Toxic Phytoplankton Blooms in the Sea
T.J. Smayda and Y. Shimizu, editors

INTRACELLULAR DISTRIBUTION OF SAXITOXIN IN *Alexandrium fundyense*

GREGORY J. DOUCETTE AND DONALD M. ANDERSON
Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543 USA

ABSTRACT

The intracellular distribution of saxitoxin (STX) in the marine dinoflagellate *Alexandrium fundyense* (strain GTCA29) was investigated using immunocytochemical methods. A non-toxic isolate of *Alexandrium tamarense* (strain PGT183) was included as a control for all experiments. Specimens were prepared using both chemical fixation followed by embedding in either an epoxy- (Spurr) or polar acrylic-based (LR White, Lowicryl K4M) resin, and cryo-fixation techniques. A total of four antibodies against STX (aSTX) were tested. Immunofluorescent labelling of non-osmicated, Spurr-embedded material by all aSTX's was associated with the permanently-condensed chromosomes of *A. fundyense*. The STX specificity of this staining pattern was verified by negative non-toxic, non-immune and STX pre-incubation controls. Immunocytochemical analysis by electron microscopy confirmed the chromosomal localization of STX. In contrast, LR White-embedded toxic and non-toxic specimens showed labelling of the nucleoplasm. Osmication of either Spurr- or LR White-embedded material eliminated virtually all staining. Non-osmicated samples embedded in Lowicryl K4M yielded no nuclear labelling. Both toxic and non-toxic cryo-fixed specimens exhibited labelled chromosomes with not only aSTX but also non-immune serum. We conclude that: 1) STX occurs in association with the chromosomes of chemically-fixed, Spurr-embedded *A. fundyense*, 2) osmication and/or embedding in polar acrylic resins precludes immunolabelling of STX, and 3) a non-specific DNA-immunoglobulin interaction prohibits the localization of STX in cryo-fixed dinoflagellates. Because of potential antigen redistribution during chemical fixation, the *in vivo* distribution of STX remains uncertain.

INTRODUCTION

Saxitoxin (STX) and its eleven derivatives are the causative agents of paralytic shellfish poisoning (PSP). While PSP is attributable to members of several marine dinoflagellate genera (*Alexandrium*, *Gymnodinium* and *Pyrodinium*), certain of the saxitoxins are also produced by a freshwater cyanobacterium (*Aphanizomenon flos-aquae*, [1]), a red alga (*Jania* sp., [2]), and possibly several species of bacteria [3]. Given the diversity of organisms capable of synthesizing these neurotoxins, coupled with the occurrence of both toxic and non-toxic strains within a single species, efforts to assign a metabolic role(s) to such compounds become especially difficult. One approach to the question of biological function has been to determine where a compound occurs within a cell or organism. The immunocytochemical approach to subcellular localization is based on an antibody's ability to recognize and bind a given antigen, followed by visualization of the antigen-antibody complex. The quality of results obtained depends largely on maintaining the molecular structural integrity and spatial cellular distribution of the antigen, as well as its accessibility to antibodies. Conventional electron microscopy fixation and embedding protocols are generally considered essential for optimal structural

preservation. However, since the end result of these techniques (e.g. chemical crosslinking, solvent extraction, etc.) may not be compatible with the aims of immunolocalization work, alternative methods have been explored. Examples include omission of OsO_4, improvements to acrylic plastics, and use of cryotechniques such as rapid specimen freezing (e.g. [4]), molecular distillation drying [5], and low temperature embedding. While each of these approaches has been used with varying degrees of success depending primarily on tissue type and nature of the antigen, procedures incorporating cryotechniques are thought to yield the most satisfactory results.

The only previous attempt employing immunocytochemistry to determine the intracellular location of STX in a dinoflagellate [6] involved a standard chemical fixation and embedding in an epoxy resin. The authors observed STX labelling within the nucleus, on or close to the permanently-condensed chromosomes. In view of recent evidence demonstrating a temporal relationship between toxin synthesis/degradation and the S phase of the cell cycle [7], we sought to verify the nuclear localization of STX. Our approach was to determine whether changes to the protocol of Anderson and Cheng [6] would yield a similar toxin distribution pattern in the same dinoflagellate species. Principal modifications to specimen preparation were grouped into two categories. The first retained application of chemical fixatives while varying embedding plastics among epoxy- (Spurr) and acrylic-based (LR White) resins, including a low temperature formulation (Lowicryl K4M). The second involved use of cryotechniques in an attempt to minimize the potential problems associated with chemical fixation noted above. In addition to these changes a total of four antibodies against STX were tested during this study, including that employed by Anderson and Cheng [6].

MATERIALS AND METHODS

Batch cultures of the toxic *Alexandrium fundyense* Balech (strain GTCA29) and the non-toxic *Alexandrium tamarense* Balech (strain PGT183) were maintained in K medium ([8], modified according to [9]) at 16°C on a 14:10 light:dark cycle (ca. 250 $\mu E/m^2/s$). Samples for immunolabelling were harvested during exponential growth and prepared using the chemical fixation and cryofixation protocols given below.

One group of chemically-fixed specimens was exposed first to 4% paraformaldehyde (PFA)/0.5% glutaraldehyde (GTA) in buffered filtered seawater (BFSW, pH 7.7) for 5 min at 4°C, after which the sample was diluted to half-strength with BFSW and allowed to stand an additional 5 min. Cells were either post-fixed in 2% OsO_4 for 1 h at 4°C and then dehydrated, or simply dehydrated. Embedding was done in Lowicryl K4M at -8°C or in Spurr at 60°C. The second chemical fixation employed 4% PFA/0.5% GTA in PIPES buffer (pH 7.2) at 4°C for 45 min followed by either 1% OsO_4 for 5 min at 4°C and dehydration, or dehydration only. These samples were embedded in LR White at 50-55°C.

Cryo-preparation of specimens was performed according to the method of Linner et al. [10] employing the LifeCell CF100™ cryo-fixation system and MDD-C™ console molecular distillation dryer (LifeCell Corp.).

Immunolabelling protocols for chemically- and cryo-fixed samples can be outlined as follows: chemically-fixed specimens were first blocked (10% normal goat serum/5% ovalbumin) and then treated with a STX antibody (aSTX) or normal serum, followed by a goat or rabbit antibody (Ab) against the appropriate animal. Secondary Ab's were

labelled with FITC for epifluorescence (EPI) light microscopy (LM) or 15 nm colloidal gold for transmission electron microscopy (TEM). Cryo-fixed specimens were first oxidized, blocked, and then stained with a primary Ab, followed by an FITC-labelled secondary Ab. Primary antisera included polyclonal (Pc) and monoclonal (Mc) Ab's against STX -keyhole limpet hemocyanin or -bovine serum albumin (BSA) conjugates raised in rabbit (Pc; two Ab's: BIOM aSTX, [11]; USAR aSTX, J.F. Hewetsen, pers. comm.), burro (Pc; USAB aSTX, J.F. Hewetsen, pers. comm.), or mouse (Mc; SFBR aSTX, [12]), normal rabbit serum (NRS, Sigma Chem. Co.), and normal mouse serum (NMS, Sigma Chem. Co.) Only BIOM aSTX was employed in stains of cryo-fixed material. Experiments were also performed in which the BIOM aSTX was pre-incubated with STX (10 μg/ml, chemically-fixed, Spurr-embedded specimens; 400 μg/ml, cryo-fixed specimens) and/or sheared salmon sperm DNA (1 mg/ml, cryo-fixed specimens) to determine the effect on immunolabelling.

Agarose gel electrophoresis was performed on phage lambda *HIND* III DNA fragments following their incubation with 0.1 M phosphate-buffered saline (PBS), BIOM aSTX, NRS, or BSA.

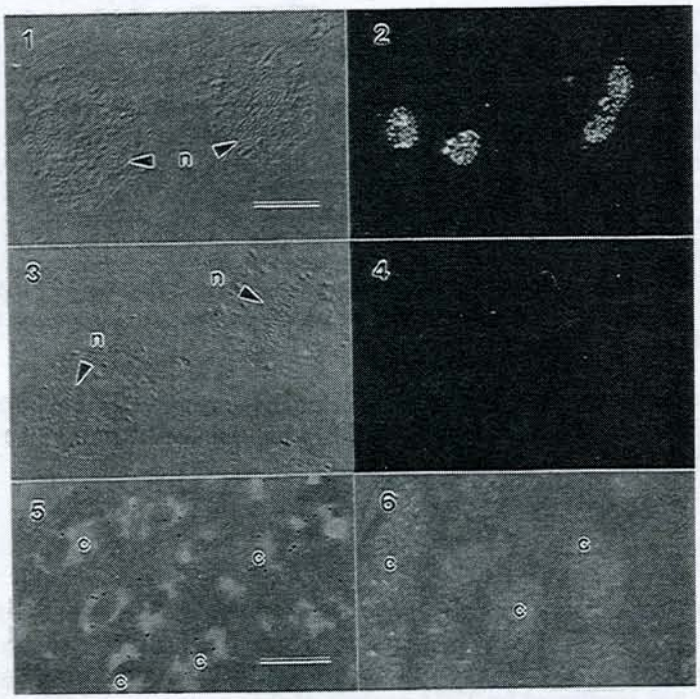
RESULTS

Immunofluorescent labelling of chemically-fixed, non-osmicated *Alexandrium fundyense* (toxic) embedded in Spurr resin appeared to be associated with the dinoflagellate chromosomes, regardless of which aSTX was used (Figs. 1,2). This observation was confirmed by TEM analysis of the same material (Fig. 5). Incubating each aSTX with STX prior to its application eliminated this staining pattern. *Alexandrium tamarense* (non-toxic), showed no labelling by any aSTX in samples examined by LM (Figs. 3,4) or EM (Fig. 6). Replacing an aSTX with the analogous normal (i.e. non-immune) serum yielded negative results for both toxic and non-toxic isolates.

In contrast to the results obtained with Spurr-embedding, both *A. fundyense* and *A. tamarense* prepared without osmium and embedded in LR White exhibited labelling of the nucleoplasm. This staining pattern, which was clearly not STX-specific (i.e. no difference between toxic and non-toxic specimens), caused chromosomes to appear as silhouettes. Non-osmicated material embedded in Lowicryl K4M had no nuclear labelling. However, staining was frequently observed in what appeared to be storage granules, irrespective of strain toxicity. Osmication of either Spurr- or LR White-embedded samples eliminated both STX- and nonSTX-specific label.

Immunolabelling of *A. fundyense* and *A. tamarense* prepared by cryotechniques produced chromosome labelling within the nucleus of both toxic and non-toxic isolates (data not shown). Furthermore, NRS controls for both strains gave a staining pattern indistinguishable from that using BIOM aSTX. Pre-incubation of BIOM aSTX with STX did not reduce the labelling of chromosomes, while exposure of the Ab to sheared salmon sperm DNA eliminated this staining.

Gel electrophoresis of phage lambda DNA fragments following incubation with BIOM aSTX or NRS showed a 25% and 17% decrease, respectively, in mobility of the largest fragment (23.13 kb) relative to the PBS control. Incubation with BSA at total protein concentrations similar to BIOM αSTX and NRS had a minimal effect on the DNA fragment migration pattern.



FIGS. 1,2,5. *A. fundyense*. FIGS. 3,4,6. *A. tamarense*. FIGS. 1-4. LM micrographs (scale bar = 10 μm). FIGS. 5,6. TEM micrographs (scale bar = 0.5 μm). FIG. 1. Section of cells revealing permanently-condensed chromosomes in the nucleus (n). FIG. 2. EPI LM micrograph of cells shown in FIG. 1 stained with BIOM aSTX (FITC label). Chromosomes are clearly labelled. FIG. 3. Sectioned cells exhibiting nucleus (n) and chromosomes. FIG. 4. EPI LM micrograph of cells in FIG. 3 (treatment as in FIG. 2). No FITC label is apparent. FIG. 5. Section showing chromosomes (c) stained with BIOM aSTX (colloidal gold label). FIG. 6. Section treated as in FIG. 5. Note absence of gold label on chromosomes (c).

DISCUSSION

The principal goal of this study was to verify using an array of immunolabelling techniques, the association of saxitoxin with the chromosomes of *Alexandrium fundyense* as reported by Anderson and Cheng [6]. Our results from the application of four STX antibodies to chemically-fixed, non-osmicated material embedded in Spurr epoxy resin clearly show chromosomal localization at both the LM and TEM levels. The STX-specific nature of this distribution pattern was confirmed not only by negative non-toxic (*Alexandrium tamarense*) and non-immune controls, but also by the absence of labelled chromosomes in the toxic isolate when antibodies were exposed to STX prior to their use. In addition, it is noteworthy that potential artifacts caused by etching and/or oxidation

before staining, as used by Anderson and Cheng [6] were eliminated by simply avoiding these steps. Thus, we are confident in concluding that STX is associated with the permanently-condensed dinoflagellate chromosomes in *A. fundyense* prepared according to this protocol. The only disagreement of any consequence between the present findings and those of Anderson and Cheng [6] is the fact that osmication precluded STX localization in our study but not in theirs. Since the benefits of osmium in terms of structural preservation are well-known and the effects of its omission on dinoflagellate chromosomes are obvious (see Figs. 5,6), additional work is warranted to explain this discrepancy.

Polar acrylic resins (e.g. LR White, Lowicryl K4M) are often preferred over epoxy resins for immunocytochemical work due to their hydrophilic nature. That such plastics are miscible with water to varying degrees allows for only partial dehydration with organic solvents prior to embedding and the polymerized resin requires no pretreatment (e.g. etching) to facilitate penetration of antibody solution into the specimen. Given these characteristics our inability to obtain STX-specific labelling with either LR White or Lowicryl K4M was quite unexpected. We are left only to conclude that these resins may interact with saxitoxin in such a way as to "mask" this antigen from antibody detection.

Clearly, a major shortcoming of immunocytochemical studies employing samples exposed initially to aqueous phase chemical fixatives is the possibility of antigen redistribution. We attempted to minimize this problem through the use of cryo-fixation and molecular distillation drying techniques. However, even though the BIOM aSTX produced chromosome labelling in *A. fundyense*, similar results were also obtained with non-toxic *A. tamarense* cells. In addition, non-immune controls for both isolates yielded a staining pattern indistinguishable from that of the STX antibody. While these data clearly are indicative of non-specific binding in the sense that a substance other than STX is being labelled, the chromosome-associated nature of the labelling pattern appears to be quite specific. The observation that pre-incubation of the antibody did not affect this pattern, yet reaction with "foreign" (i.e. non-dinoflagellate) DNA eliminated it suggests that a portion of the dinoflagellate DNA molecule itself is being bound by the various sera. It is, in fact, recognized that nucleic acids exhibit a strong non-specific affinity for immunoglobulins [13]. Our gel electrophoresis data also support the idea of a non-specific DNA-immunoglobulin interaction. A more general effect of non-globulin proteins appears unlikely due to the negligible change in DNA fragment migration pattern following treatment with BSA.

The DNA of cryo-fixed material is presumed to approximate more closely its "natural" state, and thus may retain a strong affinity for immunoglobulins present in the various sera. The resulting non-specific background staining of DNA, enhanced by the permanently-condensed nature of the dinoflagellate chromosomes, appears to overshadow any STX-specific labelling. The absence of non-specific chromosome staining in chemically-fixed specimens is likely due to the masking or elimination of certain DNA binding sites through cross-linking or extraction effects. It is our opinion that cryo-fixation affords the best opportunity to minimize antigen redistribution artifacts, but that at least limited exposure to aldehydes may be essential to eliminate the non-specific interaction between dinoflagellate chromosomes and immunoglobulins. Thus, we are continuing to explore other cryotechniques such as molecular distillation drying followed by exposure to paraformaldehyde vapor and freeze substitution methodologies.

CONCLUSIONS

To date, our conclusions are as follows: 1) Saxitoxin is associated with the chromosomes of *A. fundyense* in certain chemically-fixed, epoxy-embedded specimens; 2) Osmication and/or embedding in polar acrylic resins precludes the immunolabelling of STX; 3) A non-specific DNA-immunoglobulin interaction, which obscures or masks any STX-specific labelling, occurs in cryo-fixed *Alexandrium* specimens. Chemical fixation minimizes this DNA-immunoglobulin interaction, possibly via aldehyde crosslinking or solvent extraction effects; 4) A reduction in the electrophoretic mobility of non-dinoflagellate DNA fragments, following incubation with sera that produced chromosome-associated labelling in cryo-fixed specimens, supports the idea of a non-specific DNA-immunoglobulin interaction; 5) The unique, permanently-condensed nature of dinoflagellate chromosomes may preclude the use of "gentle" fixation methods (e.g. certain cryotechniques) in studies attempting to localize antigens occurring in close association with these structures.

ACKNOWLEDGEMENTS: This work was supported in part by an NSERC Postdoctoral Fellowship, by the Florence and John Schumann Foundation, and by NSF grant OCE 8911226. Contribution No. 8115 from the Woods Hole Oceanographic Institution.

REFERENCES

1. Carmichael, W.W. in: Handbook of Natural Toxins, A.T. Tu ed. (Marcel Dekker, N.Y. 1988) pp.121-147.
2. Kotaki, Y., M. Tajiri, T. Oshima, and T. Yasumoto, Bull. Jap. Soc. Sci. Fish. 49,283-286 (1983).
3. Kodama, M., T. Ogata, and S. Sato, Agric. biol. Chem. 52,1075-1077 (1988).
4. Phillips, T.E. and A.E. Boyne, J. Electron Microsc. Tech. 1,9-29 (1984).
5. Livesey, S.A. and J.G. Linner, Nature 327,255-256 (1987).
6. Anderson, D.M. and T. P.-O. Cheng, J. Phycol. 24,17-22 (1988).
7. Anderson, D.M., D.M. Kulis, and G.J. Doucette, in prep.
8. Keller, M.D., R.C. Selvin, W. Claus, and R.R.L. Guillard, J. Phycol. 23,633-638 (1987).
9. Anderson, D.M., D.M. Kulis, J.J. Sullivan, S. Hall, and C. Lee, Mar. Biol. 104,511-524 (1990).
10. Linner, J.G., S.A. Livesey, D.S. Harrison, and A.L. Steiner, J. Histochem. Cytochem. 34,1123-1135 (1986).
11. Carlson, R.E., M.L. Lever, B.W. Lee, and P.E. Guire, in: Seafood Toxins, ACS Symposium Series 262, E.P. Ragelis ed. (Amer. Chem. Soc., Wash. 1984) pp.181-192.
12. Huot, R.I., D.L. Armstrong, and T.C. Chanh, J. Toxicol. Environ. Health 27,381-393 (1989).
13. Nau, D.R. and J.T. Baker, BioChromatography 5,62-73 (1990).