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Comparison of Toxicity Between Populations of *Gonyaulax tamarensis* of Eastern North American Waters

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Isolates of the dinoflagellate *Gonyaulax tamarensis* were established from benthic cysts or motile cells collected along the north-western Atlantic coast from the Bay of Fundy to Long Island. All clones were grown under the same conditions and assayed in a blind test for toxin content and composition. Differences in toxin content (μmouse unit per cell) spanning two orders of magnitude were found, with decreasing toxicity from north to south. Some isolates had undetectable toxin levels. The low toxicity of southern strains of *G. tamarensis* may explain the historical absence of paralytic shellfish poisoning (PSP) in areas where cysts and motile cells have been reported. The cause of the observed geographic pattern is unknown, but it does suggest that there may be an environmentally-determined southern limit to the regional PSP problem.

Qualitative data on the toxin composition of some clones indicate that saxitoxin, neosaxitoxin and gonyautoxins -II, -III and -IV are generally present. A more quantitative approach (i.e. one which examines each isolate for all of the 12 *Gonyaulax* toxins) is needed to fully utilize the potential of toxin composition in discriminating between strains.

Introduction

Paralytic shellfish poisoning (PSP) in New England coastal waters has been linked to the toxins produced by *Gonyaulax tamarensis* Lebour (= *Protogonyaulax tamarensis* Taylor), a marine dinoflagellate (Prakash *et al.*, 1971). This unicellular alga overwinters in the sediments as a cyst (Dale, 1977; Anderson & Wall, 1978). Upon vernal warming, the cysts germinate, inoculating the overlying waters with a population of asexually dividing motile cells available to filter-feeding shellfish (Anderson & Morel, 1979).

Anderson *et al.* (1982) described the distribution of *G. tamarensis* cysts in sediments along the coast of southern New England as a baseline record of the population's geographic range. Cysts were found not only in estuarine and coastal locations where PSP

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outbreaks are common, but also in some areas of Connecticut and Long Island waters with no PSP history. This historical absence of shellfish toxicity in areas where the toxic organism is known to be present could be the result of (1) its recent introduction to the region (Anderson *et al.*, 1982), (2) its inability to develop into large blooms, or (3) the low toxicity of local strains. The first possibility seems unlikely since no dangerous levels of PSP have been reported to this date, now four years after the initial discovery of cysts (Anderson, unpublished). The second alternative is inconsistent with recent data on *G. tamarensis* populations reaching the potentially dangerous concentration of 100 000 cells l^{-1} in some Long Island estuaries (Schrey *et al.*, 1984). The last hypothesis is supported by reports of intraspecific variation in toxin content for various *G. tamarensis* clones (Schmidt *et al.*, 1978; Alam *et al.*, 1979; Schmidt & Loeblich, 1979; Oshima *et al.*, 1982*a*,*b*).

The main objective of this paper is to evaluate the toxin content of unialgal cultures of *G. tamarensis* established from cysts and motile cells isolated from locations between Long Island Sound and the Bay of Fundy. This work allows us to re-examine the relationship between levels of shellfish toxicity and the abundance and toxicity of the dinoflagellate. This geographical distribution of toxic organisms superimposed on the distribution of cysts in the sediments also provides data of a predictive nature for potential outbreaks of PSP and their severity in given areas.

Methods

Following a survey looking for *G. tamarensis* in the sediments of southern New England and Long Island, individual cysts were isolated by micropipette and germinated in f/2 medium with no added silicate (Guillard & Ryther, 1962) at 20°C. Site selection and sediment collection details are given by Anderson *et al.* (1982). Twenty-three *G. tamarensis* cultures were established from cysts in this manner. Eleven more were established through isolation of individual motile cells from water samples during bloom season. All clones were identified on the basis of known morphological criteria established by light microscopy for the vegetative cell and/or the benthic cyst (Dale, 1977; Anderson & Wall, 1978). Details of the isolation method and origin of each clone are presented in Table 1.

In order to insure a completely blind test of clonal toxicity, each strain was assigned a code name at the Woods Hole Oceanographic Institution and then delivered to the University of Rhode Island for culturing and toxin analysis. Furthermore, some of the clones were delivered several times throughout the project under different code names as a test of the precision in toxin analyses.

Cultures were grown in batch mode in two 3-l Fernback flasks, each containing 1·5 l of medium f without silicon addition at a salinity of 30‰. The cells received *ca.* 150 $\mu E\ m^{-2}\ s^{-1}$ of irradiance (cool-white fluorescent lamps) under a 17-h photoperiod at 12°C (± 1°C). Of the 34 clones, only three, Gonyaulax #2, Gonyaulax #7 and PPbl, were bacteria-free as tested with medium OZR without agar addition (Sieburth, 1971). Cell numbers were determined using a Palmer-Maloney chamber or by counting the entire content of several 5-µl aliquots. A minimum of 200 cells were counted for each isolate.

The cultures were harvested during late exponential or early stationary phase growth by filtration on a column of packed glass wool. The cells were extracted for two successive 30-min intervals in ethanol (80%, pH 2·5 and 40%, pH 3·5). The alcohol extract was evaporated under vacuum, redissolved in water and adjusted to pH 4·5–5·0 prior to the

TABLE 1. Origin and cellular toxicity (with standard error, SE) of cultured isolates of *Gonyaulax tamarensis* from New England and eastern Canadian waters

Isolates	Origin	Type of Parent cell[a]	Toxicity (μmouse units cell^{-1}) (SE)
Gonyaulax ♯2	Bay of Fundy, N.B. (45°N)	M	289
Gonyaulax ♯7	Bay of Fundy, N.B. (45°N)	M	254
GtMEM10	Lubec, ME (44°50′N)	T	127 (25)
GtMEF10	Lubec, ME (44°50′N)	T	178 (28)
GtME8	Machiasport, ME (44°30′N)	C	108
GtME6	Petit Manan Point, ME (44°20′N)	C	89
GtMEM21	Monhegan Island, ME (43°45′N)	T	79
GtME20	Monhegan Island, ME (43°45′N)	C	105
GtME1	York River, ME (43°15′N)	C	148
GtG9B	Gloucester Harbor, MA (42°35′N)	C	87
GtSP1	Salt Pond, Eastham, MA (41°45′N)	M	39 (5)
Gtm243	Town Cove, Eastham, MA (41°45′N)	C	Undetectable
Gtm242	Town, Cove, Eastham, MA (41°45′N)	C	Undetectable
Gtm253	Mitchell R, Orleans, MA (41°45′N)	C	37 (5)
Gtm240B	Mill Pond, Orleans, MA (41°45′N)	C	83 (9)
GtMP	Mill Pond, Orleans, MA (41°45′N)	C	70 (6)
GtMP4	Mill Pond, Orleans, MA (41°45′N)	C	96
GtMP9	Mill Pond, Orleans, MA (41°45′N)	C	63 (5)
GtMP21	Mill Pond, Orleans, MA (41°45′N)	C	63
GtMMP103	Mill Pond, Orleans, MA (41°45′N)	M	48
GtMMP104	Mill Pond, Orleans, MA (41°45′N)	M	17 (5)
GtMMP117	Mill Pond, Orleans, MA (41°45′N)	M	95
GtCH4	Nantucket Sound, off Chatham, MA (41°40′N)	M	237 (59)
Gt270	Hyannis Harbor, MA (41°35′N)	C	73
GtPPbl	Perch Pond, Falmouth, MA (41°30′N)	M	43
GtPPkF	Perch Pond, Falmouth, MA (41°30′N)	P	36
GtC	Palmer Cove, Groton, CT (41°20′N)	M	5 (1)
GtCN-1	Palmer Cove, Groton, CT (41°20′N)	P	5
GtC-2	Mumford Cove, Groton, CT (41°20′N)	C	7 (4)
GtLI18-C	Mattituck Creek, NY (41°N)	M	34
GtLI-11	Mud Creek, Moriches Bay, NY (40°45′N)	P	16
GtLI-15	Mud Creek, Moriches Bay, NY (40°45′N)	P	41
GtLI12-A	Mud Creek, Babylon, NY (40°40′N)	M	37 (9)
GtLI12-C	Mud Creek, Babylon, NY (40°40′N)	M	16

[a]The clonal cultures were started from different types of parent cells: M, motile cell isolated from a plankton sample; C, benthic cyst isolated from a sediment sample; T, one of the tetrad cells from a germinated cyst; P, planomeiocyte (newly germinated cell before the first division).

mouse assay (Association of Official Analytical Chemists, 1980). Standardization of the assay was carried out by injecting mice with a range of concentrations of pure toxin and recording the time of death. Toxicity is reported in terms of μmouse units* (μm.u.) per cell, representing the mean of six individual mouse tests. When the remaining extract contained at least 1000 m.u., it was rinsed with chloroform and processed for column chromatography (Bio-Gel P-2) as described previously (Alam *et al.*, 1979). Toxic fractions were identified against a mixture of standard toxins by thin-layer chromatography (Whatman LHP-K) in a solvent system of pyridine:ethyl acetate:water:acetic acid

*One mouse unit is defined as the amount of toxin necessary to kill a 20-g mouse in 15 min (=0·18 μg saxitoxin equivalent) (Shimizu, 1978).

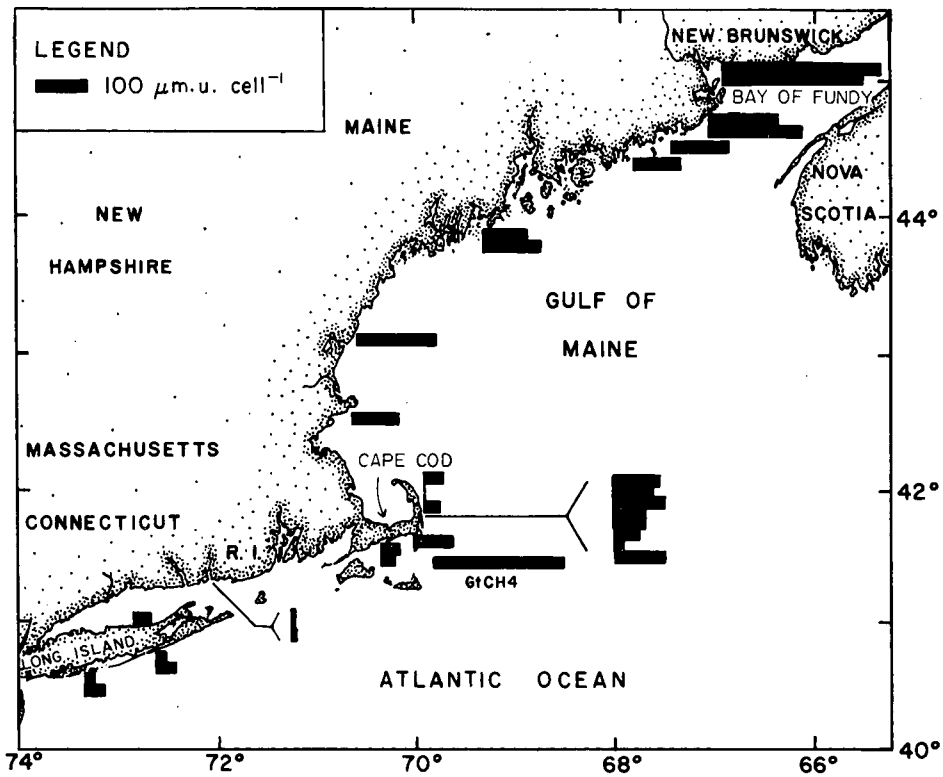

Figure 1. Distribution of toxicity among isolates of *G. tamarensis* collected from the Bay of Fundy to Long Island Sound.

(15:10:4:3). To prevent hydrolysis and transformation of toxins by epimerization, the pH of the samples was kept close to 3 at all steps except for the Bio-Gel P-2 chromatography when the pH was raised to approximately 5.

One clone (Gtm240B) was analyzed five times in a blind test to verify the reproducibility of the analytical procedure. Variation in toxicity between samples originating from the same location was tested by analyzing eight clones from a given estuary (Mill Pond, Orleans, MA), five of which had been isolated from benthic cysts and three from motile cells.

A culture of the diatom *Skeletonema costatum* (clone Sk6C) served as a non-toxic control; it was assayed for toxicity and an extract of 10^9 cells failed to elicit any PSP symptoms in the mice tested.

Results

Toxin content

Toxicity values ranged from 289 μm.u. cell^{-1} to undetectable for *G. tamarensis* clones isolated from sediments or waters between Long Island Sound and the Bay of Fundy, a 5° latitudinal difference (Table 1, Figure 1). All values greater than 100 μm.u. cell^{-1} but one (GtCH4) were found above the 43°N latitude, in areas of recurrent PSP (Figure 2).

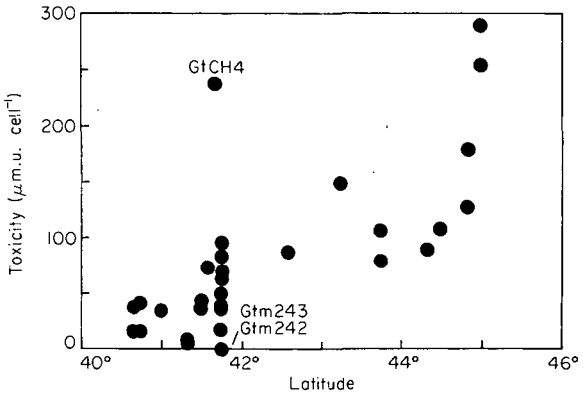

Figure 2. Toxicity values of isolates of *G. tamarensis* as a function of their latitude of origin.

Clones originating from Long Island and Connecticut (below 41°30'N) were of low toxicity, generally less than 40 μm.u. cell^{-1}. The intermediate latitudinal range had low but more variable toxin scores. No toxicity was detected in two clones (Gtm242 and Gtm243) isolated as cysts from the sediments of a protected cove on the east side of Cape Cod.

The data strongly suggest a decrease in the toxin content per cell for populations from north to south (Figure 2). Toxicity values correlate, with latitudinal distance with a coefficient of 0·82, GtCH4, Gtm242 and Gtm243 not included (see p. 407).

Variation within a sample, estimated by repeated analysis of Gtm240B, gave an average toxin content of 83 ± 9 μm.u. cell^{-1} (one standard error about the mean). The eight isolates from Mill Pond averaged 72 ± 11 μm.u. cell^{-1}, with the five clones started from benthic cysts averaging 75 ± 6 μm.u. cell^{-1} and the three clones started from motile cells, 53 ± 23 μm.u. cell^{-1}. An analysis of variance showed no significant differences ($P < 0{\cdot}05$) between Mill Pond clones obtained from cysts and the replicates of Gtm240B (considered here as individual samples) (Table 2). Similarly, the observed variation in toxicity in the Mill Pond clones obtained from motile cells was not significantly different ($P < 0{\cdot}05$) from the variation within a sample or within the cyst population. Thus, the observed variation in toxicity between samples from a given estuary was not significantly different from the variation within a sample.

Toxin composition

Six of the 12 major toxins known to occur in the *Gonyaulax* group, saxitoxin, neosaxitoxin, and the gonyautoxins-I, -II, -III and -IV (Shimizu, 1984) were identified by thin-layer chromatography (TLC) and the appropriate purified standards. Toxins that are weakly adsorbed on Bio-Gel P-2 (C1, C2 or GTX-VIII, C3 and C4) were not recovered, and separation of GTX-V and -VI (also known as B1 and B2; Hall, 1982) was not attempted. The toxin profile (or composition) of nine clones is given on a qualitative basis in Table 3.

Saxitoxin, neosaxitoxin and 3 gonyautoxins (-II, -III and -IV) were generally recovered from all clones. Gonyautoxin-II (GTX-II) and gonyautoxin-IV (GTX-IV) do not separate well from each other in the solvent system used so they are reported here as one indistinguishable pair. In two cases (GtME1 and GtG9B), spots of identifiable shape and color were found in the proper order on the chromatogram but R_f values were

TABLE 2. An analysis of variance for toxin content of clones from a given location where the variance within a clone (Gtm240B) and the variance between clones either obtained from benthic cysts or from motile cells were tested against each other

Sources of variation	Variance	
Gtm240B vs. *clones from benthic cysts*		
between samples	161·6	$F=1·92$
within samples	309·5	where $F_{(8,1)}=239$ (5%)
Gtm240B vs. *clones from motile cells*		
between samples	1696·9	$F=2·16$
within samples	786·5	where $F_{(1,6)}=6·0$ (5%)
Clones from benthic cysts vs. *clones from motile cells*		
between samples	911·1	$F=1·40$
within samples	652·5	where $F_{(1,6)}=6·0$ (5%)

TABLE 3. Toxin profile of some isolates of *Gonyaulax tamarensis*[a]

Isolates	STX	neoSTX	GTX-II/-IV	GTX-III	GTX-I
Gonyaulax ♯2	+	+	+	+	−
Gonyaulax ♯7	+	+	+	+	+
GtMEM10	+	+	+	+	+
GtME1	+	?	?	+	−
GtG9B	?	+	+	+	+
Gtm240B	+	+	+	+	+
GtMMP117	+	+	+	+	−
GtCH4	+	+	+	+	?
GtPPb1[b]	+	+	+	+	+

[a] +, present; −, absent or below detection; ?, spot present but questionable identification.
[b] The toxin profile for GtPPb1 is from Alam *et al.* (1979).

sufficiently different from that of the reference to make us uncertain of the presence of some toxins. However, trace amounts of salts in the Bio-Gel P-2 purified sample could have been responsible for the altered migration rate of the toxins on the TLC plate. Gonyautoxin-I (GTX-I) was often missing from our analyses. GTX-I is relatively unstable and difficult to recover due to its poor fluorescence yield under UV and the interference of a pigment spot of slightly lower R_f value (Shimizu, 1979).

Discussion

Variations in toxin content from 289 μm.u. cell^{-1} to undetectable levels were observed among 34 strains of *G. tamarensis* isolated from waters or sediments between New Brunswick, Canada, and Long Island, NY. These toxicities were highest for the northern isolates and lowest for those in the south. A general north-to-south trend of decreasing toxicity is suggested. With the repeated analysis of clone Gtm240B (83 ± 9 μm.u. cell^{-1} (SE)) providing an indication of reproducibility, we conclude that

the observed two-order-of-magnitude difference in toxin content among the isolates is real and does not result from the sampling or the analytical procedures.

One clear exception to the observed toxicity pattern was clone GtCH4 (237 μm.u. $cell^{-1}$) a culture established from a motile cell in a water sample collected two miles south of Cape Cod, an area where water from the Gulf of Maine can be advected (Anderson *et al.*, 1982). It is possible that this isolate is not representative of its region of origin but instead is from an imported population. This suggests caution in interpreting toxicity data obtained from motile cell isolates, especially where large-scale movement of water can be implicated in population transport.

Gtm242 and Gtm243 were also anomalous as neither had detectable toxicity. Both were tested three times, always with negative results. They were identified as *G. tamarensis,* though the cells were generally smaller than other isolates. Furthermore, they did not always grow well under our culture conditions. Loeblich and Loeblich (1975) and Yentsch *et al.* (1978) have also reported non-toxic *G. tamarensis.*

Before discussing the implications of the observed north-to-south variation in toxicity, it is important first to examine the validity of this pattern. An initial concern is whether one can characterize the toxicity of an isolate by a single number when it is known that the toxin content of some *G. tamarensis* strains can vary by as much as two- to four-fold during the stages of growth in batch culture or under different environmental conditions (Prakash, 1967; White, 1978; White & Maranda, 1978; Schmidt & Loeblich, 1979; Hall, 1982; Singh *et al.*, 1982). We selected the transition period between late log phase and early stationary phase growth as the best time to harvest the cultures. The studies mentioned above and our own data generally show a decrease in toxicity from early log phase to stationary phase growth, with the amount of toxin per cell remaining relatively constant from late log phase on. Conditions of salinity, temperature, light and nutrients were constant throughout.

A second concern is whether the analysis of clonal cultures under defined light, temperature and nutrient conditions provides an accurate estimate of the toxin content of populations in the natural environment. Stated differently, even though all clones grew under the chosen laboratory conditions, we do not know whether these conditions were optimal for the growth of all isolates. As mentioned above, culture effects could explain two- to four-fold variations in toxin content between isolates – but not the two-order-of-magnitude difference we observed.

A third concern is whether one isolate is representative of the region from which it was collected. Our toxicity analyses of samples originating from a given estuary (Mill Pond) suggest that in certain locations, clones obtained from either cysts or motile cells can represent regional populations. This representation would be strongest in those areas where the *G. tamarensis* populations are patchy or localized – in effect the area extending from Cape Cod to Long Island (Anderson *et al.*, 1982). Further north, the species is more uniformly distributed, with widespread cyst accumulations in estuarine and deeper coastal waters (Lewis *et al.*, 1979; Anderson *et al.*, 1982; White & Lewis, 1982; Thayer *et al.*, 1983) so single isolates are less likely to be representative of specific bloom populations in that region. One also sees from clone GtCH4 that a motile cell collected from nearshore waters could deviate markedly from the apparent trend when advective processes are involved.

Despite a high degree of correlation between toxin content and latitudinal distance, it may not be prudent to describe the toxin data in Fig. 2 as a trend given our reservations about the link between an isolate and a region. A conservative conclusion would be that

G. tamarensis populations along the north-west Atlantic coast can be divided into two groups on the basis of toxin content and geography – a northern, high toxicity group from New Brunswick to the north of Cape Cod and a southern group with low toxicity from Cape Cod to Long Island. A general north-to-south trend of decreasing toxicity is suggested but requires more data for verification.

Our results are consistent with the general pattern of PSP episodes within the western north Atlantic region. Major outbreaks with high shellfish toxin levels are common in Maine and New Brunswick, whereas the southern waters have less frequent, lower toxicity closures (Hurst, 1979; Anderson *et al.*, 1982; Anderson, unpublished). The absence of shellfish closures in Long Island and Connecticut, despite the documented presence of *G. tamarensis* cysts (Anderson *et al.*, 1982) and motile cells (Schrey *et al.*, 1984), is consistent with the low toxicity of the isolates from that region. A bloom with cell concentrations one to two orders of magnitude higher than those occurring to the north would be required to reach PSP quarantine levels.

It is interesting to note that cultures of *G. tamarensis* originating from estuaries on the east coast of Japan show a similar geographic pattern in their toxicity. In a north-to-south trend, two clones from Funka Bay (42°30′N) contained 143 and 100 μm.u. cell^{-1}, respectively, whereas 20 clones from Ofunato Bay (39°N) ranged between 27 and 67 μm.u. cell^{-1} for an average of 38 ± 3 (SE) (Oshima *et al.*, 1982*a*,*b*; Nishihama, personal communication). One clone from Owase Bay (30°N) had a toxin content of 25 μm.u. cell^{-1} but its identification as *G. tamarensis* is uncertain (Hashimoto *et al.*, 1976; Fukuyo, 1979). Several toxicity values can be found for clones originating from the eastern North Pacific waters (Shimizu, 1979; Hall, 1982) but the presence of two additional species (*G. catenella* and *G. acatenella*, both closely resembling *G. tamarensis*) creates a taxonomic problem that restricts comparisons.

The reasons for the geographic pattern that we observed in *G. tamarensis* toxicity are unknown. One explanation for the pattern in Fig. 1 is that the clonal differences are real and that environmental factors have segregated low toxicity populations in the southern portion of our region and high toxicity strains to the north. Physiological variability among clones of the same species has been demonstrated for several taxonomic groups of the phytoplanktonic community (Brand, 1981; Gallagher, 1982). Differences in growth rates, temperature and salinity requirements, nutrient uptake kinetics, luminescence of clones grown under similar conditions have all been used to distinguish between ecotypes (Guillard & Ryther, 1962; Carpenter & Guillard, 1971; Underhill, 1977; Schmidt *et al.*, 1978) and certain of these ecotypes can be separated genetically by electrophoretic analysis of enzymes (Gallagher, 1980; Hayhome & Pfiester, 1983). The logical inference is that certain *G. tamarensis* genotypes may be more suited for growth in the southern region than others and that the observed phenotypic variations (toxin content) are an indirect result of selection for these strains.

Alternatively, it is also possible that the toxin distribution in Fig. 1 is a reflection of the pattern of species dispersal. If *G. tamarensis* was introduced to the southern region relatively recently compared to the more established northern populations (Anderson *et al.*, 1982), then advective dispersal from one or two low toxicity populations (e.g. from Cape Cod) could explain the Connecticut and Long Island toxin distribution without invoking environmental selection.

One way to distinguish isolates from each other might be through toxin composition (Shimizu, 1979; Hall, 1982). The nine toxin profiles in Table 2 show no differences between isolates as five of the six recovered toxins were present in all isolates tested. The

sixth toxin (GTX-I) is too unstable to be used as a discriminating character. It is clear that our qualitative analysis of toxin composition was insufficient to resolve clonal differences and that a quantitative analysis including all 12 toxins is required. Since the 12 toxins do not have all the same degree of potency (Genenah & Shimizu, 1981; Hall, 1982), a quantitative toxin profile could potentially explain the observed differences in overall toxicity among isolates.

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