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Paralytic toxin profile of the marine dinoflagellate *Gymnodinium catenatum* Graham from the Mexican Pacific as revealed by LC-MS/MS

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The paralytic shellfish toxin (PST) profiles of *Gymnodinium catenatum* Graham have been reported for several strains from the Pacific coast of Mexico cultured under different laboratory conditions, as well as from natural populations. Up to 15 saxitoxin analogues occurred and the quantity of each toxin depended on the growth phase and culture conditions. Previous analysis of toxin profiles of *G. catenatum* isolated from Mexico have been based on post-column oxidation liquid chromatography with fluorescence detection (LC-FLD), a method prone to artefacts and non-specificity, leading to misinterpretation of toxin composition. We describe, for the first time, the complete toxin profile for several *G. catenatum* strains from diverse locations of the Pacific coast of Mexico. The new results confirmed previous reports on the dominance of the less potent sulfocarbamoyl toxins (C1/2); significant differences, however, in the composition (e.g., absence of saxitoxin, gonyautoxin 2/3 and neosaxitoxin) were revealed in our confirmatory analysis. The LC-MS/MS analyses also indicated at least seven putative benzoyl toxin analogues and provided support for their existence. This new toxin profile shows a high similarity (> 80%) to the profiles reported from several regions around the world, suggesting low genetic variability among global populations.

Keywords: toxin profile; tandem mass spectrometry; benzoyl analogues; paralytic shellfish toxins; *Gymnodinium catenatum*

Introduction

The toxic marine dinoflagellate *Gymnodinium catenatum* is a conspicuous inhabitant of warm–temperate, subtropical and tropical waters, with a wide global distribution, including the Iberian Peninsula, Pacific coast of North America, Southeast Asia and Australia (reviewed by Hallegraeff et al. 2011). This species is occasionally responsible for incidents of shellfish poisoning caused by the consumption by humans of shellfish contaminated with paralytic shellfish toxins (PSTs) produced by the dinoflagellate. Although *G. catenatum* was first described from material collected in the Gulf of California in 1939 (Graham 1943), it was not until the 1980s that it was associated with shellfish poisoning (Mee et al. 1986). Among the PSTs, up to 15 analogues of the neurotoxin saxitoxin have been reported for strains isolated from the coast of Mexico (Gárate-Lizárraga et al. 2005; Band-Schmidt et al. 2006, 2010; Bustillos-Guzmán et al. 2012). Recently, at least four benzoyl saxitoxin analogues have been tentatively identified from two Mexican strains (Bustillos-Guzmán et al. 2011). Proton magnetic nuclear resonance (^{1}H-NMR) spectroscopy proved the presence of benzyl rings from *G. catenatum* toxin extracts (Durán-Riveroll et al. 2013).

The lack of certified analytical standards is one of the main critical limitations for qualitative and quantitative analyses of novel saxitoxin analogues, other than the common carbamoyl and N-sulfocarbamoyl derivatives found in dinoflagellates, certain cyanobacteria and accumulated in shellfish (Vale 2011). This has imposed a particular constraint on the confirmatory structural analysis of recently discovered benzoyl analogues (Negri et al. 2007; Vale 2008b, 2010). In many previous studies, lack of toxin standards has compromised both quantification and confirmatory structural analysis, particularly if results are inferred from available toxin response factors of other analogues, such as saxitoxin. The pre- and post-column oxidation HPLC methods are the most common chemical methods for paralytic shellfish poisoning (PSP) analysis. Despite the many benefits of each, which include an increased sensitivity especially for non-1N-hydroxylated toxins and less variability in the results, they also present some drawbacks (Rodríguez et al. 2010; DeGrasse et al. 2011). For instance, when PST analysis is conducted by liquid chromatography with fluorescence detection (LC-FLD), misidentification may also occur if two or more toxins have the same retention time. Finally, when extraction and clean-up methods are not efficient, auto-

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fluorescent matrix compounds can compromise toxin identification resulting from phantom peaks in post-column oxidation LC-FLD detection (Luckas 1990; Baker et al. 2003; Martins et al. 2003; Krock et al. 2007), but in addition there are fluorescent interferences that only become visible after oxidation (Krock, unpublished data). Application of advanced analytical techniques based upon comparative ion masses for PSTs by LC-MS/MS (e.g. Krock et al. 2007) has done much to alleviate many of these analytical constraints on confirmatory structural analysis of PSTs.

This paper describes, for the first time, the complete PST profile for several *G. catenatum* strains from several regions of the Pacific coast of Mexico, as revealed by LC-MS/MS. This new profile of hydrophilic toxins is compared with other profiles of *G. catenatum* from diverse locations in order to detect toxin patterns and possible relationships among worldwide populations.

Materials and methods

Strains and culture conditions

Eight strains of *G. catenatum* were brought into uni-algal culture from six locations along the Pacific coast of Mexico: Bahía Concepción, Bahía de Topolobampo, Bahía de La Paz, Bahía de Mazatlán, Bahía de Manzanillo and Lázaro Cárdenas. Details of strains are provided in Table 1.

Strains were cultivated in GSe medium (Blackburn et al. 2001) with earthworm soil extract, and also in modified f/2 medium (Guillard 1975), modified by adding 10^{-8} M selenium (H_2SeO_3) and lowering the copper ($CuSO_4$) concentration (10^{-8} M), with seawater obtained from the Ensenada de La Paz (salinity 34). Cultures in two media allowed us to compare changes in the toxin profile of *G. catenatum* when grown under different nutrient conditions. The cultures were maintained at 24–26 ± 1°C under 150 µmol m^{-2} s^{-1} illumination from cool white fluorescent lights on a 12:12 light:dark cycle in a controlled environment chamber. Both seawater and nutrient stock solutions for growth media were sterilised by filtration (0.22 µm). Cultures were maintained in 50 ml and 1400 ml Erlenmeyer flasks for 9–18 days (until late exponential growth phase) after which cells were harvested by filtration (GF/F, Whatman International, Maidstone, UK) and immediately frozen (–20°C).

Only one strain of *G. catenatum* (BAPAZ5) was additionally cultivated in a 19 L glass flask with 10 L culture media to have sufficient biomass to set up the LC-MS/MS method and corroborate the production of the less abundant toxins. Seawater was sterilised by adding 0.017 g l^{-1} 90% calcium hypochlorite ($Ca(ClO)_2$); after 24 h, 0.119 g l^{-1} sodium thiosulfate were added to neutralise the chlorine. Cells were inoculated into the culture flask after 24 h, adding 1.5 L of actively growing culture. Cultures were maintained without supplementary aeration under the same conditions mentioned above. Cells were collected on a 20 µm mesh sieve after 15 days of culturing and then gently centrifuged to eliminate excess of water.

Extraction and analyses of paralytic shellfish toxins

Filters were lyophilised, and extracted in 500 µl of 0.05 M acetic acid and disrupted with a Microson XL ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA) for 60 s. All extracts were centrifuged at 5000*g* for 10 min and filtered with 13 mm diameter syringe filters (0.22 mm pore size PVDF Millex membrane, EMD Millipore, Billerica, MA, USA; and Sao Paulo, Brazil) prior to LC-MS/MS analysis.

Table 1. *Gymnodinium catenatum* strains from different locations on the Mexican Pacific.

Strain (code)	Place and year of isolation	Isolated by …[a]
GCCV-6	Bahía Concepción, B.C.S., 2000 (26° 40′ LN and 111° 50′ LW)	C. Band-Schmidt[1]
GCCV-7	Bahía Concepción, B.C.S. 2000 (26° 40′ LN and 111° 50′ LW)	C. Band-Schmidt[1]
BAPAZ-5	Bahía de La Paz, B.C.S., 2007 (24° 27′ LN and 110° 30′ LW)	C. Band-Schmidt[1]
BAPAZ-7	Bahía de La Paz, B.C.S., 2007 (24° 27′ LN and 110° 30′ LW)	C. Band-Schmidt[1]
GCMV-7	Bahía de Mazatlán, 2006 (23° 13′ LN and 106° 26′ LW),	C. Band-Schmidt[1]
62L	Lázaro Cárdenas, Michoacán, 2005 (17° 58′ LN and 102° 05′ LW)	M. C. Rodríguez-Palacio[2]
G7	Manzanillo, Colima, 2010 (19° 06′ LN and 104° 22′ LW)	S. Quijano-Scheggia[3]

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Reagents were purchased from Merck (Darmstadt, Germany). The certified standard calibration solutions of PSTs were purchased from the Institute of Marine Biosciences, Certified Reference Materials Program, National Research Council of Canada (Halifax, NS, Canada). Enantiomeric pairs (such as sulfocarbamoyl toxins types C1 and C2) were expressed as sums because they easily epimerise under extraction and analytical conditions. Even though they can be determined individually, their measured ratios do not necessarily reflect the original composition in plankton. PSTs were quantified by external calibration with toxin standards. Toxin sulfocarbamoyl toxin type B2 was expressed as sulfocarbamoyl toxin type B1 equivalents, sulfocarbamoyl toxins type C3 and decarbamoyl gonyautoxin 1 (dcGTX1) were expressed as gonyautoxin 1 (GTX1) due to the lack of standards for these toxins.

Mass spectral experiments to confirm the identity of the PSP toxin components, to quantify them and to detect GC toxins were performed on a triple quadrupole mass spectrometer (API 4000 QTrap, AB Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray interface, coupled to a liquid chromatograph (model 1100, Agilent, Waldbronn, Germany). The liquid chromatograph included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B) and temperature-controlled column oven (G1316A). Mass spectrometric analyses for PSTs were performed according to the hydrophilic interaction liquid ion chromatography (HILIC) method described by Diener et al. (2007) with slight modifications. The analytical column (150 × 4.6 mm) was packed with 5 µm ZIC-HILIC stationary phase (Merck, Darmstadt, Germany) and maintained at 35°C. The flow rate was 0.7 ml min^{-1} and gradient elution performed with eluent A (2 mM formic acid and 5 mM ammonium formate in acetonitrile in water (80:20 v/v)) and eluent B (10 mM formic acid and 10 mM ammonium formate in water). A total of 5 µl of sample was injected and gradient elution for HILIC with MS detection was performed as presented in Table 2. LODs of the toxins are given in the Table 3.

Table 2. HPLC gradient programme for HILIC-MS/MS.

Time (min)	A (%)	B (%)
0	80	20
5	65	35
10	60	40
20	55	45
24	55	45
25	80	20
45	80	20

Note: Eluent A: 2 mM formic acid and 5 mM ammonium formate in acetonitrile in water (80:20 v/v). Eluent B: 10 mM formic acid and 10 mM ammonium formate in water. The mobile phase flow is 0.7 ml min^{-1}.

Table 3. Limits of detection (LODs) for the HILIC-MS/MS method.

Toxin	LOD (pg on-column)
dcSTX	56
dcNEO	7
STX	10
NEO	72
dcGTX2	1125
dcGTX3	473
B1	92
GTX2	24
GTX3	62
C1	508
C2	24
GTX1	370
GTX4	17

Table 4. Mass transitions (*m/z*) (Q1 > Q3 mass) and their respective paralytic shellfish toxin.

Mass transition	Toxin	Collision energy (V)
257 > 156	dcSTX	30
257 > 196	dcSTX	30
273 > 255	dcNEO	30
300 > 204	STX	30
300 > 282	STX	30
316 > 196	NEO	30
316 > 298	NEO	30
353 > 273	dcGTX2/3	30
369 > 289	dcGTX1/4	30
377 > 257	GC3	30
380 > 282	B1	30
380 > 300	B2	30
393 > 273	GC6	30
396 > 298	B2, C1/2, GTX2/3	30
396 > 316	B2, C1/2, GTX2/3	30
412 > 314	C3/4, GTX1/4	30
412 > 332	C3/4, GTX1/4	30
457 > 377	GC3b	30
473 > 393	GC1/2, GC6b	30
489 > 409	GC1a/2a, GC4/5	30
553 > 473	GC1b/2b,	30
569 > 489	GC4b/5b	30

Selected-reaction monitoring (SRM) experiments were performed in positive-ion mode. The transitions are listed in Table 4. Dwell times of 100–200 ms were used for each transition. For these studies, the source parameters were: curtain gas (30 psi), temperature (650°C), ion spray voltage (5000 V), gas 1 and 2 (70 psi), interface heater (on), collision gas (high), declustering potential (66 V), entrance potential (10 V), collision energy (30 V), and collision cell exit potential (12 V).

Comparison of hydrophilic toxin profiles

The relative composition of PSTs of the strains that were examined, expressed as molar percentages (% mol), was

converted to a matrix of relative abundance, with pooled epimeric pairs and based upon the scoring criteria of Negri et al. (2001). The arbitrarily defined categories were: not detected (0), < 5% (1), 5%–40% (2) and > 40% (3). Toxin profiles of strains from the Pacific coast of Mexico were compared with those of strains from other regions by accessing published data sets (Oshima et al. 1993; Méndez et al. 2001; Negri et al. 2001; Holmes et al. 2002; Camino-Ordás et al. 2004; Park et al. 2004; Montoya et al. 2006; Mohammad-Noor 2010; Oh et al. 2010; Costa et al. 2012). All toxins (in % mol) were also converted to their relative abundance as in Negri et al. (2001) (see the Appendix). The matrix was compared by a Bray–Curtis single-link cluster analysis with the BioDiversity Professional Beta, 2.0 software (McAleece 1997). The toxin analogues deoxysaxitoxin (doSTX), C5/6, and hydroxy-benzoyl analogues 1 and 2 (GC1/2), as described by Negri et al. 2003), and other benzoyl variants were not included in the cluster analysis because they were not analysed or included in the cited papers. In a few cases where the complete array of toxins (including doSTX, C5/6 and benzoyl toxins) was considered in the calculation of individual contributions (% mol basis), we recalculated the data by assuming no contribution of doSTX, C5/6 and benzoyl toxins to the toxin profile.

Results

Toxin profiles

A typical chromatogram of cell extracts mainly showed decarbamoyl family toxins: decarbamoyl neosaxitoxin (dcNEO), decarbamoyl gonyautoxin 1/4 (dcGTX1/4), and the less potent sulfocarbamoyl toxins B1, B2, C1/2 and C3/4 (Figure 1). The N-sulfocarbamoyl component C1/2 was the most abundant, with an average content > 85% on a molar basis (Figure 2). Decarbamoyl toxins generally represented a mean average content < 5%. No clear differences in molar toxin composition for the strains were evident from a comparison of growth on different media.

Benzoyl profile

We searched for the ions of known hydroxybenzoate PSP analogues previously reported by Vale (2008b) and found five hydroxy-benzoyl analogues (GC1/2, GC3 and GC4/5) and two sulfated benzoyl analogues (GC1b/2b) (Figure 3). We thereby confirm the presence of hydroxy-benzoyl toxins in the two *G. catenatum* strains we analysed previously (Bustillos-Guzmán et al. 2011; Durán-Riveroll et al. 2013). As with hydrophilic saxitoxin (STX) analogues, there was no clear evidence of an effect of the growth medium on the relative contribution of the benzoyl group toxins to the total toxin composition (data not shown).

The lack of benzoyl toxin standards did not permit the determination of the appropriate response factor for quantification. In terms of relative abundance (area per cell) peaks were compared (Figure 3). There was a trend of increasing cell quotas of all benzoyl toxins from north to south. An opposite gradient is also evident when comparing toxin ratios in the same strains (Figure 4), most evident among toxins GC4–5/1–2 and GC4–5/3, which vary from 3.4 to 16 and from 3 to 13.1, respectively.

Cluster analysis

Cluster analysis grouped all *G. catenatum* strains from Mexico at 88.8% similarity, with the exception of one strain from Colima (Figure 5). This strain contained only N-sulfocarbamoyl toxins C1/2, and is thus more similar (87%) to a strain from Galicia, Spain, than to others from

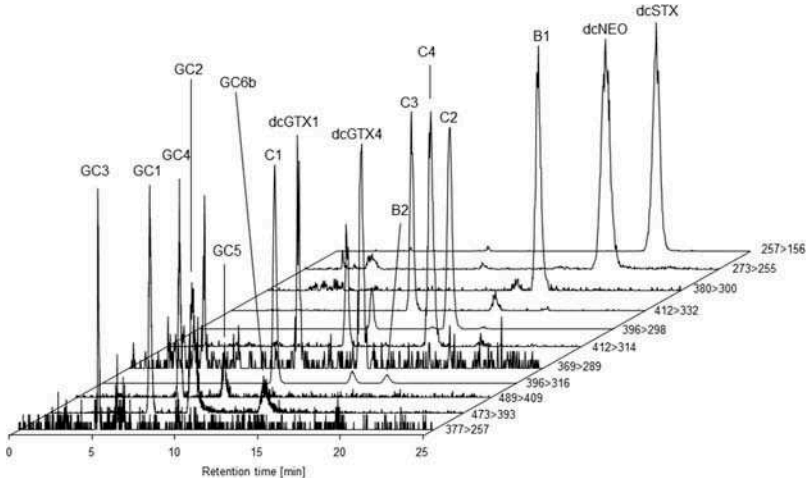
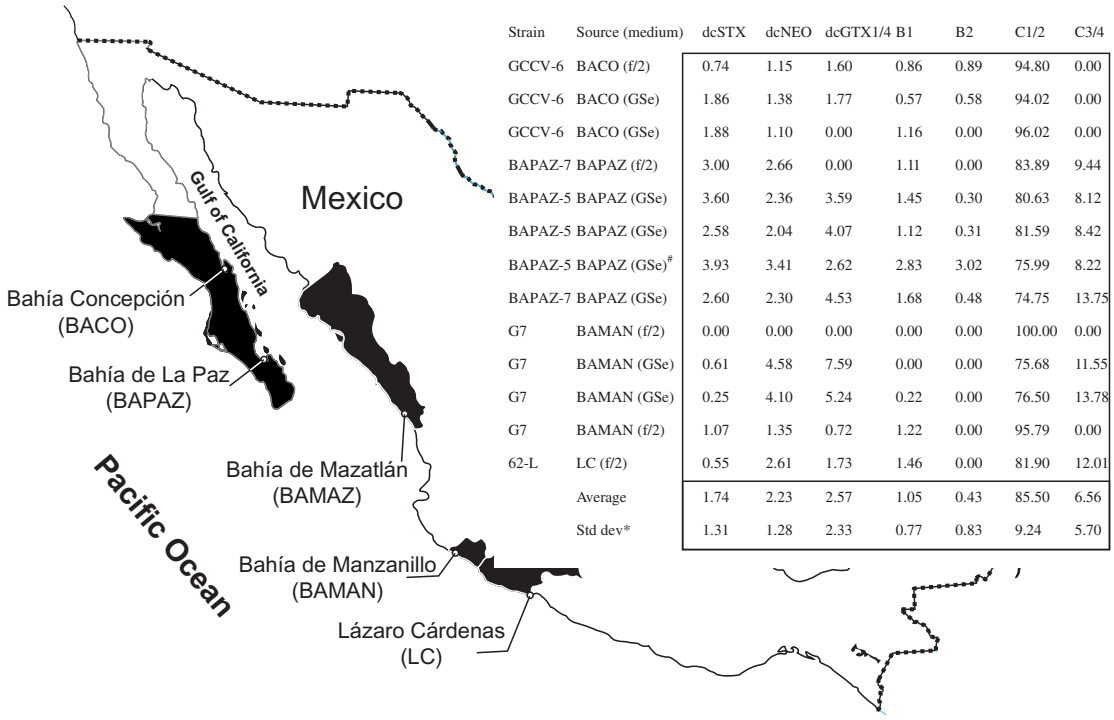


Figure 1. LC-MS/MS SRM chromatogram of strain BAPAZ5 (Q1 mass > Q3 mass). Each ion trace is normalised to 100% base peak, i.e. peak sizes of different ion traces do not reflect relative toxin abundances. The identities of B1, C1, C2, dcNEO and dcSTX have been confirmed by comparison of CID spectra and retention times with analytical standards. All other compounds are inferred from literature data and molecular masses.



Strain	Source (medium)	dcSTX	dcNEO	dcGTX1/4	B1	B2	C1/2	C3/4
GCCV-6	BACO (f/2)	0.74	1.15	1.60	0.86	0.89	94.80	0.00
GCCV-6	BACO (GSe)	1.86	1.38	1.77	0.57	0.58	94.02	0.00
GCCV-6	BACO (GSe)	1.88	1.10	0.00	1.16	0.00	96.02	0.00
BAPAZ-7	BAPAZ (f/2)	3.00	2.66	0.00	1.11	0.00	83.89	9.44
BAPAZ-5	BAPAZ (GSe)	3.60	2.36	3.59	1.45	0.30	80.63	8.12
BAPAZ-5	BAPAZ (GSe)	2.58	2.04	4.07	1.12	0.31	81.59	8.42
BAPAZ-5	BAPAZ (GSe)#	3.93	3.41	2.62	2.83	3.02	75.99	8.22
BAPAZ-7	BAPAZ (GSe)	2.60	2.30	4.53	1.68	0.48	74.75	13.75
G7	BAMAN (f/2)	0.00	0.00	0.00	0.00	0.00	100.00	0.00
G7	BAMAN (GSe)	0.61	4.58	7.59	0.00	0.00	75.68	11.55
G7	BAMAN (GSe)	0.25	4.10	5.24	0.22	0.00	76.50	13.78
G7	BAMAN (f/2)	1.07	1.35	0.72	1.22	0.00	95.79	0.00
62-L	LC (f/2)	0.55	2.61	1.73	1.46	0.00	81.90	12.01
	Average	1.74	2.23	2.57	1.05	0.43	85.50	6.56
	Std dev*	1.31	1.28	2.33	0.77	0.83	9.24	5.70

*Standard deviation; #massive culture.

Figure 2. Relative abundances (%) of PSTs on a molar basis. dcSTX, decarbamoyl saxitoxin; dcNEO, decarbamoyl neosaxitoxin; dcGTX1/4, decarbamoyl gonyautoxin 1/4; B1, N-sulfocarbamoyl toxin B1; B2, N-sulfocarbamoyl toxin B2; C1/2, N-sulfocarbamoyl toxin C1/2; and C3/4, N-sulfocarbamoyl toxin C3/4. The growth medium is shown in parentheses.

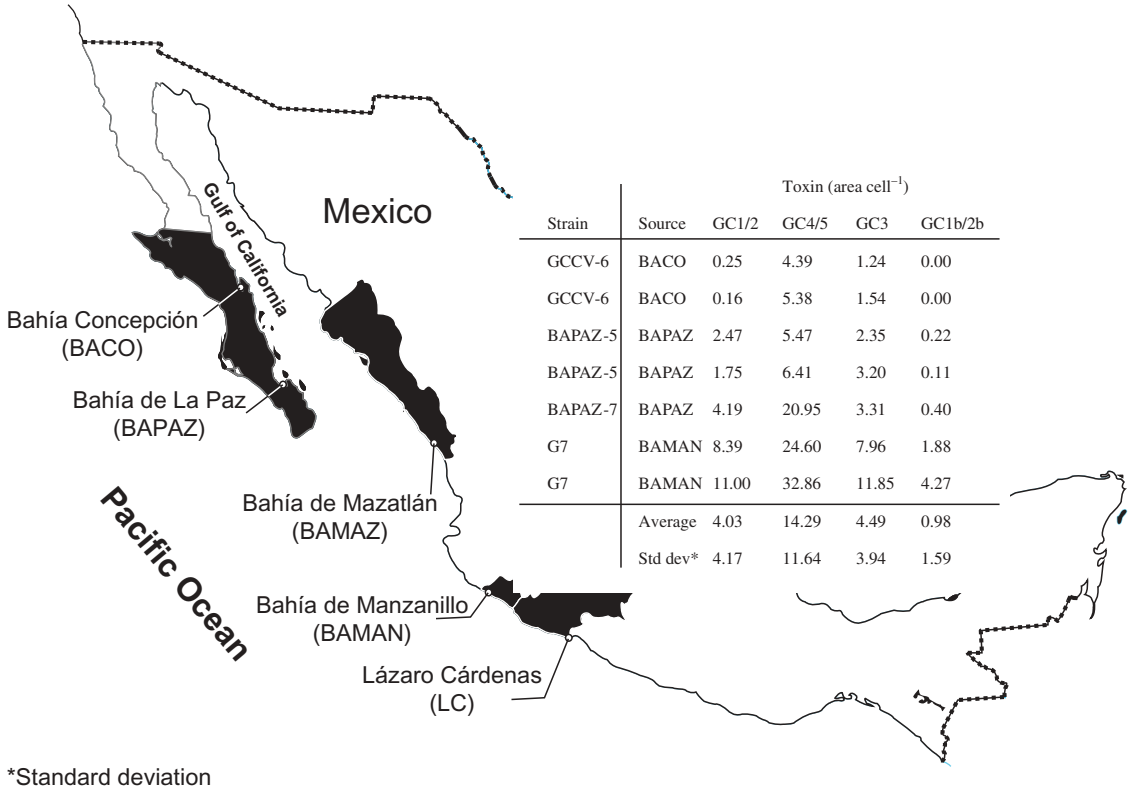
Mexico. A toxin profile consisting of 97% C1/2 is characteristic of the Galician strain (Camino-Ordás et al. 2004).

Discussion

Analytical determination of toxin profiles

We could confirm the presence of only 10 saxitoxin analogues among the strains analysed by LC-MS/MS. This is surprising because at least 15 PST have been recorded from among diverse strains in Mexico (Band-Schmidt et al. 2005, 2006; Gárate-Lizárraga et al. 2005). In contrast to previous studies, we could not confirm the presence of decarbamoyl gonyautoxins 2 and 3 (dcGTX2/3), STX, GTX2/3 or NEO. The absence of NEO merits particular mention because strains in Mexican coastal waters were considered to be the only strains to produce this analogue in significant quantities – up to 46% in a mol basis (reviewed by Band-Schmidt et al. 2010; Hallegraeff et al. 2011).

In general, the LC-MS/MS method in all configurations has a rather low sensitivity for non-N1-hydroxylated PSTs, such as dcGTX2 (LOQ = 77 pg on-column) particularly in comparison with LC-FLD approaches. These toxins could therefore have been simply undetected in low biomass samples and remained below the detection limit. Nevertheless, this is less likely because the LC-MS/MS method also failed to detect dcGTX2/3 in the BAPAZ 5 sample from the mass culture that contained a much higher cell equivalent concentration (Table 4). It is more plausible that dc GTX2/3 are absent or only present in low quantities in Mexican *G. catenatum* strains. In any case, STX and NEO have been recurrently reported in strains from Mexico (Band-Schmidt et al. 2005; Gárate-Lizárraga et al. 2005) based upon LC-FLD analysis. In previous studies, however, the concentrations of these two analogues varied with the composition of the growth medium. Nevertheless, the specific reason for this discrepancy remains unresolved from the growth rate-dependant shifts in toxin composition. Indeed the apparent absence of toxin profile stability within a strain of *G. catenatum* is highly unusual among dinoflagellates (see the review by Cembella 1998). In most cases, a major shift in quantitative toxin composition (usually to fewer STX analogues and less complex derivatives) only occurs in response to extreme growth conditions, such as in advanced senescence or high nitrogen starvation. As cited in the review by Band-Schmidt et al. (2010), STX was usually absent from strains when cultivated in modified f/2 medium, but at maximum concentrations (0.8% mol) when cultivated in GSe medium. Concentration of NEO also varied



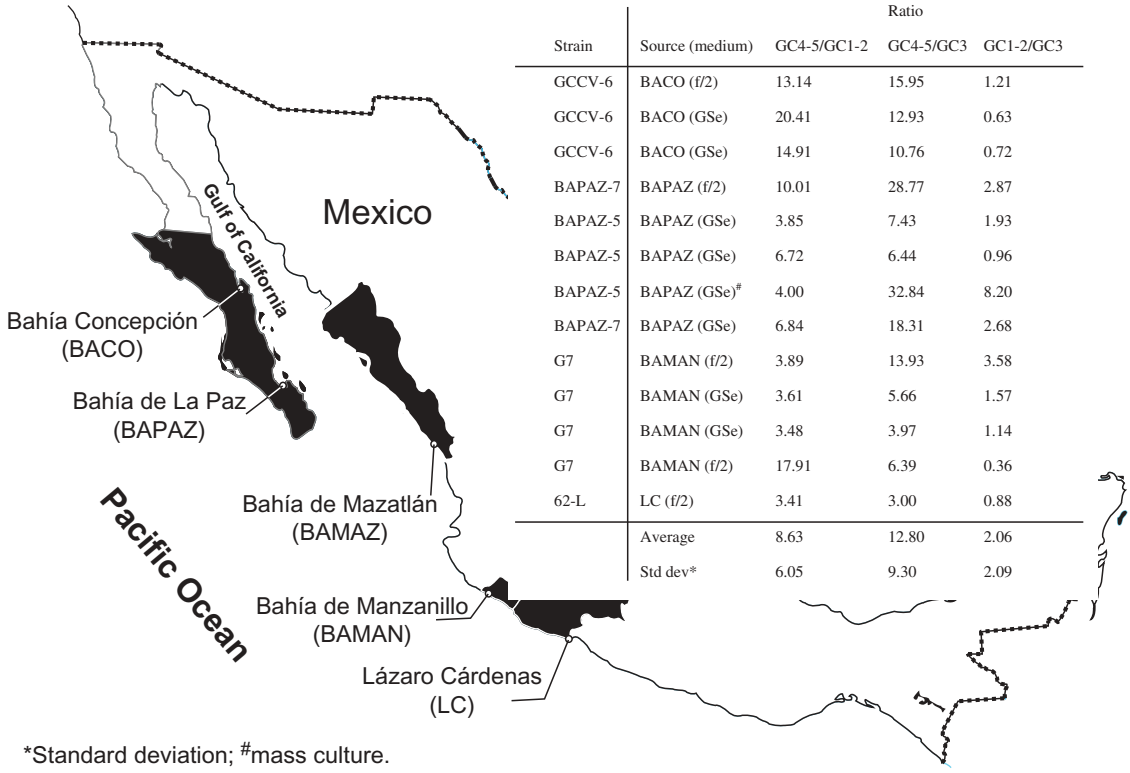
Strain	Source	Toxin (area cell^{-1}) GC1/2	GC4/5	GC3	GC1b/2b
GCCV-6	BACO	0.25	4.39	1.24	0.00
GCCV-6	BACO	0.16	5.38	1.54	0.00
BAPAZ-5	BAPAZ	2.47	5.47	2.35	0.22
BAPAZ-5	BAPAZ	1.75	6.41	3.20	0.11
BAPAZ-7	BAPAZ	4.19	20.95	3.31	0.40
G7	BAMAN	8.39	24.60	7.96	1.88
G7	BAMAN	11.00	32.86	11.85	4.27
	Average	4.03	14.29	4.49	0.98
	Std dev*	4.17	11.64	3.94	1.59

*Standard deviation

Figure 3. Relative benzoyl toxin profiles (chromatographic peak area per cell) for some strains of *G. catenatum* isolated from diverse sites in Mexico. BACO, Bahía Concepción; BAPAZ, Bahía de La Paz; and BAMAN, Bahía de Manzanillo. All strains were cultivated in GSe medium. GC1/2, hydroxy-benzoyl analogues 1 and 2; GC4/5, hydroxy-benzoyl analogues 4 and 5; and GC1b/2b, sulfated benzoyl analogues 1b and 2b.

considerably (from 0.0 to 46.3% mol), depending on the strain and the growth medium (see the review by Band-Schmidt et al. 2010). Whereas wide variation in toxin composition between strains is not unexpected, the lack of stability within a strain is remarkable if it is a function of biological factors.

This begs the question regarding the reliability of LC-FLD to an analytical approach to qualitative and quantitative analysis of PST profiles in dinoflagellates. Although both pre- and post-column oxidation LC-FLD methods have been subjected to rigorous inter-laboratory comparison (DeGrasse et al. 2011) and are widely used in analysis of known PST analogues in routine toxin control in shellfish monitoring programmes, the methods are subject to several serious drawbacks when used to resolve complex toxin profiles. This is especially true where novel and structurally undefined analogues may be present, and for which reference standards are lacking. These issues are related to the failure of LC to resolve all PST analogues and the lack of confirmation of peak identifications via fluorescence detection (Luckas 1990). For example, with the post-column oxidation method (Yu et al. 1998), we followed in the previous PST analyses of *G. catenatum* strains from Mexico (Band-Schmidt et al. 2010, and references therein). dcNEO and NEO would not have been resolved because they have the same retention time. The recurrent finding of NEO may actually correspond to dcNEO, as suggested by the results. Furthermore, the B2 toxin in these strains also contribute to the 'NEO peak' because the method of Yu et al. (1998) requires an additional analysis after oxidation to transform B2 to NEO for its indirect detection and quantification. Misidentification of peaks of fluorescent molecules, other than toxins (false positives) – either as pre- or post-column oxidation products – has also undoubtedly led to frequent misinterpretations of PST analysis with fluorescence-based detection methods (Baker et al. 2003; Biré et al. 2003; Krock et al. 2007; Soto-Liebe et al. 2010). A case of inconsistencies involving PST analogues produced by the toxigenic cyanobacterium *Cylindrospermopsis raciborskii* T3 is described by Soto-Liebe et al. (2010). All published PST profiles, based solely on LC-FLD, including our own previous analyses of *G. catenatum* strains, are subject to misinterpretation and must be interpreted with caution. A second independent detection method, such as MS, must therefore be included to confirm the identity of the chromatographic peaks for critical analysis.



Strain	Source (medium)	Ratio GC4-5/GC1-2	Ratio GC4-5/GC3	Ratio GC1-2/GC3
GCCV-6	BACO (f/2)	13.14	15.95	1.21
GCCV-6	BACO (GSe)	20.41	12.93	0.63
GCCV-6	BACO (GSe)	14.91	10.76	0.72
BAPAZ-7	BAPAZ (f/2)	10.01	28.77	2.87
BAPAZ-5	BAPAZ (GSe)	3.85	7.43	1.93
BAPAZ-5	BAPAZ (GSe)	6.72	6.44	0.96
BAPAZ-5	BAPAZ (GSe)#	4.00	32.84	8.20
BAPAZ-7	BAPAZ (GSe)	6.84	18.31	2.68
G7	BAMAN (f/2)	3.89	13.93	3.58
G7	BAMAN (GSe)	3.61	5.66	1.57
G7	BAMAN (GSe)	3.48	3.97	1.14
G7	BAMAN (f/2)	17.91	6.39	0.36
62-L	LC (f/2)	3.41	3.00	0.88
	Average	8.63	12.80	2.06
	Std dev*	6.05	9.30	2.09

Figure 4. Relative toxin/chromatographic peak area ratios for benzoyl toxins of *G. catenatum* isolated from diverse sites in Mexico. BACO, Bahía Concepción; BAPAZ, Bahía de La Paz; BAMAN, Bahía de Manzanillo; and LC, Lázaro Cárdenas. In parentheses are the culture medium. GC1/2, hydroxy-benzoyl analogues 1 and 2; GC4/5, hydroxy-benzoyl analogues 4 and 5; and GC1b/2b, sulfated benzoyl analogues 1b and 2b.

Benzoyl toxins

The precautionary approach to the interpretation of toxin profiles also applies to the benzoyl toxins reported from several strains of *G. catenatum* (Negri et al. 2007; Vale 2008a, 2008b, 2010), including strains from the coast of Mexico (Bustillos-Guzmán et al. 2011). These studies used different analytical approaches. For example, Bustillos-Guzmán et al. (2011) detected benzoyl toxins by comparing LC-FLD chromatogram peaks with similar retention times with those from a *G. catenatum* strain from Portugal. Further separation by net charge in a carboxylic acid resin cartridge and analysis by automated pre-column periodate oxidation revealed the sulfated and sulfonated STX analogues (C1/2 and C3/4) in the first fraction (charge: 0); the benzoylated GTXs were observed in the second fraction (charge: +1) and the non-sulfonated analogues eluted in the last fraction (charge: +2). The presence of benzoyl toxins in a *G. catenatum* strain collected at Manzanillo, Colima, Mexico, was subsequently confirmed by NMR detection of aromatic protons (Durán-Riveroll et al. 2013). Significantly, the sulfated benzoyl analogues GC1b/2b were recorded only in strains collected in Bahía de La Paz (BAPAZ 5 and 7) and Bahía de Manzanillo (G7). These sulfated benzoyl analogues had previously been recorded only in a strain from Portugal (Vale 2008b). The presence of benzoyl toxins as well as its gradient variation should be considered with caution because we only have data of four strains. Also, if these gradients are related to the geographical origin of strains, this would have to be confirmed by further research with more strains collected in each area. More detailed studies on the physiology of the production of benzoyl analogues in a larger number of strains are now underway in our laboratory.

These benzoyl saxitoxin analogues merit special attention because they have been detected in many *G. catenatum* strains, representing an important fraction, up to 42% of the toxin composition (Negri et al. 2007). Yet the significance of these compounds with respect to their toxicity, structural diversity, biosynthesis and bio-geographical distribution in dinoflagellates and marine food webs remains largely unresolved. For example, Negri et al. (2003, 2007) considered only the contribution of GC1–3 to the toxin composition of *G. catenatum*. The search for benzoyl toxin accumulation in bivalves shellfish, crabs and sardines during blooms of

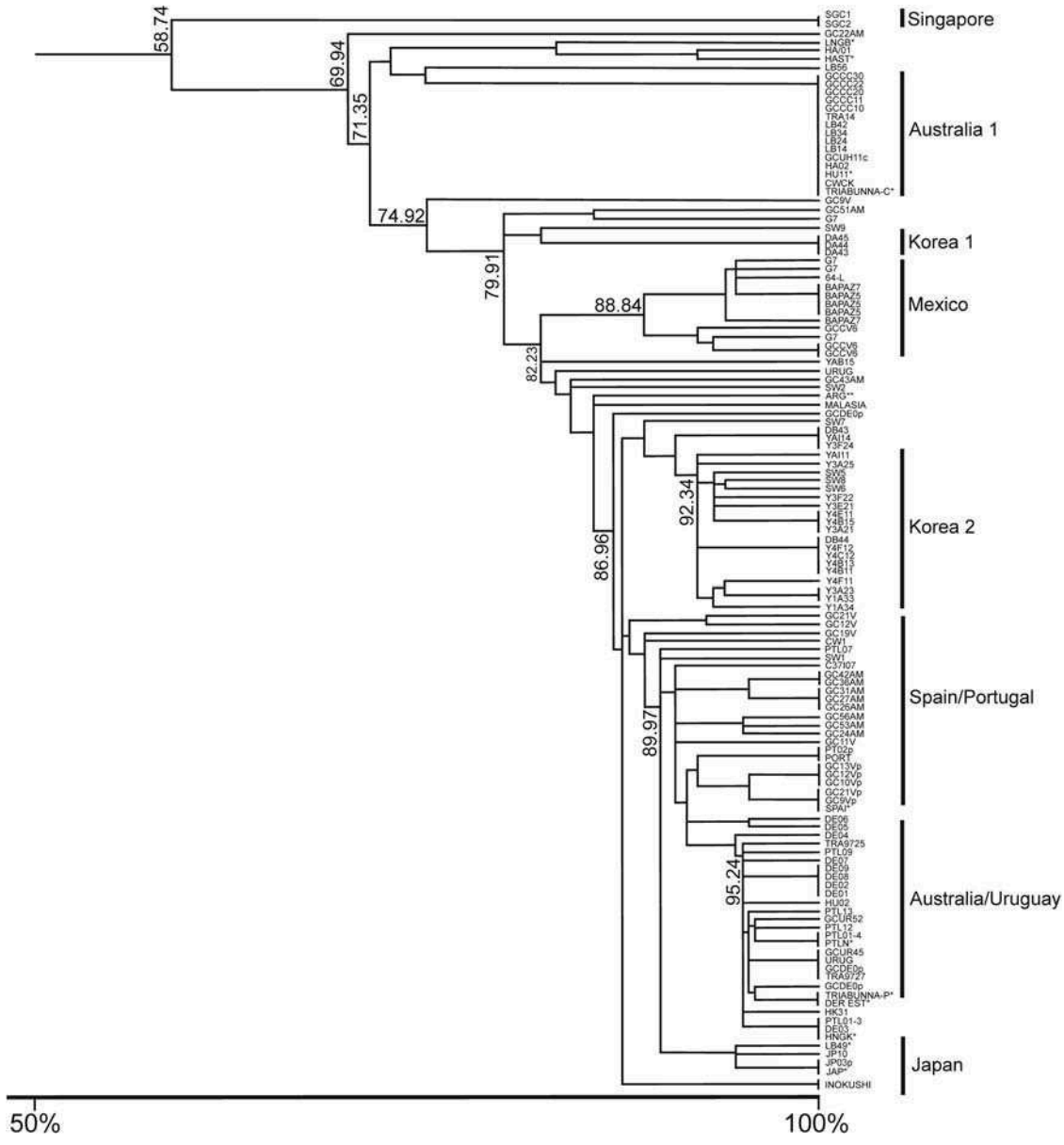


Figure 5. Bray–Curtis single link cluster analyses of similarity (%) of PST profiles from globally diverse strains. See the Appendix for data and strain codes. Country groups are given at the right hand side.

G. catenatum revealed that these analogues were either absent or represented a minor component of the N1-H-containing toxins (Vale 2008a). A non-specific carbamoylase activity converts benzoyl toxins into the decarbamoyl analogues, as was confirmed when digestive glands of some bivalves were incubated with semi-purified GC toxins. The risk to human health of metabolic biotransformation of GC toxins in shellfish must be carefully considered, especially in areas where *G. catenatum* blooms are common. For example, Mexican food safety regulations for PSP only require the mouse bioassay for seafood security analysis. Since benzoyl toxins can be converted by bivalves, the mouse bioassay should assure detection of their net toxicity contribution. Nevertheless, from the perspective of toxin research, analysis of benzoate toxins is essential to clarify the sources as well as the food vectors to humans. In this context, it is important to point out that most LC methods miss the presence of the benzoyl fraction because of its hydrophobic side and longer retention time than common hydrophilic analogues (Vale 2008a).

As mentioned above, the medium influences the hydrophilic (Band-Schmidt et al. 2010) and benzoyl toxin profile (Vale 2008a). However, in this study no clear differences were noted in strains cultured in GSe or modified f/2 media.

Comparison of toxin profiles

Cluster analysis grouped all *G. catenatum* strains from Mexican coastal waters with 88.84% similarity, with the exception of one strain from Colima (Figure 5). This strain contained only N-sulfocarbamoyl toxins C1/2 and thus is more similar (87%) to a strain from Galicia, Spain (Camino-Ordás et al. 2004), than to other strains from Mexico. A toxin profile consisting of 97% C1/2 is characteristic of this Galician strain. Both the Colima and Galician strains have 79.91% similarity to the toxin profiles of other Mexican and Korean strains. Less similar profiles are those of Australian strains derived from cysts, and considered by Hallegraeff et al. (2011) as aberrant profiles. Malaysian strains are also less similar to those from Mexico because the former strains contain a higher molar % of GTX 1–4 toxins (Mahammad-Noor 2010). In any case, most of the published *G. catenatum* toxin profiles demonstrate a high degree of toxin relatedness, with similarity > 80%, indicating a relatively low phenotypic diversity in toxin composition.

This high similarity of toxin profiles agrees with studies of alloenzyme variations and analysis of small and large subunit ribosomal DNAs that both show very little genetic variation in *G. catenatum* over its global range (Bolch et al. 1999; Holmes et al. 2002). Nevertheless, *G. catenatum* strains from the Pacific coast of Mexico can still be clearly distinguished from strains from other geographic regions on the base of the presence dcGTX1/4 and the high relative contribution of the N-sulfocarbamoyl toxins C1/2 (on average 85% of total composition). In any case, all toxin profiles of *G. catenatum* strains must be corroborated by complementary techniques, such as MS, to confirm these results.

In conclusion, we confirmed the presence of benzoyl and hydrophilic saxitoxin analogues, based on specific collision-induced dissociation (CID) mass transitions, in diverse strains isolated from the coast of Mexico. The biogeographic distribution of toxin profiles of *G. catenatum* extend over an important geographical zone of the Mexican Pacific coast, more than 1300 km long, from the middle of the Gulf of California to Lázaro Cárdenas, Michoacán. The toxin profiles were rather unexpectedly complex in that at least seven benzoyl analogues were detected, together with 10 hydrophilic STX analogues. Among the hydrophilic analogues, the absence of the carbamoyl group was the most significant finding. Although benzoyl toxins have been previously detected in two Mexican strains of *G. catenatum*, the present study confirms for the first time the presence of these toxins by LC-MS/MS. This work also reports benzoyl toxins in other strains from diverse geographical zones of the Mexican Pacific. In addition, in three of these strains, we found unreported benzoyl toxins, namely GC1a/b and GC5. Further analysis of the benzoyl group is clearly needed to establish a possible relationship among diverse strains of *G. catenatum* from widespread geographical regions. In some cases, this will require reanalysis of toxin profiles with advanced confirmatory methods. Finally, the implications of the accumulation and biotransformation of the benzoyl analogues on the toxic effect in diverse trophic interactions should be reconsidered in the context of risk assessment of seafood consumption.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix

	STRAIN ORIGIN/CODE	ISOLATE	C1/C2	C3/C4	GTX1/4	GTX2/3	GTX5	GTX6	dcGTX2/3	dcSTX	STX	C5/6***	doSTX	NEOstx	dcNEO	dcGTX1/4	GC1+2	GC3	REFERENCE**
JAPAN	Inokushi Bay (mar)	P	79.8			0.2	18		0.3		1.7								1
	Inokushi Bay (jul)	P	83.3			2.3	10.6		0.3		3.1								1
	JAPAN*	P	83.5	–		0.4	7.5	3.1	4.9	0.6	–	–	–						2
	SETO INLAND SEA/JP03[P]	P	83.4	–	–	0.7	6.4	2.6	4.4	1.0	–						1.2	0.4	3
	SENZAKI BAY/JP10[P]	P	74.7	–	–	0.4	11.5	4.9	7.6	0.1	–						0.2	0.6	
CHINA	CHINA/HNGK*	M	40.0	35.5		0.3	4.0	1.1	16.4	2.5	–	+	–						2
	DEEP BAY, HK/HK31	M	18.2	16.2	–	0.1	1.8	0.5	7.5	1.2	–					–	32.8	21.8	3
SINGAPORE	SGC1	P			52.8	37.2	–	–	–	–	1.7	nd	nd	8.3	–	–	nd	nd	4
	SGC2	P			50.4	37.75	–	–	–	–	3.3	nd	nd	8.2	–	–	nd	nd	5
	Y1A34	C	15.38	3.85		76.92	–		3.85	–				–					6
KOREA	Y1A33	C	30.56	11.11		55.56	–		2.78	–				–					
	Y3A21	C	20.00	–		60.00	–		20.00	–				–					
	Y3A23	C	25.29	6.90		65.52	–		2.30	–				–					
	Y3A25	C	31.55	–		63.69	–		3.27	–				1.49					
	Y3E21	C	40.35	–		49.12	–		10.53	–				–					
	Y3F22	C	60.00	–		20.00	–		20.00	–				–					
	Y3F24	C	1.71	–		97.56	–		0.73	–				–					
	Y4B11	C	8.82	–		88.24	–		2.94	–				–					
	Y4B13	C	36.59	–		58.54	–		4.88	–				–					
	Y4B15	C	25.00	–		69.44	–		5.56	–				–					
	Y4C12	C	13.46	–		84.62	–		1.92	–				–					
	Y4E11	C	28.57	–		64.29	–		7.14	–				–					
	Y4F11	C	46.88	6.25		43.75	–		3.13	–				–					
	Y4F12	C	12.12	–		84.85	–		3.03	–				–					
	Y4I11	C	59.09	–		36.36	–		4.55	–				–					
	Y4I14	C	1.77	–		95.76	–		2.47	–				–					
	DA43	C	24.24	–		1.01	71.72		3.03	–				–					
	DA44	C	22.95	–		0.82	72.95		3.28	–				–					
	DA45	C	17.78	–		2.22	75.56		4.44	–				–					
	DB43	C	2.41	–		95.18	–		2.41	–				–					
	DB44	C	8.20	–		88.52	–		3.28	–				–					
	SW1	P	42.86	0.95		2.86	35.24		13.33	4.76				–					
	SW2	P	25.93	–		3.70	37.04		18.52	14.81				–					
	SW5	P	77.33	–		1.33	–		20.00	1.33				–					
	SW6	P	50.00	–		16.67	–		30.00	3.33				–					
	SW7	P	8.33	–		8.33	–		66.67	16.67				–					
	SW8	P	43.75	–		6.25	–		12.50	37.50				–					
	SW9	P	16.13	3.23		6.45	67.74		6.45	–				–					
	CW1	P	80.60	–		1.49	11.94		5.97	–				–					
	YELLOW SEA, Y4B15	M	18.6	1.3	57.1	22.6	–	–	0.4	–	–						–	–	3
MALAYSIA		P	13			28.7	4.00	1.1	16.4	2.50									7
AUSTRALIA	DERWENT ESTUARY*	P	21.3	66.3		0.4	1.8	0.3	8.7	1.0	0.2	+	+						2
	PT LINCOLN/PTLN*	M,P	14.8	67.7		0.6	2.5	0.4	7.2	6.9	–	+	–						
	TRIABUNNA–P*	P	14.8	70.8		0.3	1.1	0.5	10.2	2.4	0.03	+	–						
	TRIABUNNA–C*	C	–	–		–	–	–	100.0	–	–	+	–						
	HASTINGS BAY/HAST*	C	–	–		–	–	–	77.4	20.1	2.5	+	–						
	LONG BAY/LNGB*	C	–	–		0.7	–	–	95.7	2.9	0.8	+	–						
	LONG BAY/LB49*	C	77.0	–		2.1	2.6	1.4	16.2	0.6	–	–	–						
	COWAN CREEK/CWCK*	C	–	–		–	–	–	100.0	–	–	+	–						

(*continued*)

Appendix. Continued.

	STRAIN ORIGIN/CODE	ISOLATE	C1/C2	C3/C4	GTX1/4	GTX2/3	GTX5	GTX6	dcGTX2/3	dcSTX	STX	C5/6***	doSTX	NEOstx	dcNEO	dcGTX1/4	GC1+2	GC3	REFERENCE**
	HUON ESTUARY/HU11*	C	–	–		–	–	–	100.0	–	–	+	–						
	HUON ESTUARY/HU02	P	27.0	68.4	–	0.1	1.6	1.7	0.7	0.5									8
	DERWENT ESTUARY/DE01	P	68.0	22.7	–	0.4	2.8	1.3	0.7	4.0									
	DERWENT ESTUARY/DE02	P	77.2	14.3	–	0.8	3.0	1.2	1.2	2.3									
	DERWENT ESTUARY/DE03	C	71.9	11.8	–	1.2	1.3	1.9	9.2	2.7									
	DERWENT ESTUARY/DE04	P	78.3	20.1	–	0.3	0.2	–	0.9	0.2									
	DERWENT ESTUARY/DE05	P	31.9	56.7	0.2	0.4	7.6	0.8	0.3	2.1									
	DERWENT ESTUARY/DE06	P	20.9	70.3	0.1	–	7.0	0.5	0.1	1.2									
	DERWENT ESTUARY/DE07	P	70.0	17.1	–	0.4	9.5	1.1	1.5	0.5									
	DERWENT ESTUARY/DE08	P	79.3	15.3	–	0.5	2.1	0.3	0.8	1.7									
	DERWENT ESTUARY/DE09	P	71.8	20.1	–	0.3	4.1	0.8	0.8	2.1									
	HASTING BAY/HA01	C	–	–	–	–	–	–	8.9	6.6	0.8						68.2	15.4	3
	HASTING BAY/HA02	C	–	–	–	–	–	–	5.8	–	–						68.0	26.2	
	HUON ESTUARY/GCHU11c	C	–	–	–	–	–	–	6.9	–	–						63.2	29.9	
	LONG BAY/LB14	C	–	–	–	–	–	–	4.0	–	–						53.0	43.0	
	LONG BAY/LB24	C	–	–	–	–	–	–	3.2	–	–						56.4	40.3	
	LONG BAY/LB34	C	–	–	–	–	–	–	20.6	–	–						45.7	33.7	
	LONG BAY/LB42	C	–	–	–	–	–	–	8.4	–	–						50.7	41.0	
	LONG BAY/LB56	C	–	–	–	1.3	–	–	13.3	–	–						49.1	36.3	
	SPRING BAY/TRA14	C	–	–	–	–	–	–	0.3	–	–						70.6	29.0	
	COWAN CREEK/GCCC10	C	–	–	–	–	–	–	1.6	–	–						45.5	52.9	
	COWAN CREEK/GCCC11	C	–	–	–	–	–	–	1.3	–	–						62.6	36.1	
	COWAN CREEK/GCCC20	C	–	–	–	–	–	–	0.5	–	–						72.7	26.8	
	COWAN CREEK/GCCC22	C	–	–	–	–	–	–	0.8	–	–						69.2	30.0	
	COWAN CREEK/GCCC30	C	–	–	–	–	–	–	1.0	–	–						63.6	35.3	
	PORT LINCOLN, PTL07	M	1.0	38.8	–	–	0.3	0.1	2.2	3.6	–						40.9	13.1	
	PORT LINCOLN, PTL09	M	2.2	29.4	–	0.1	0.2	–	4.9	0.4	–						44.5	18.3	
	PORT LINCOLN, PTL12	M	3.3	29.1	–	1.0	2.8	0.4	2.3	2.3	–						40.4	18.4	
	PORT LINCOLN, PTL13	M	3.4	35.7	–	0.1	0.2	0.1	0.5	3.3	–						38.3	18.4	
	SPRING BAY/TRA9725	M	3.5	71.5	–	0.5	2.0	0.7	4.3	0.8	–						13.2	3.3	
	SPRING BAY/TRA9727	M	11.0	61.4	–	0.2	0.2	0.3	12.2	3.3	–						10.5	1.0	
	PORT LINCOLN, PTL01–3	P	25.0	8.0	–	0.4	1.0	0.1	6.2	1.0	–						40.5	17.7	
	PORT LINCOLN, PTL01–4	P	3.7	31.8	–	0.2	1.6	0.1	4.7	3.9	–						35.3	18.8	
	DERWENT ESTUARY/GCDE02[P]	P	28.3	12.7	–	–	1.0	0.5	3.0	0.8	3.0						36.4	17.0	
	DERWENT ESTUARY/GCDE06[P]	P	10.7	27.4	0.03	0.1	1.1	0.1	4.1	1.7	0.1						40.5	14.2	
	DERWENT ESTUARY/GCDE09[P]	P	8.5	41.7	–	0.2	1.1	0.1	4.9	0.3	–						30.6	12.7	
MEXICO	BAHIA CONCEPCION/GCCV–6	P	94.8				0.86	0.89		0.74					1.15	1.6			This study
	BAHIA CONCEPCION/GCCV–6	P	94.02				0.57	0.58		1.86					1.38	1.77			
	BAHIA CONCEPCION/GCCV–6	P	96.02				1.16	0		1.88					1.1				
	BAHIA DE LA PAZ/BAPAZ7	P	83.89	9.44			1.11	0		3					2.66				
	BAHIA DE LA PAZ/BAPAZ5	P	80.63	8.12			1.45	0.3		3.6					2.36	3.59			
	BAHIA DE LA PAZ/BAPAZ5	P	81.59	8.42			1.12	0.31		2.58					2.04	4.07			
	BAHIA DE LA PAZ/BAPAZ5	P	75.99	8.22			2.83	3.02		3.93					3.41	2.62			
	BAHIA DE LA PAZ/BAPAZ7	P	74.75	13.75			1.68	0.48		2.6					2.3	4.53			
	COLIMA/G7	P	100																
	COLIMA/G7	P	75.68	11.55						0.61					4.58	7.59			
	COLIMA/G7	P	76.5	13.78			0.22			0.25					4.1	5.24			
	COLIMA/G7	P	95.79				1.22			1.07					1.35	0.72			

(*continued*)

Appendix. Continued.

	STRAIN ORIGIN/CODE	ISOLATE	C1/C2	C3/C4	GTX1/4	GTX2/3	GTX5	GTX6	dcGTX2/3	dcSTX	STX	C5/6***	doSTX	NEOstx	dcNEO	dcGTX1/4	GC1+2	GC3	REFERENCE**
	MICHOACAN/62–L	P	81.9	12.01			1.46			0.55					2.61	1.73			
URUGUAY	Punta del Este		71.5	8.5	0.6	1	6	9.5	1					0.1					9
	URUGUAY/URUG	M	36.1	43		0.3	5	2.4	10.9	2.4	–	+		–					2
	PUNTA DEL ESTE/GCUR45	M	30.7	41.4	–	0.2	4.1	1.7	10.5	1.8	–						8.4	1.2	3
	PUNTA DEL ESTE/GCUR52	M	32.1	34.4	–	0.3	4.5	2.4	8.5	2.2	–						13.5	2.2	
ARGENTINA	Phytoplankton sample		**82.0**	–	5.4	9	–	–	0.14	–			–			–	–		10
SPAIN	GC9V	P	7.88	–	–	–	21.37	14.68	–	11.78	10.4	–	20.02	13.88					11
	GC11V	P	10.27	10.22	–	–	16.56	23.89	1.17	1.67	2.57	–	33.65	–					
	GC12V	P	14.14	8.47	0.91	0.91	16.13	40.49	0.95	0.87	1.68	–	16.37	–					
	GC19V	P	63.62	–	–	–	26.1	2.79	0.96	0.26	2.65	–	3.53	–					
	GC21V	P	10.19	14.28	1.23	1.23	12.42	23.08	1.22	0.61	4.29	–	32.69	–					
	GC22AM	P	16.43	–	–	–	15.33	–	1.08	–	–	+	11.89	50.92					
	GC24AM	P	38.5	15.64	–	–	7.75	23.13	1.25	0.97	–	–	12.74	–					
	GC26AM	P	32.54	14.18	–	–	12.34	25.39	1.29	1.26	–	+	11.15	1.85					
	GC27AM	P	22.69	13.06	–	–	22.39	18.21	0.74	0.47	–	+	18.45	3.98					
	GC31AM	P	16.61	14.38	–	–	18.3	31.89	0.92	0.42	–	+	15.14	2.34					
	GC36AM	P	16.07	5.83	–	–	22.97	16.46	2.56	0.27	–	+	29.35	6.5					
	GC42AM	P	9.37	10.68	–	–	19.85	27.19	0.73	0.51	–	+	24.85	6.79					
	GC43AM	P	26.86	17.22	–	–	–	–	3.84	1.86	–	+	50.23	–					
	GC51AM	P	67.95	–	–	–	–	–	1.8	–	–	–	30.25	–					
	GC53AM	P	34.5	12.92	–	–	1.6	10.24	1.49	0.11	–	+	38.76	–					
	GC56AM	P	25.25	11.7	–	–	2.44	10.48	2.21	0.6	–	+	45.69	1.63					
	SPAIN/SPAI*	P	22.7	15.3		2.0	29.2	22.8	4.5	3.5	–	+	–						2
	RIA DE VIGO/GC9V ^{p}	P	7.0	7.7	–	0.4	19.3	14.7	0.5	2.4	–						37.6	10.4	3
	RIA DE VIGO/GC10V^{p}	P	14.4	7.5	–	1.8	17.2	8.4	3.7	1.8	–						36.4	8.7	
	RIA DE VIGO/GC12V^{p}	P	11.1	5.4	–	1.2	8.5	16.0	2.3	1.5	–						33.9	20.1	
	RIA DE VIGO/GC13V^{p}	P	14.1	5.1	–	1.8	17.8	9.4	4.2	1.8	–						31.3	14.1	
	RIA DE VIGO/GC21V^{p}	P	10.9	5.1	–	0.2	22.7	19.9	2.5	2.8	–						25.2	10.7	
PORTUGAL	C37I07 Cascais Bay	P	34.3	17.1			23.6	16.2	3.2	4.1					1.5				12
	PORT	P	31.8	10.4		1.8	26.9	15.1	8.1	5.5	0.6	+	–						2
	AGUDA/PT02^{p}	P	14.5	6.0	–	1.0	12.9	5.9	4.0	2.5	0.35						35.3	17.6	3

Annex. Toxin profile of worldwide *Gymnodnium catenatum* strains and phytoplankton samples (mol%). *, average from several strains, p, phytoplankton cells; +, present but unquantified; –, not found; nd, not detected; M, mixed; P, plankton; C, cysts. **1. Oh et al. (2010). 2. Negri et al. (2001). 3. Negri et al. (2007). 4. Holmes et al. (2004). 5. Holmes et al. (2001). 6. Park et al. (2004). 7. Mahammad–Noor (2010). 8. Oshima et al. (1993). 9. Méndez et al. (2004). 10. Montoya et al. (2006). 11. Camino–Ordás et al. (2004). 12. Costa et al. (2012). ***, toxins C5 and C6 may correspond to GC1 and GC2, respectively, according to Camino–Ordás et al. (2004).