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PARALYTIC SHELLFISH POISONING IN NORTHWEST SPAIN: THE TOXICITY OF THE DINOFLAGELLATE *GYMNODINIUM CATENATUM*

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D. M. ANDERSON, J. J. SULLIVAN and B. REGUERA. Paralytic shellfish poisoning in northwest Spain: the toxicity of the dinoflagellate *Gymnodinium catenatum*. *Toxicon* **27**, 665–674, 1989.—The highly productive mussel fishery in the Rias Bajas region of northwest Spain has experienced several outbreaks of paralytic shellfish poisoning (PSP) beginning in 1976. In this study, similarities in the HPLC analyses of extracts from toxic shellfish, plankton tows and cultured dinoflagellates from the Rias Vigo and Pontevedra clearly indicate that *Gymnodinium catenatum* Graham is the organism responsible for recent PSP episodes. The toxin profile of the dinoflagellate contains an unusually high proportion of the low potency sulfocarbamoyl toxins (*ca.* 90–95 mole %), although a major portion of the overall toxicity is due to the more potent saxitoxin that is present at 5–10% of the total. Toxin profiles of shellfish showed approximately the same composition as that of the dinoflagellate, although the shellfish contained several carbamate toxins (GTX I, GTX II, GTX IV and NEO) that were not detected in *G. catenatum* culture extracts. The shellfish also contained decarbamoyl toxins (dc-GTX II and dc-GTX-III) at approximately 2% of the total profile. Since these were not detected in the dinoflagellate, their presence reflects either chemical or enzymatic conversion within the shellfish.

INTRODUCTION

THE GALICIAN Rias Bajas of northwestern Spain are the sites of intensive raft mussel culture, providing a major portion of the world's supply of the blue mussel. It was thus a major setback when, in the fall of 1976, an epidemic of paralytic shellfish poisoning (PSP) occurred in several European countries, with the source of the toxin being mussels from the Rias Vigo and Pontevedra (LUTHY *et al.*, 1978; LUTHY, 1979). Analysis of the mussels detected saxitoxin and several of its derivatives (LUTHY *et al.*, 1978), but the causative planktonic organism was not identified. PSP was subsequently detected in 1981, 1985, 1986 and 1987. Fortunately, during the latter 3 years an extensive field program was already underway to study dinoflagellate populations and their interactions with the unique hydrography of the region (FRAGA *et al.*, 1988). During each of these three PSP episodes, two dinoflagellate species were dominant in the Ria de Vigo—*Gymnodinium*

catenatum and *Alexandrium affine,* both of which were suspected source organisms for the paralytic shellfish toxin. The former species had been linked to PSP in Mexico in 1979 (MEE *et al.,* 1986) and the latter is closely related to other well known toxic species in the genus *Alexandrium* (=*Protogonyaulax*). In this paper, we compare the toxin characteristics of shellfish to those of plankton samples and cultures of the two dinoflagellate species and demonstrate that *G. catenatum* is the toxin progenitor in that region of Spain.

MATERIALS AND METHODS

Dinoflagellates

Gymnodinium catenatum strains GC1V, GC3V and GC11V and *Alexandrium affine* strain AA71V were used in this study. GC3V, GC11V and AA71V were established from cyst germinations using sediment collected from the Ria de Vigo in 1985. GC1V was established from a motile cell isolated from the plankton during the 1985 bloom in Ria de Vigo.

All cultures were grown in 2 liter flasks containing 1 liter of seawater-based K medium (KELLER and GUILLARD, 1985) at 20°C under a 14:10 h light:dark cycle (*ca.* 250 μEinst./m^2/sec provided by cool white fluorescent bulbs). Cells were harvested in mid-to-late exponential growth as follows: Cultures were divided into 4–250 ml aliquots and chilled to 4°C to help concentrate the cells by retarding swimming activity. Once cooled, the samples were centrifuged at 4°C for 25 min at 2700 × ***g***. After the supernatant was removed by aspiration, the 'pellet' was successively transferred to smaller centrifuge tubes (50 ml then 15 ml) until the contents of the four 15 ml tubes could be combined into one tube. This number of centrifugation steps was necessary because *G. catenatum* only formed loosely packed pellets due to its mucilaginous secretions.

Alexandrium affine cells were harvested by pouring the culture through a 10 μm mesh net and then backwashing the concentrate into a 15 ml centrifuge tube which was then centrifuged at 1700 × ***g*** for 3 min at room temperature. After the final centrifugation, cells were extracted with 1–3 ml of 0.05 M acetic acid, depending upon pellet size (final concentration 0.025 M), and stored frozen at −20°C. Prior to HPLC analysis, the samples were thawed and frozen three times to lyse all cells for efficient toxin extraction, then centrifuged at 1700 × ***g*** for 3 min. Four hundred microliters of the supernatant was filtered through 0.45 μm Gelman LC3S filters into a teflon capped, borosilicate auto-analyzer vial.

Natural dinoflagellate populations were collected in vertical hauls of a 20 μm mesh plankton net at Limens (Ria de Vigo) on 25 September 1986 (four tows) and from Aldan (Ria de Pontevedra) on 21 October 1986 (one tow). *G. catenatum* was the dominant species in these five samples. An additional tow was collected on 17 November 1986, when *A. affine* was dominant, although some *G. catenatum* were also present in low numbers. Plankton tows were sedimented by gentle centrifugation and the seawater aspirated and discarded. The cell pellet was suspended in a small volume of 1 N acetic acid and frozen. Samples for HPLC analysis were shipped on dry ice to the U.S.A.

Mussels

Mussels (*Mytilus galloprovincialis;* SANJUAN *et al.,* in press) were collected from rafts at Limens (Ria de Vigo) on 23 September and 13 October, from Aldan on 28 October (Ria de Vigo), and from Bueu (Ria de Pontevedra) on 28 October 1986. One hundred grams of mussel meat were blended for 2 min with 100 ml of 0.1 N HCl without boiling in a minor modification of the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC) (1975) method. The mixture was centrifuged and the supernatant frozen. Samples for HPLC analysis were shipped on dry ice to the U.S.A.

Toxin analysis

Prior to HPLC analysis, the shellfish extracts were thawed, centrifuged, and a small volume diluted with 9 volumes of water. The sample was centrifuged (1000 × ***g***) and a small portion of the supernatant (*ca.* 1 ml) passed through a 10,000 mol.wt cutoff ultrafiltration membrane (YM-10, Amicon Corp., Danvers, MA, U.S.A.). All sample extracts were analyzed for toxin composition by HPLC (SULLIVAN and WEKELL, 1988) using the conditions specified in Table 1. Briefly, the method involves separation of the various PSP toxins by ion-interaction chromatography with detection by fluorescence following post-column oxidation with periodate. Standards containing the 12 sulfocarbamoyl and carbamate toxins (Fig. 1) were utilized for qualitative peak assignments and for quantitation of the carbamate toxins in the sample extracts. To determine the levels of the various sulfocarbamoyl toxins, a portion of the extract was adjusted to 0.2 N HCl and heated at 100°C in a sealed vial to hydrolyze the sulfocarbamoyl toxins to their carbamate form. The quantities of the sulfocarbamoyl toxins were then derived from the difference in the carbamate toxin concentrations before and after hydrolysis. The levels of decarbamoyl GTX II and decarbamoyl GTX III were calculated assuming that the responses of the carbamate and decarbamoyl toxins were the same. For the purposes of this study, this assumption was necessary since no standards were available for the decarbamoyl toxins.

TABLE 1. CONDITIONS UTILIZED FOR HPLC SEPARATION OF THE PSP TOXINS

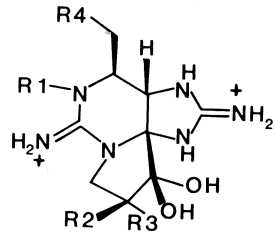
Column:	Hamilton Co., PRP-1, 15 cm × 4.1 mm, 10 μm packing		
Column: Flow	1.3 ml/min		
Temp.	35°C		
Mobile phase A:	Water with 1.5 mM each hexane and heptane sulfonate and 1.5 mM ammonium phosphate (as PO4), pH 6.7.		
Mobile phase B:	25% acetonitrile with 1.5 mM ea. hexane and heptane sulfonate and 6.25 mM ammonium phosphate (as PO4), pH 7.0		
Gradient conditions:	Time (minutes)	%A	%B
	0	100	0
	4	100	0
	11	70	30
	17	10	90
	17.5	0	100
	18.5	0	100
	19	100	0
Post column reaction system:			
Pump A:	0.5 ml/min flow of 5 mM periodate in 0.1 M sodium phosphate at pH 7.8. Reaction in 1.0 ml coil held at 90°C		
Pump B:	0.3 ml/min flow of 0.75 M nitric acid		
Fluorescence detector:	Excitation—340 nm (15 nm slit) Emission—400 nm (20 nm slit)		

In addition to the HPLC analysis, mouse bioassays were used to determine the toxin content of culture extracts. These samples were extracted according to the ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (1975) procedure, with no modifications.

RESULTS

Blooms

During November 1985, the detection of PSP toxins in shellfish from Ria de Vigo and Ria de Pontevedra coincided with the sudden appearance and numerical dominance of the dinoflagellates *G. catenatum* and *A. affine* in the plankton (FRAGA *et al.*, 1988). Maximum concentrations of *G. catenatum* and *A. affine* were approximately 5 and 8×10^4 cells per liter, respectively. Monitoring for toxins in commercial shellfish by mouse bioassay showed that clams, cockles, mussels and other bivalves were contaminated with PSP toxins (up to 3990 μg STX per 100 g). From September to November 1986, dense blooms of *G. catenatum* occurred once again in Vigo and Pontevedra, with toxin levels reaching 2640 μg STX per 100 g. *G. catenatum* was the dominant dinoflagellate species starting around 25 September, with other dinoflagellates (*Ceratium horidum, C. fusus, C. candelabrum, Protoperidinium depressum* and *P. divergens*) and a few diatoms at background concentrations. *Heterosigma akashiwo* was also dominant in September, but disappeared by late October. Diatoms were more abundant than dinoflagellates, but in some sections of the rias, *G. catenatum* concentrations were as high as 2×10^6 cells/liter. In November, *G. catenatum* concentrations declined to less than 10^4 cells/liter as *A. affine* became the dominant species.



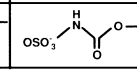
Carbamate Toxins	N-Sulfocarbamoyl Toxins	Decarbamoyl Toxins	R1	R2	R3
STX	B1	dc-STX	H	H	H
NEO	B2	dc-NEO	OH	H	H
GTX I	C3	dc-GTX I	OH	H	OSO_3^-
GTX II	C1	dc-GTX II	H	H	OSO_3^-
GTX III	C2	dc-GTX III	H	OSO_3^-	H
GTX IV	C4	dc-GTX IV	OH	OSO_3^-	H
		HO	← R4		

FIG. 1. STRUCTURES OF THE PSP TOXINS.
Several of the toxins have not been reported in the literature (dc-NEO, dc-GTX I, dc-GTX IV), but are likely to occur in selected shellfish species based on the documented existence of the other decarbamoyl toxins. Abbreviations: STX-saxitoxin; NEO-neosaxitoxin; GTX-gonyautoxin; DC-decarbamoyl.

HPLC toxin analysis

HPLC analysis of a mixed toxin standard showed that the carbamate toxins (Fig. 1) could be resolved sufficiently for individual quantification (Fig. 2). Figure 3 illustrates the effects of acid hydrolysis on a chromatogram of a typical *G. catenatum* extract. The

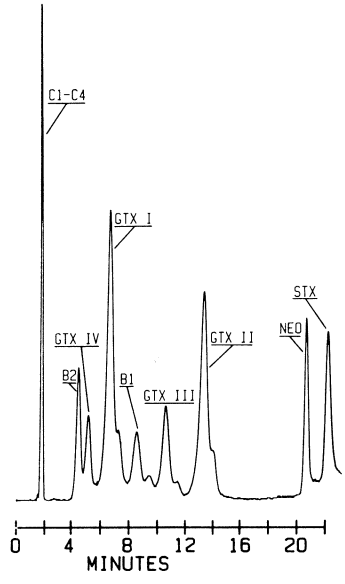


FIG. 2. HPLC CHROMATOGRAM OF THE CARBAMATE AND SULFOCARBAMOYL PSP TOXIN STANDARDS. Conditions as per Table 1. Quantity injected in pmoles: C1/C2 = 9, B2 = 34, GTX IV = 12, GTX I = 60, B1 = 20, GTX III = 3, GTX II = 9, NEO = 34, STX = 19.

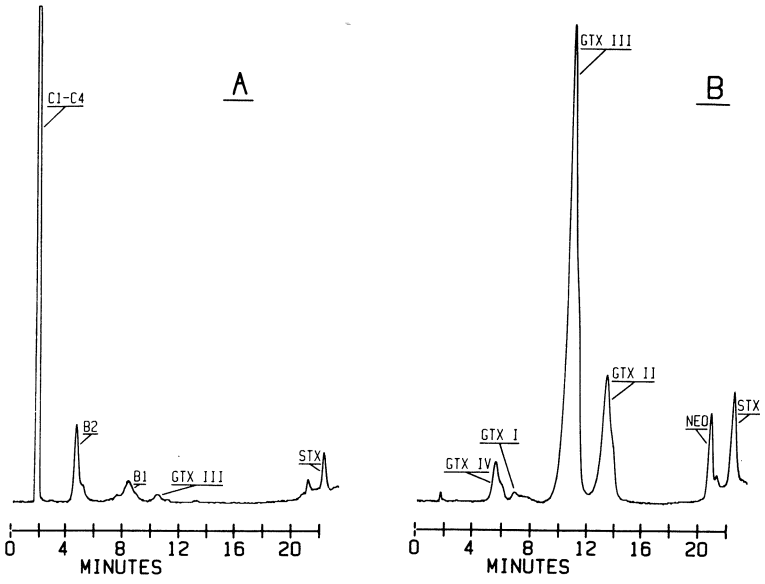


FIG. 3. HPLC CHROMATOGRAM OF A TYPICAL *G. catenatum* EXTRACT BEFORE (A) AND AFTER (B) HYDROLYSIS OF THE SULFOCARBAMOYL TOXINS TO THE CARBAMATE FORM (5 μl OF AN EXTRACT AT 10^5 cells/ml).

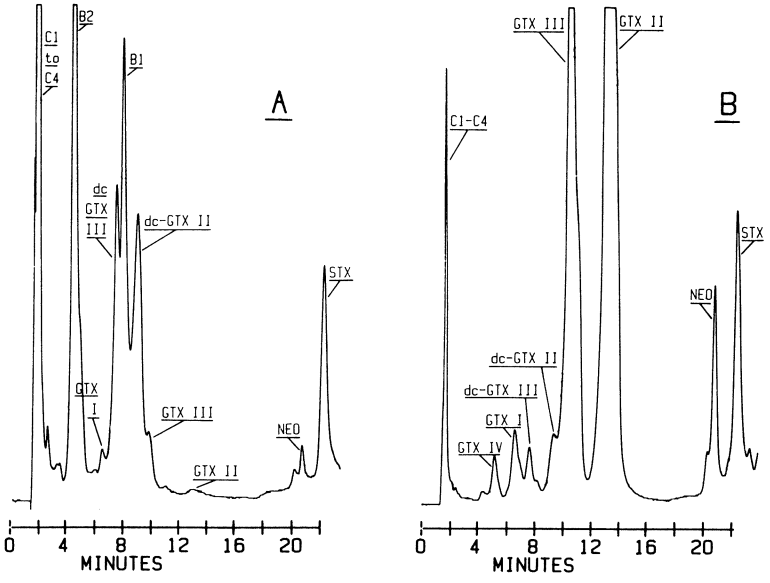


FIG. 4. HPLC CHROMATOGRAM OF A TYPICAL VIGO SHELLFISH SAMPLE EXTRACT BEFORE (A) AND AFTER (B) HYDROLYSIS OF THE SULFOCARBAMOYL TOXINS TO THE CARBAMATE FORM.
A. Five microliters of an extract at 500 mg tissue per ml; B. Five microliters of an extract at 125 mg tissue per ml.

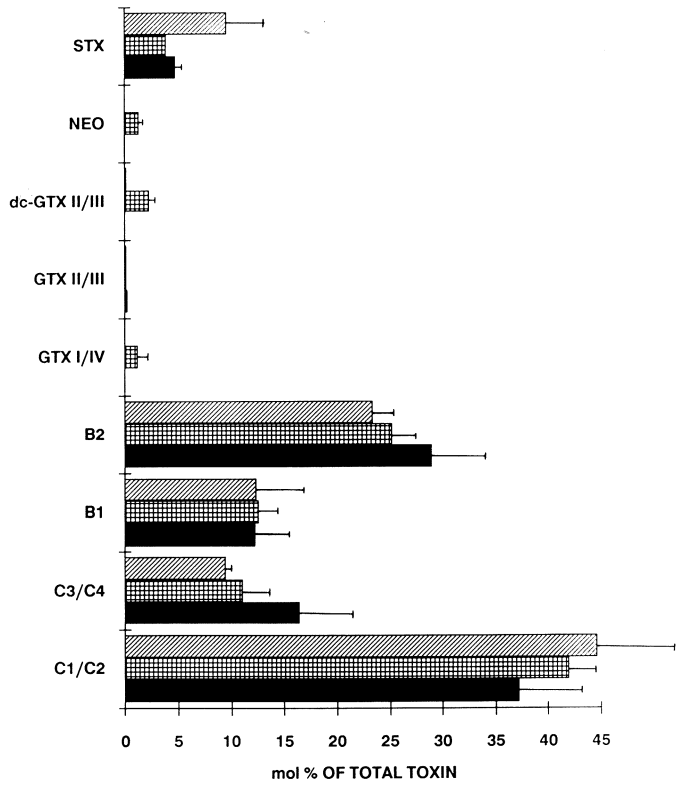


FIG. 5. COMPARISON OF THE AVERAGE TOXIN COMPOSITION OF THE SHELLFISH (CHECKERED COLUMNS, $n=5$), FIELD *G. catenatum* POPULATIONS FROM PLANKTON TOWS (SOLID COLUMNS, $n=5$) AND CULTURED *G. catenatum* (DIAGONAL COLUMNS, $n=2$).
Error bars denote one standard deviation.

substantial increase in the peak heights of the carbamate toxins after hydrolysis documents the dominance of the sulfocarbamoyl derivatives in these samples (*ca.* 90–95% of the total). This pattern was observed both for the extracts from cultured *G. catenatum* and for the plankton tows from the blooms of that species in September 1986 (Fig. 5). The dominant toxins in these dinoflagellate samples were C1/C2 and B2. Applying the potencies of each of the individual toxins (HALL and REICHARDT, 1984) to the HPLC results, we calculate the average overall toxicity of *G. catenatum* as 7.5×10^{-6} μg STX equiv. per cell (range $7.1–7.8 \times 10^{-6}$). Mouse bioassay results yielded an estimate of 20×10^{-6} μg STX equiv. per cell for this species (J. Hurst, personal communication). The bioassay samples were extracted using the standard AOAC (1975) method with boiling in HCl, which would be expected to hydrolyze some of the sulfamates to their more potent carbamate forms. Our HPLC samples were not boiled.

Cultured *A. affine* was judged to be non-toxic using both HPLC and mouse bioassay analysis. A plankton tow during the *A. affine* bloom in November 1986 contained trace amounts of toxins, presumably from the few remaining *G. catenatum* cells still present. It is also noteworthy that a small species of *G. catenatum* (strain GC3V) which was isolated from a cyst in Ria de Vigo sediments was non-toxic.

Toxin profiles of shellfish collected in the two rias during the 1986 bloom contained PSP toxins with approximately the same composition as the dinoflagellate samples (Fig. 4). However, in addition to the sulfocarbamoyl toxins, the shellfish contained trace levels of several carbamate toxins which were not apparent in the dinoflagellates (GTX I, GTX II, GTX IV, NEO). In addition, the shellfish contained decarbamoyl toxins (dc-GTX II and dc-GTX III) at approximately 2% of the total profile.

DISCUSSION

Similarities between the toxin composition of natural plankton samples, cultured dinoflagellates, and toxic shellfish all indicate that *G. catenatum* is the species responsible for recent paralytic shellfish poisoning outbreaks in the Rias Bajas region of northwest Spain (Fig. 5). Not only do the toxin profiles of that dinoflagellate match those of the shellfish, but the other co-occurring suspect organism. *A. affine* was shown to be non-toxic. We used HPLC to analyze shellfish from only the 1986 episode, but the presence of *G. catenatum* in high numbers when PSP was detected in the Rias Vigo and Pontevedra in the fall of 1976 (ESTRADA *et al.*, 1984), 1985 (FRAGA *et al.*, 1988) and 1987 (unpublished observations) suggests a common toxin progenitor for these other years.

Our efforts to establish the link between the toxic shellfish and a source organism were facilitated by HPLC analysis which provides toxin composition 'fingerprints'. As demonstrated by Hall (Ph.D. thesis, University of Alaska, 1982) and BOYER *et al.* (1987), the mixture of saxitoxin and its derivatives in a dinoflagellate strain does not vary significantly with growth stage or nutrient status. The situation is not quite as simple when shellfish extracts are involved, since SHIMIZU and YOSHIOKA (1981) demonstrated the bioconversion of paralytic shellfish toxins in scallop tissues, and SULLIVAN *et al.* (1983) and SULLIVAN (1982) described enzymatic decarbamoylation of these same toxins by the clam *Prototheca staminea*.

Fortunately, in our study the toxin profiles of shellfish collected in the vicinity of the dinoflagellate blooms contained PSP toxins with approximately the same composition as the dinoflagellates, both cultured and wild (Fig. 5). The shellfish did, however, contain trace amounts of certain carbamate and decarbamoyl toxins which were not detected in the dinoflagellates. The source of these additional toxins is probably *G. catenatum*, with the difference in the toxin profiles being a result of either chemical or enzymatic conversion of the sulfocarbamoyl forms. It is of note that enzymatic decarbamoylation in the clam *P. staminea* converts the vast majority of acquired toxin into the decarbamoyl form (SULLIVAN *et al.*, 1983) whereas in the Spanish shellfish samples, only about 2–3% of the total toxin was decarbamoylated. As decarbamoyl toxins were not detected in the dinoflagellate extracts, decarbamoylation presumably occurred in the shellfish following ingestion.

The presence of the carbamate PSP toxins in the shellfish but not in *G. catenatum* is likely due to either hydrolysis of the sulfocarbamoyl form to the carbamate form (a conversion that occurs at $pH < 2$: HALL *et al.*, 1980) or to selective retention of the carbamate form in the shellfish. Under the HPLC conditions utilized in this study, the decarbamoyl forms of saxitoxin and neosaxitoxin were not separated and individually quantified. If present, these toxins (dc-STX and dc-NEO) would co-elute with NEO and STX. The reported levels of NEO and STX therefore represent the sum total of the decarbamoyl and carbamate forms. Based on the presence of the decarbamoyl forms of

GTX II and GTX III in the shellfish, it is possible that a substantial amount of the reported levels of NEO and STX may be due to the decarbamoyl forms of these toxins. Alternate HPLC conditions would be required to separate and individually quantify dc-NEO and dc-STX. Similarly, the HPLC is not capable of separating dc-GTX I or dc-GTX IV.

Paralytic shellfish toxins were first detected in Spanish mussels in 1976, and some analyses were conducted to characterize those toxins (LUTHY *et al.*, 1978; LUTHY, 1979). Due to analytical limitations at that time and to the somewhat degraded condition of the shellfish samples analyzed (Y. Shimizu, personal communication), it is difficult to compare our HPLC composition data from the most recent blooms with the published compositions from 1976. For example, LUTHY *et al.* (1978) and LUTHY (1979) separated and identified toxins using column and thin layer chromatography, finding STX, GTX I, II, III, IV, V and an unknown toxin. GTX V (an equivalent and older name for toxin B1) was the most abundant. Our analyses of *G. catenatum* isolates from the 1985 bloom showed relatively low amounts of the four gonyautoxins, but B1 and STX were present in significant quantities. It would be unwise to conclude that the 1976 PSP outbreak was or was not caused by *G. catenatum*, given the different methods of toxin separation and analysis and the potential for biological transformations in the degraded 1976 samples. However, it is noteworthy that the outbreak occurred in October and November, the same months as the episodes in 1985, 1986 and 1987 (FRAGA *et al.*, 1988; unpublished observations). Also, during the 1976 outbreak, *G. catenatum* was present in the Ria de Pontevedra at concentrations exceeding 20,000 cells/liter (ESTRADA *et al.*, 1984).

The toxin composition and content of the Spanish *G. catenatum* are noteworthy for several reasons. First, the toxin profile of this species has an unusually high proportion (95%) of sulfocarbamoyl toxins on a mole percent basis (Fig. 5) compared to PSP-producing *Alexandrium* (= *Protogonyaulax*) cultures (S. Hall, Ph.D. thesis, University of Alaska, 1982; CEMBELLA *et al.*, 1987). These are low potency toxins, however, and account for only 26% of the total toxicity (in STX equivalents) in cultured *G. catenatum*. In contrast, STX accounts for 73% of the toxicity in our two isolates of this species, even though it represents an average of approximately 10% of the total toxins. This type of toxin profile has major public health implications, since the relatively low potency sulfamate toxins can be converted under acidic conditions to the much more potent carbamate toxins (N. H. Proctor, Ph.D. thesis, University of California, San Francisco, 1973; S. Hall, Ph.D. thesis, University of Alaska, 1982). Uncertainties that must be resolved include the extent to which this conversion occurs during the extraction for the standard mouse bioassay (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, 1975) relative to that occurring either at the low pH of the human stomach or when the shellfish are pickled in vinegar, a common method for mussel preparation in the Rias Bajas region. Depending on the extent of these interconversions, the mouse bioassay could either overestimate or underestimate the human oral potency of shellfish containing *G. catenatum* toxins.

Toxin composition analysis also allows comparisons to be made between the Spanish strains of *G. catenatum* and those causing PSP in Tasmanian (OSHIMA *et al.*, 1987) and Japanese shellfish (IKEDA *et al.*, 1988). A number of similarities are immediately apparent. Much as we observed, the Tasmanian *G. catenatum* cultures produced predominantly sulfocarbamoyl toxins (98–99 mole per cent) whereas these comprised only 77–93% of the total in the shellfish (90–95 and 81%, respectively, in our samples). The Japanese *G. catenatum* produced predominantly sulfocarbamoyl toxins as well, although data were

only presented qualitatively (IKEDA *et al.*, 1988). Several carbamate toxins (GTX I, GTX II and GTX III) were found in Tasmanian shellfish but not in *G. catenatum* cells from that region, again in agreement with our results. Also, dc-STX was 10–40 times higher in mussels than in the cultured dinoflagellate (OSHIMA *et al.*, 1987). We did not measure dc-STX, but did see dc-GTX II and dc-GTX III in shellfish but not dinoflagellates from Vigo.

There are, however, some differences between the two *G. catenatum* populations that have been analyzed in the most detail, notably in the relative proportion of individual toxins. For example, the Tasmanian strains contained virtually no STX, compared to 5–10% in our field dinoflagellates and cultures. Also, OSHIMA *et al.* (1987) found that toxin composition varied between laboratory and field populations of *G. catenatum* (especially in the relative proportions of the C1/C2 vs C3/C4 toxins) whereas our culture analyses were quite similar to those of the plankton tows (Fig. 5).

We consider these differences to be slight, however, and view the Tasmanian and Spanish *G. catenatum* populations and shellfish toxicity patterns as remarkably similar, given the large distances that separate these regions. It is noteworthy that in both regions, the apparent historical absence of PSP in shellfish or of *G. catenatum* in plankton records has led to speculation that the species may have been introduced—perhaps by man's activities via ship ballast water, shellfish importation or some similar long distance transport (ANDERSON, 1988; HALLEGRAEFF *et al.*, 1988). Such transport would have been facilitated by the species ability to form resistant resting cysts as described by ANDERSON *et al.* (1988).

This dinoflagellate is not abundant globally, having been reported only in Mexico (GRAHAM, 1943; MOREY-GAINES, 1982), Argentina (BALECH, 1964), Spain (ESTRADA *et al.*, 1984), Tasmania (HALLEGRAEFF and SUMNER, 1986) and Japan (IKEDA *et al.*, 1988). There is a clear need to compare isolates from these different regions using HPLC toxin composition analysis, isozyme electrophoresis or other markers. It may be that *G. catenatum* isolates will be genetically similar compared to populations of the toxic *Alexandrium tamarense/catenella* complex which are widely distributed globally and are genetically diverse (CEMBELLA *et al.*, 1987; SAKO *et al.*, 1988).

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