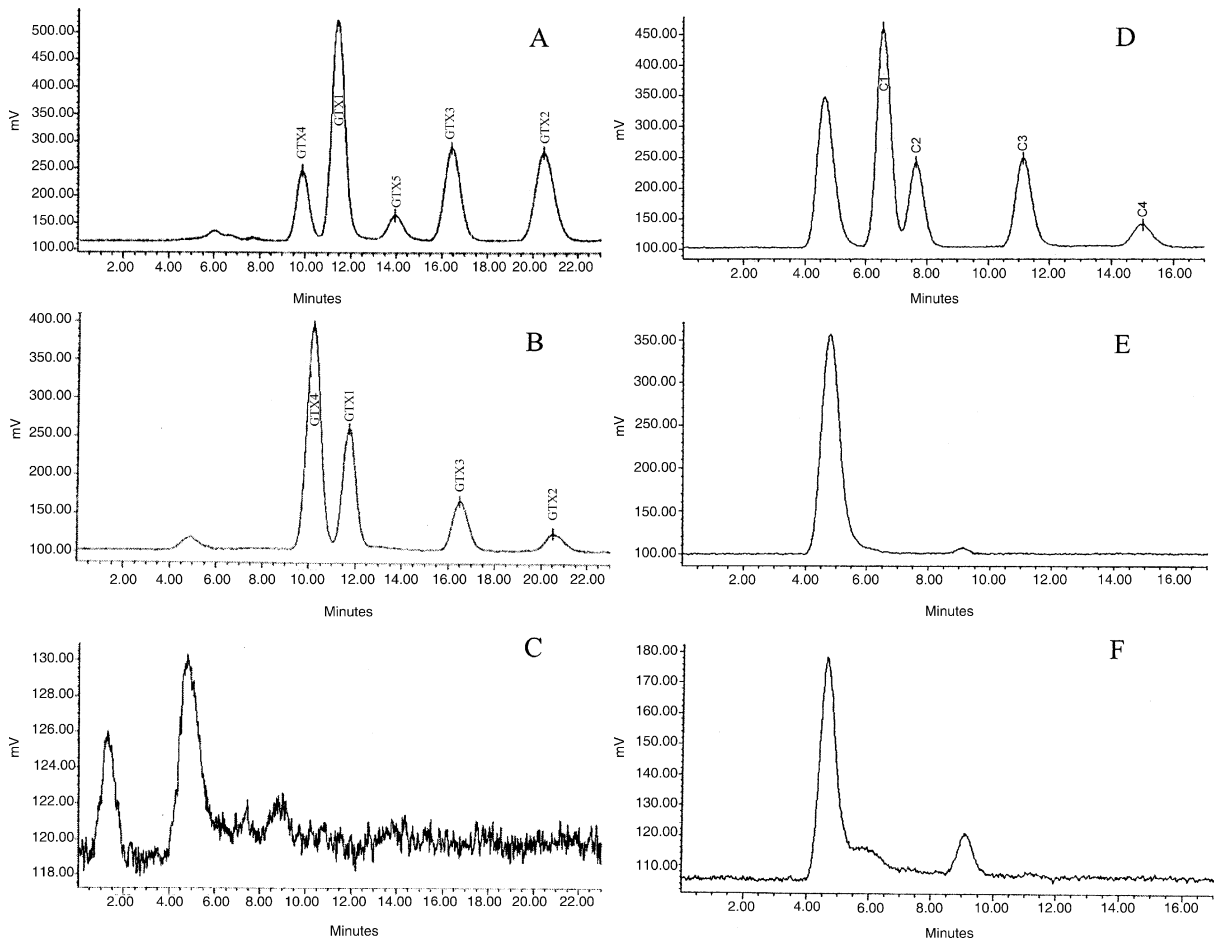

Fig. 4. HPLC analysis for GTX—(left) and C-toxins (right) in f/2 medium. Panels A and D refer to the standard mixture, B and E are 18-1T extracts and C and F refer to the 18-1NT culture.

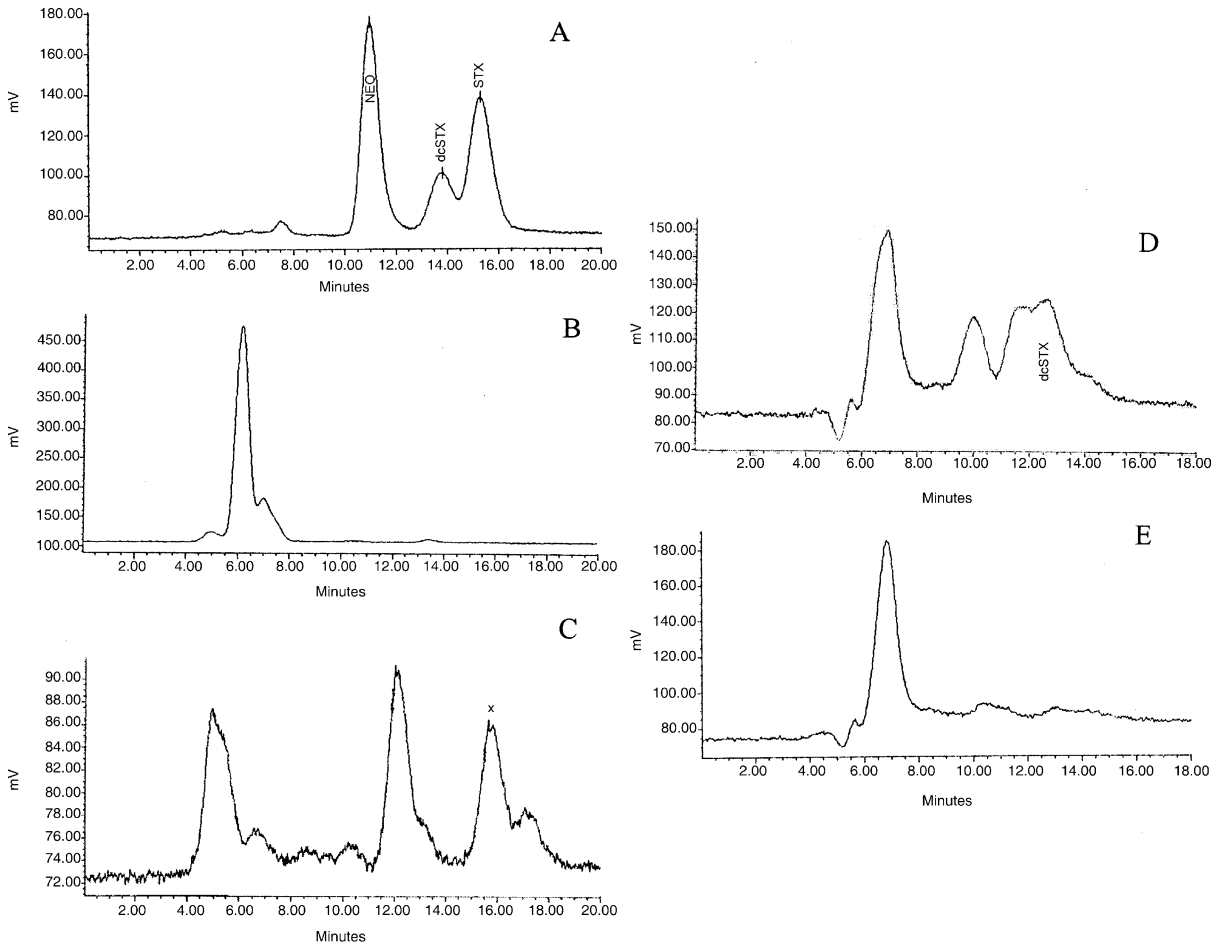

Fig. 5. HPLC analysis for neoSTX, dcSTX and STX for 18-1T and 18-1NT (B and C) in f/2 medium, and standard mixture (A). Putative STX peak in 18-1-NT extracts is labelled as 'x'. Results of the one hour hot hydrolysis in HCl (1:1 v/v) are shown in panel D (STX standard) and E (18-1NT extract).

was not STX, both sample and standard (containing only STX) were incubated at 100 °C in concentrated HCl (1:1 v/v) for 1 h. HPLC analysis of the concentrated, hotacid-treated samples showed different behaviors for the standard and 18-1NT sample (Fig. 5). For the hydrolyzed STX standard, the peak corresponding to STX disappeared and was converted to a compound with a retention time equivalent to that of decarbamoyl STX (dcSTX). The 18-1NT sample peak also disappeared, but did so without giving rise to dcSTX like compound. These differences in behavior following hydrolysis, plus the difference in retention times between peak X and STX lead to the conclusion that the former is in fact an 'impostor toxin' (Sato, 1998).

Analysis of 18-1T cultures grown in f/2 versus ASP7 media showed the former to be more toxic with 3.62 fmol cell^{-1} versus 1.22 fmol cell^{-1} in ASP7. The toxin profiles obtained (Fig. 6) were similar: both samples analyzed had GTX 1,4 as main toxins present, with lower quantities of GTX2,3 also detected. The mole percentages of each toxin differed slightly, as GTX2,3 accounted for 4% of the toxin content of the culture in ASP7 versus 7% in the culture grown in f/2. For 18-1NT, no peaks were ascribed to PSP toxins in ASP7 media.

Mouse neuroblastoma assay. In this assay, a comparison of sodium channel blocking (SCB) activity between the cell extracts of both cultures and a known non-toxic dinoflagellate, *Scrippsiella* sp. was performed. The MNA was used as a qualitative assay due to its high sensitivity for toxin detection by comparing the samples with the control. SCB activity, significantly higher than that of the controls, was detected for *A. lusitanicum*18-1T once cytotoxic effects were diluted out. The SCB activity detected could not be explained solely as matrix effects, as it differed significantly from that of the control extracts (Fig. 7, left) and the parental strain (Fig. 7, right). The residual levels of SCB activity seen in the controls are common in non-toxic extracts and media and correspond to the effect of co-extracted salts and other cellular components that have an

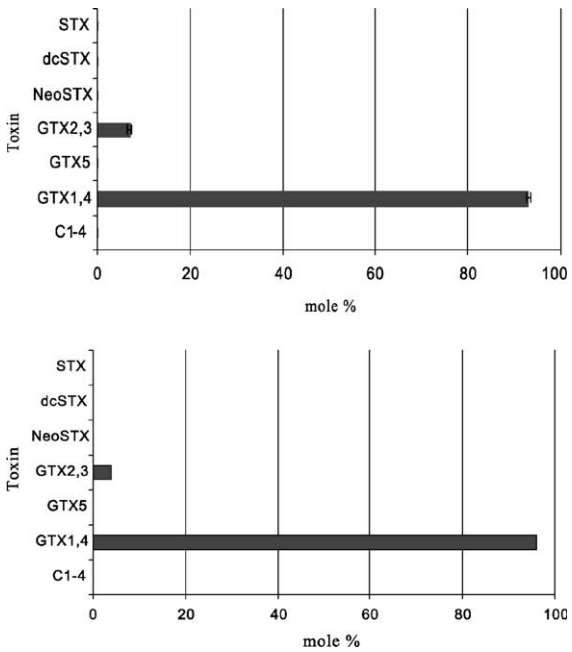

Fig. 6. Comparison of the toxin profile of the 18-1T *A. lusitanicum* cultures grown in different media in mol%. Top: toxin profile in mol% of 18-1T culture grown in f/2 medium. Bottom: toxin profile of the same culture in ASP7 medium.

effect on mouse neuroblastoma cells (Gallacher and Birkbeck, 1992).

Lower dilutions of the 18-1T sample showed a different behavior when compared to the negative control and the higher dilutions of this sample (Jellet et al., 1992). These effects can be ascribed to cytotoxic effects that could be diluted out of the sample. The cell pellets obtained from 18-1T subcultures growing in f/2 and ASP7 media showed similar results, as SCB activity was positively detected in both cases. However, the 18-1T culture grown in ASP7 media demonstrated a lower SCB activity, around 1/8th the value observed for cells growing in f/2.

No SCB activity ascribed to neurotoxins was detected for the 18-1NT culture, as the activity detected was always consistently lower than or equal to that of the control (Fig. 7, right). This result holds true for both growth media tested.

4. Discussion

We have demonstrated, for the first time, that saxitoxin production is not a constitutive, stable characteristic of *Alexandrium*, but can be lost during routine culture maintenance over a prolonged interval. Results clearly show a divergence, both in growth and toxicity, in two cultures derived from the same parental *A. lusitanicum* culture, originally isolated in 1962. Despite the fact both 18-1NT and 18-1T were grown and tested under the same conditions, no toxicity could be detected in the former, while the latter was always toxic, conserving the same toxin composition and roughly the same toxin content as the original 18-1 parental isolate, as analyzed in the past by Cembella et al. (1987), Alvito et al. (1995) and Mascarenhas et al. (1995). This striking loss of toxicity was confirmed by three independent toxin detection methods: the standard AOAC mouse bioassay, the mouse neuroblastoma assay and HPLC. Considering that the MNA has a higher level of sensitivity than HPLC (Gallacher and Birkbeck, 1992) and that the extracts assayed were from concentrations of cells well above the sensitivity limits described in the literature (Oshima, 1995), it is unlikely that the 18-1NT strain has low, undetected levels of toxicity, but this is of course possible. A number of independent sequence analyses of different rRNA domains also demonstrated that the toxic and non-toxic cultures are genetically identical for those markers examined. This strongly argues that the loss of toxicity is not due to a culturing artifact or mistake, such as culture mislabeling or accidental contamination with a different, non-toxic, species. These results and their implications are discussed in more detail below.

The longstanding view that saxitoxin production is a stable constitutive characteristic of an *Alexandrium* strain is refuted by the results obtained for our 18-1NT culture. Until our present study, only changes in the toxin content and more rarely in the toxin profile had been reported in the literature,

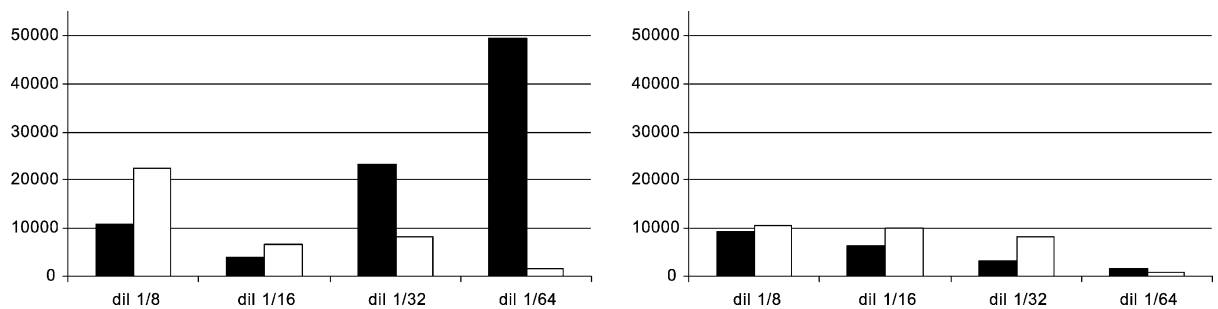

Fig. 7. Sodium channel blocking activity of dinoflagellate extracts (in black) and non-toxic control, *Scripsiella* (in white) obtained by mouse neuroblastoma assay. All cultures were grown in f/2 medium. Left: 18-1T; Right: 18-1NT.

while the ability to produce PSP toxins was never fully lost and it was never possible to elicit toxin production in non-toxic strains. This is therefore the first report of a species of *Alexandrium* losing the ability to produce toxins and it is also the first time any saxitoxin producer from any genus has ever become non-toxic. Three independent methods for toxin detection were used to confirm the loss of toxicity, each with different sensitivity limits and different principles of detection. Although the mouse bioassay has a detection limit of only 0.5 μM (Thibault et al., 1991), the HPLC system used has a sensitivity threshold of 14, 8, 3, and 14 nM for GTX1 to 4 in a 10 μl injection, respectively (D. Kulis, unpub. data). The qualitative assay (MNB) is approximately two orders of magnitude more sensitive than the mouse bioassay (Gallacher and Birkbeck, 1995). Within these limits, we are confident 18-1NT does not produce PSP toxins. The different toxin extraction methods, detection limits and toxin standards used on these methods explain the general lack of numerical agreement in the amount of toxin analyzed, though all three methods were in agreement that 18-1T was toxic and 18-1NT non-toxic. Samples for toxin analysis were always taken in exponential phase, when toxin content is highest in *Alexandrium* cultures (Anderson et al., 1990b; Franco et al., 1995; Mascarenhas et al., 1995), and large numbers of cells were extracted to ensure that instrument sensitivity or limits of detection were not issues. One putative STX peak was detected in extracts of 18-1NT using HPLC, but this peak did not behave like STX upon acid hydrolysis, nor was any SCB activity detected in extracts from this culture using the highly sensitive MNA. Similarly, the mouse bioassay did not detect any toxicity in 18-1NT. Furthermore, in our analysis of 18-1T and in all previous HPLC studies of the 18-1 strain of *A. lusitanicum*, STX has never been detected. We are therefore confident that peak X (Fig. 5) is an impostor toxin (Sato and Shimizu, 1998) and that 18-1NT is non-toxic. The biochemical nature of compound X is not known; it was present in only the non-toxic cultures, and has not been seen in HPLC analysis of other non-toxic *A. minutum* analyzed with the same methods and instrument (unpub. data).

The proof that the ability to produce toxin was completely lost was essential to our study. The partial reduction of toxin content in long-term cultures has been previously reported (Hansen et al., 2003) and still constitutes an argument for constitutive toxin production. Our present results, however, clearly show that it is possible for *Alexandrium* to lose the ability to produce saxitoxins.

The toxin profile of the 18-1T subculture was comparable to that previously obtained for the original *A. lusitanicum*18-1 isolate (Mascarenhas et al., 1995; Cembella et al., 1987; Alvito et al., 1995). In all cases, GTX1,4 are dominant, with lower proportions of GTX2,3 also present. This toxin profile has therefore remained constant after 40 years in culture in 18-1T, but was dramatically altered in 18-1NT.

The loss of toxicity in the 18-1NT strain seems to be associated with a reduction in growth capability. The maximum cell yield, as well as the growth rate, were lower for this non-toxic strain compared to the toxic 18-1T strain in both media types tested. The growth rate and cell yield of cells of both strains kept in ASP7 were consistently lower than for the same cultures grown in f/2.

Toxin content and to a lesser extent, toxin profile also differed for 18-1T cells kept in the two media types. 18-1T culture had lower levels of toxicity when grown in the artificial ASP7 medium, achieving toxin content values that were lower than ever reported for this strain (Cembella et al., 1987; Mascarenhas et al., 1995). Low toxicity levels for *A. lusitanicum* subcultures kept in different growth media have been reported previously (Reguera et al., 1993). It is unclear what limits dinoflagellate growth and toxin production in ASP7. This medium differs from f/2 in some characteristics, namely nitrate and phosphate concentrations but the most significant difference probably relates to the artificial seawater base of ASP7 and either the lack of some unknown micronutrient or the presence of toxic contaminants from the reagent salts. These results demonstrate that toxin production could not be restored by growing the 18-1NT clone in optimal growth media (f/2), nor did 18-1T become non-toxic in the artificial media. This evidence strongly supports our view that the different media the clones were kept in was not responsible for the observed change. Growth of the 18-1NT clone could be improved by transferring it into f/2 media, but it never regained the ability to produce saxitoxins.

The observed differences in growth and toxicity might suggest that the two subcultures are not derived from the same parental strain, as might happen due to contamination or mislabeling of the parent culture during laboratory handling and culture transfers, for example. Throughout the interval in question, however, only a single *Alexandrium* strain (18-1) was cultured in the Laboratorio de Microbiologia e Ecotoxicologia where the loss of toxicity was first observed (Pereira et al., 2000). In other words, there was no morphologically similar culture to be mislabeled or to serve as a contaminant. Furthermore, our morphological and sequencing data both argue that 18-1NT and 18-1T have identical origins. Detailed thecal plate examinations showed that both cultures are morphologically identical, and that both have the distinctive taxonomic characteristics of *A. lusitanicum*. Sequencing of the D1/D2 region of rDNA also showed the two clones to be indistinguishable over 665 bp. In comparison, a detailed examination of rDNA data for *A. minutum* and *A. lusitanicum* (Scholin et al., 1994) showed these two morphologically similar species to differ by a single base pair over this same region. This single base difference between *A. lusitanicum* and *A. minutum* clones was also observed in this study. Using the same analysis, our

A. lusitanicum 18-1NT and 18-1T subcultures' D1/D2 sequences were identical.

The nucleotide sequences of the D8/D10 hypervariable domain of the Lsu rDNA were identical for the two clones, as was also found when the highly variable ITS1, 5.8S and ITS2 regions of rDNA were sequenced. Furthermore, when the ITS sequences of the 18-1NT and 18-1T strains were matched to the sequence for *A. lusitanicum* described by Penna and Magnani (1999) and posted in GenBank, a 100% match was found. All of these sequencing efforts argue that the 18-1NT and 18-1T are indeed from the same parental stock culture, and are *A. lusitanicum*. Nuclear ribosomal DNA is an ideal phylogenetic marker, as it possesses the same function in all organisms and has highly conserved regions (Adachi et al., 1996). Due to these characteristics, if 18-1T and NT are derived from the same culture and kept apart for only 10 years, base pair differences are not expected for these rDNA regions.

Other, more variable, regions of the genome, however, could have suffered and accumulated mutations over this time period. In view of the present results, we are left to speculate how the changes in toxicity and growth may have arisen. We find it noteworthy that the loss of toxicity is also associated with reduced growth potential. If indeed loss of toxicity is due to a mutation in a gene (or genes) involved in the toxin biosynthetic pathway, then the mutation responsible for this change (if such is indeed the case), is also affecting some pathway or pathways critical to cell growth and/or division. The fact that the parental culture has remained non-toxic for at least 4 years presents an argument for mutation rather than differential regulation at the gene expression level and that the loss of toxicity is not a transient characteristic.

Another possibility relates to the idea first proposed by Silva (1962) that bacteria are involved in saxitoxin production, either by autonomous toxigenesis or by supplying necessary metabolites. This concept has been advanced in several studies (see Gallacher and Smith, 1999 for a review). The occurrence of intracellular bacteria underneath the theca of the original 18-1 culture has been observed by electron microscopy (Franca et al., 1995) and bacteria have been isolated from aliquots of both 18-1NT and 18-1T cultures (data not shown). It is therefore possible that the continued antibiotic treatment of only the 18-1 culture eliminated associated bacteria essential for toxin production. Previous reports suggest the importance of associated bacteria in dinoflagellate (Doucette and Powell, 1998) and even diatom toxicity (Bates et al., 1995). In the case of dinoflagellates, however, authors report the production of substantially less toxin, rather than the total loss of toxicity in axenic cultures. The total loss of dinoflagellate toxicity due to removal of associated bacteria has never been reported and is therefore unlikely, but still possible, to provide an adequate explanation for our present results.

On the other hand, the sustained use of antibiotics could have influenced the cells in other ways. Despite affecting mainly protein synthesis and the prokaryotic cell wall, some of the antibiotics contained in the AM9 mixture with which the 18-1 culture was treated have been shown to be detrimental to algal cells. In particular, streptomycin caused sustained loss of pigmentation in *Chlorella* (Dube, 1952) and changes in chloroplast morphology and number, and a reduction in growth in *Euglena*, even after multiple transfers in streptomycin-free culture medium (Provasoli et al., 1948). Chloramphenicol, another antibiotic in the AM9 mixture, is reported to inhibit the growth of *Euglena* even at low concentrations (Miyoshi and Tsubo, 1969). It also can affect amino acid incorporation in the chloroplast system and inhibit chlorophyll formation. Interestingly, in that study, cells bleached by the chloramphenicol treatment never regained their green pigmentation after more than 2 years of transfers in medium without the antibiotic.

The effect of antibiotic treatments on dinoflagellate cells has not been documented, so it is possible, but still speculative, that prolonged exposure to the AM9 antibiotic mixture could have caused changes in the parental 18-1 culture, rendering it non-toxic. At that time, the 18-1T subculture was being maintained at another culture facility, but without the monthly antibiotic treatments (G. Doucette, pers. comm.), and thus it would not have been exposed to a similar stressor. The reduction in growth of *Euglena* observed by (Miyoshi and Tsubo, 1969) and (Provasoli et al., 1948) agrees with our own observation of reduced growth in 18-1NT, which was exposed to antibiotics for a longer time period.

The mechanisms underlying the reported changes in toxin production and growth are presently under investigation for the 18-1NT and 18-1T cultures.

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