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Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries

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Abstract. Paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* Graham were investigated as a possible biochemical marker to distinguish different geographic populations of this species. Isolates obtained between 1986 and 1988 from Japan, Tasmania (Australia) and Galicia (Spain) were cultured under similar conditions and the toxins produced were analyzed using HPLC. Variations in temperature, salinity, and nitrate and phosphate levels in the culture medium had no significant effect on the toxin profile, suggesting that toxins can be used as a stable biochemical marker for this dinoflagellate. All the isolates produced mainly toxins of the N-sulfocarbamoyl group (C1–C4, gonyautoxins 5 and 6) but their relative abundance differed according to their geographic origin. Furthermore, only the Australian population produced the newly found 13-deoxydecarbamoyl toxins, and these could readily be used to distinguish the Australian populations from those of the other two countries.

Introduction

Among some ten dinoflagellates known to produce paralytic shellfish toxins, *Gymnodinium catenatum* Graham is the only unarmored species. This chain-forming dinoflagellate was first described from the Gulf of California (Graham 1943) and later reported from Argentina (Balech 1964) but without any knowledge on its potential for toxin production. The involvement of this species in causing outbreaks of shellfish poisoning was first recognized in the Galician rias in Spain (Estrada et al. 1984, Fraga and Sanchez 1985) and later also from the Pacific coast of Mexico (Mee et al. 1986). However, chemical confirmation of the toxin production by *G. catenatum* was first achieved by the present authors using specimens from Tasmania, Australia (Oshima et al. 1987). Since then, this organism has also been reported from Japan (Yuki and Yoshimatsu 1987, Ikeda et al. 1989), Portugal (Franca and Almeida 1989), Italy (Carrada et al. 1991) and Venezuela (La Barbera Sanchez 1991). In Tasmania, blooms of *G. catenatum* have caused shellfish toxicity since 1985 (Hallegraeff et al. 1989), and investigations on its resting cyst distribution (Hallegraeff et al. 1988, 1989) as well as past plankton records from the area suggested that this species may have been introduced there only recently (Hallegraeff et al. 1990). Successful sexual crossing experiments have been carried out between Tasmanian, Japanese and Spanish strains (Blackburn et al. 1989, Oshima et al. 1992), indicating that the different geographic populations are genetically very close. In order to trace possible geographical dispersal of a species, it is essential to have a means to distinguish the organism at the intraspecies level.

The development of sensitive high performance liquid chromatography (HPLC) for the analysis of paralytic shellfish toxins (Oshima et al. 1989, Sullivan 1990) has enabled the recognition of complex toxin profiles in some dinoflagellates. More than 20 analogs of saxitoxin have now been reported (see Fig. 1). The diversity of toxin profiles produced by *Alexandrium* species has been well documented and sometimes used as a biochemical characteristic to distinguish strains within the species of this genus (Boyer et al. 1986, Cembella et al. 1987, Oshima et al. 1990). Discrepancies have been reported from different countries on the toxins produced by *Gymnodinium catenatum* as well as those present in contaminated shellfish (Oshima et al. 1987, Anderson et al. 1989, Ikeda et al. 1989, Rodriguez-Vazquez et al. 1989), suggesting that the toxin profile could be a useful biochemical marker. However, no direct comparisons have been made on the *G. catenatum* toxins produced by different geographic isolates under exactly the same culture conditions.

In the present study, the effects of different temperature, salinity and nutrient levels in the culture medium on the toxin profile of *Gymnodinium catenatum* were investigated to examine its stability and consequent value as a biochemical marker. The toxins produced by different *G. catenatum* isolates from Japan, Tasmania (Australia) and Galicia (Spain) were compared and, within the Tasmanian population, a number of different bloom events were also examined.

Table 1. *Gymnodinium catenatum.* Characteristics of isloates

Culture code	Source	Isolation[a]	Culture history
DE01	Derwent Estuary, Tasmania, Australia	6 Jan 1986	Chain of 6 cells
DE02	Derwent Estuary	23 Jan 1986	Chain of 8 cells
DE03	Derwent Estuary	5 Feb 1986	Single germling cell (planomeiocyte)
DE04	Derwent Estuary	5 Feb 1986	Single cell
DE05	Derwent Estuary	8 Jan 1987	Chain of 12 cells
DE06	Derwent Estuary	8 Jan 1987	Chain of 10 cells
DE07	Derwent Estuary	8 Jan 1987	Chain of 8 cells
DE08	Derwent Estuary	15 Jun 1987	Chain of 8 cells
DE09	Derwent Estuary	15 Jun 1987	Chain of 4 cells
HU02	Huon Estuary, Tasmania, Australia	6 Jun 1986	Chain of 8 cells
HU04	Huon Estuary	12 Nov 1986	Wild resting cyst germinated, all products
HU05	Huon Estuary	15 Jun 1987	Resting cyst in HU04 germinated, all products
HU09	Huon Estuary	15 Jun 1988	Resting cyst in HU05 germinated, one of four 8-celled chains
HU10	Huon Estuary	15 Feb 1988	Resting cyst in HU05 germinated, one of four 8-celled chains
JP01	Harimanada, Kagawa, Japan	14 Sep 1985	Chain of 6 cells
JP02	Senzaki, Yamaguchi, Japan	12 Dec 1986	Single cell
SP01	Ria de Vigo, Galicia, Spain	5 Nov 1985	Chain of 6 cells

[a] JP01 isolated by S. Yoshimatsu, JP02 by T. Ikeda and SP01 by I. Bravo. All cultures from Tasmania isolated by S. Blackburn

Materials and methods

Dinoflagellate cultures

Between January 1986 and July 1987, nine clonal cultures (DE01, DE02, DE03, DE04, DE05, DE06, DE07, DE08, and DE09) of *Gymnodinium catenatum* were isolated as motile vegetative cells from the Derwent Estuary, and one clonal culture (HU02) originated from the Huon Estuary, Tasmania. A culture (HU04) was also established by germination of a wild resting cyst collected on June 1988 from the Huon Estuary, and HU05 was cultured by germination of a resting cyst formed during culture of HU04. Two clonal cultures (HU09 and HU10) were also established from vegetative cells isolated from the products of germination of a resting cyst in HU05. Cultures of *G. catenatum* from Harimanada, Kagawa Prefecture (JP01) and Senzaki Bay, Yamaguchi Prefecture (JP02), Japan as well an isolate (SP01) from Vigo, Galicia, Spain were also used in this experiment. Detailed characteristics of these isolates are summarized in Table 1.

The culture medium (GSe) consisted of filtered seawater (salinity adjusted to 28‰) autoclaved in Teflon containers, with nutrients added according to Loeblich's GPM medium (Loeblich 1975), but modified with the addition of H_2SeO_4 at 1×10^{-8} *M* final concentration (Blackburn et al. 1989). Modified media without nitrate, phosphate or both, and with salinities (20, 28, 35‰) adjusted by adding distilled water were also used. The organism was cultured for 2 to 3 wk in 50-ml Erlenmeyer flasks at temperatures of 12.5, 17 and 25 °C and at a light intensity of 80 μE m^{-2} s^{-1} (12 h light: 12 h dark cycle) and harvested at an early stationary growth phase.

Analysis of toxins

Cultured cells were collected by centrifugation at 2000 *g*, suspended in 100 to 200 μl of 0.5 N acetic acid and homogenized with three successive sonications (20 s each) using a Labsonic homogenizer (Braun, 100 W) equipped with a fine probe. The weight inside the tube was measured prior to sonication to estimate the total volume of the extract. The supernatant obtained after centrifugation at 3000 *g* for 10 min was passed through an ultrafiltration membrane (UltraFree C3GC, Millipore) and 10 μl each was subjected to analysis.

Toxin analysis was carried out on a HPLC with fluorescent detection using ion pair chromatography with post column derivatization, as reported previously (Oshima et al. 1989), with slight modifications. A silica-base reversed phase column (Develosil C-8-5, 0.46 × 15 cm, Nomura Chemical) and the following three mobile phases (flow rate 0.8 ml min^{-1}) were used for separation of the different toxin groups: (a) 2 m*M* tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for C1–C4 toxins; (b) 2 m*M* 1-heptanesulfonic acid in 10 m*M* ammonium phosphate buffer (pH 7.1) for the gonyautoxin group; and (c) 2 m*M* 1-heptanesulfonic acid in 30 m*M* ammonium phosphate buffer (pH 7.1):acetonitrile (100:5) for the saxitoxin group. The eluate from the column was continuously mixed with 7 m*M* periodic acid in 50 m*M* sodium phosphate buffer (pH 9.0) at 0.4 ml min^{-1}, heated at 65 °C by passing through a Teflon tubing (0.5 mm id, 10 m long), and then mixed with 0.5 *N* acetic acid at 0.4 ml min^{-1} just before entering the monitor. The fluoromonitor was set at an excitation wave length of 330 nm and emission wave length of 390 nm. Either a Hitachi L-6000 HPLC equipped with F-1050 fluoromonitor or an ETP KORTEC K34M equipped with Shimadzu RF-530 fluoromonitor was used. As external standard, pure toxin solutions calibrated with the nitrogen measurement by combustion analysis were used. The following abbreviations of toxins (Fig. 1) are used hereafter: STX = saxitoxin; neoSTX = neosaxitoxin; GTX1–GTX6 = gonyautoxins 1–6, dcSTX = decarbamoylsaxitoxin; dcGTX2 and dcGTX3 = decarbamoylgonyautoxins 2 and 3; doSTX = 13-deoxydecarbamoylsaxitoxin; doGTX2 and doGTX3 = 13-deoxydecarbamoylgonyautoxins 2 and 3.

Results and discussion

A representative isolate from Tasmania (DE08) and three isolates (SP01, JP01 and JP02) from other countries were cultured under different temperature and salinity conditions and nutrient concentrations. In the nitrate and phosphate deficient medium, the concentrations of the two nutrients at the time of harvesting cells were less than 0.1 and 0.8×10^{-6} *M*, respectively. The relative abundance of toxins in mol% in isolate DE08 and typical HPLC-chromatograms of the isolate are shown in Table

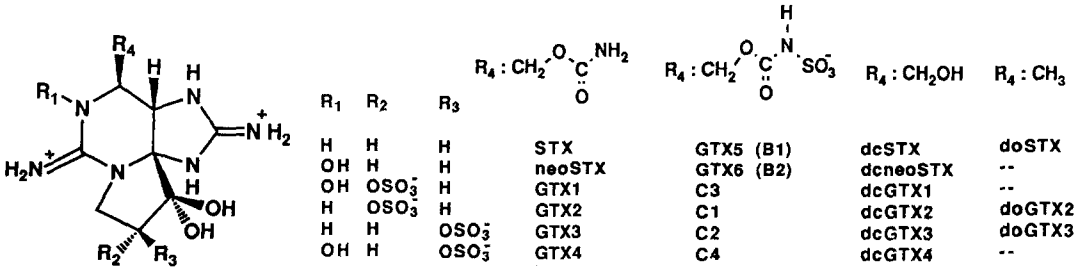

Fig. 1. Structures of paralytic shellfish toxins. For definition of toxin abbrevations see "Materials and methods – analysis of toxins"

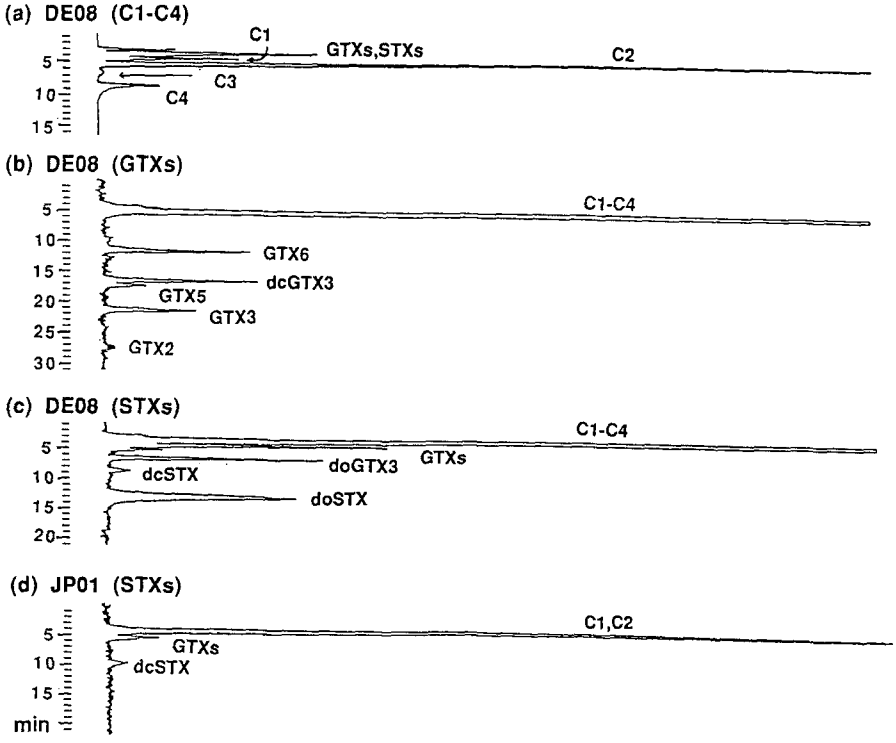

Fig. 2. *Gymnodinium catenatum.* High performance liquid chromatograms of paralytic shell fish toxins in *G. catenatum* from Australia and Japan. (a), (b) and (c) are those of the isolate DE08 from Tasmania taken using mobile phases (a), (b) and (c), respectively. (d) is that of JP01 from Harimanada, Japan taken using mobile phase (c), indicating absence of doGTX3 and doSTX in this isolate. For definitions of toxin abbreviations see "Materials and methods – analysis of toxins"

Table 2. *Gymnodinium catenatum.* Toxin composition (mol%) of an isolate from Tasmania (DE08) under various culture conditions. Full strength GSe-enriched medium as control: Full. Modified GSe medium without nitrate: (–)N; without phosphate: (–)P; or with neither: (–)N&P. For definitions of toxin abbreviations see "Material and methods analysis of toxins"

Toxins	Culture conditions				Temperature (°C)			Salinity (‰)		
	Full	(–)N	(–)P	(–)N&P	25	17	12.5	35	28	20
dcSTX	0.2	0.1	0.3	2.2	5.7	3.2	1.4	0.0	0.0	0.0
GTX2+GTX3	0.3	0.3	0.0	0.5	0.3	0.9	1.3	0.5	0.1	0.5
GTX5	0.2	0.3	0.3	0.3	0.9	1.9	1.7	0.7	0.2	0.5
GTX6	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.3	0.1	0.2
dcGTX2+dcGTX3	0.9	0.9	0.7	1.0	0.6	1.6	2.2	1.3	0.3	1.7
CT+C2	78.3	75.9	66.6	71.7	82.6	86.5	86.5	73.8	79.3	86.6
C3+C4	20.1	22.5	32.1	24.2	9.9	9.6	6.7	23.4	20.0	10.4

2 and Fig. 2, respectively. The absolute toxicity level (total toxin content cell^{-1}) was variable according to culture conditions. More than 90% of the toxins were composed of C1–C4 with trace amounts of dcSTX, GTX3, dcGTX2. STX, neoSTX, and GTX1 and GTX4 were not detected or present at less than 0.1%, and thus are not shown in Table 2. The relative abundance of β-epimers of 11-hydroxysulfate toxins (C2, C4, GTX3, dcGTX3) always exceeded those of α-epimers (C1, C3, GTX2, and dcGTX2). In actively growing cells only β-epimers were detected. The same phenomenon has been observed in *Alexandrium* species (Hall 1990, Oshima et al. 1990), indicating that the β-epimer is the first biosynthesis product and the chemically more stable α-epimer is formed grad-

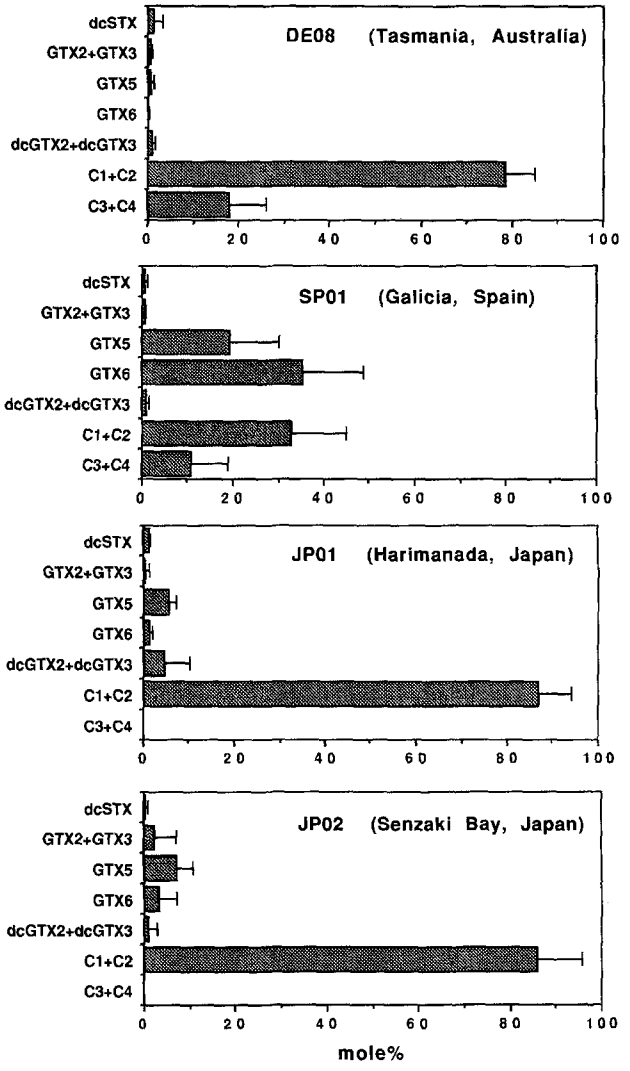

Fig. 3. *Gymnodinium catenatum.* Relative abundance of paralytic shellfish toxins in the isolates from three different countries. Average and standard deviation of toxin compositions (mol%) under seven different culture conditions (Table 2) shown for each isolate. For definitions of toxin abbreviations see "Materials and methods – analysis of toxins"

ually through keto-enol epimerization. Therefore, in the present study they were combined to simplify the comparison of toxin profiles. Recently, three new toxins were isolated from Tasmanian shellfish and characterized to be a new series of 13-deoxydecarbamoyl derivatives of STX, GTX2 and GTX3 (doSTX, doGTX2 and doGTX3, Fig. 1) (Oshima et al. 1990). These components were produced by all Tasmanian toxic isolates (Fig. 2c) but were excluded from the table due to lack of an appropriate quantitative standard.

Regardless of changes in culture conditions, the toxin profile of the Tasmanian isolate DE08 remained stable, except for a slight variation in the relative proportion of C1 + C2 vs C3 + C4 toxins. Similarly, the toxin profiles of the Spanish and Japanese isolates varied only slightly (Fig. 3), but the difference in toxin compositions of *Gymnodinium catenatum* from the three countries was significant. The Spanish isolate (SP01) is characterized by the larger proportions of GTX5 (30%) and GTX6 (20%). The two Japanese isolates from different localities (Harimanada and Senzaki Bay) showed very similar toxin profiles and were clearly distinguished from those of other countries by the complete absence of C3 and C4 toxins. Moreover, 13-deoxydecarbamoyl toxins were not detected in Japanese (Fig. 2d) or Spanish isolates. A toxin profile similar to our SP01 isolate was reported for Spanish *G. catenatum* by Anderson et al. (1989), except for a slight difference in the contents of trace toxins. Ikeda et al. (1989) also confirmed the complete absence of C3 and C4 in *G. catenatum* from Senzaki Bay, although their analysis was qualitative.

Variations in the toxin profiles of the dinoflagellate population of one geographic area were examined using *Gymnodinium catenatum* isolates from different bloom events in Tasmanian waters (Table 3). Most of the isolates showed toxin profiles (C1 + C2 68 to 79%, C3 + C4 12 to 23% and trace amounts of GTX5 and GTX6) similar to DE08 except for the HU02, DE05 and DE06 isolates which showed a larger proportion of C3 and C4 toxins (57 to 70%). Further experiments are needed to clarify whether this discrepancy is due to genetic differences or caused by unknown physiological conditions. All the isolates in Table 3 produced 13-deoxydecarbamoyl derivatives, especially doSTX, in large quantities. Assuming that this component has a fluorescent response equal to that of STX, it reached more than 50% of total toxins in most of the Tasmanian isolates.

The Tasmanian isolates HU04, HU05, HU09 and HU10, all derived from one wild resting cyst, are regarded to be nontoxic since the toxin contents were below the detection limit of our HPLC (less than 0.01 fmol cell^{-1}), compared to 50–200 fmol cell^{-1} of other toxic isolates. Successful sexual crossing experiments were carried out between these nontoxic isolates and toxic isolates (Oshima et al. 1992), indicating that they clearly belong to the same biological species. Further investigation is necessary to decide whether they are merely mutants that lost their toxicity or actually are a nontoxic subpopulation of *Gymnodinium catenatum* that exists in Tasmanian waters. The healthy growth of these isolates without toxin production may indicate that saxitoxin derivatives are secondary metabolites which are not essential for the dinoflagellates. The occurrence of nontoxic isolates in *Alexandrium tamarense* has also been reported (Boyer et al. 1986, Cembella et al. 1987).

In *Alexandrium* species, toxin composition has long been recognized as a stable or conservative property of a clone (Boyer et al. 1986, Cembella et al. 1987, Ogata et al. 1987, Oshima et al. 1990). However, recently Boczar et al. (1988) and Anderson et al. (1990) reported drastic changes in toxin composition of *Alexandrium* isolates in nutrient-stressed batch or semicontinuous culture. In our batch cultures of *Gymnodinium catenatum*, compositional changes in toxins were minor, even under nutrient-deficient conditions, and were insignificant compared to variations between the isolates from different geographic origins.

The toxin profile is an expression of enzymes involved in toxin biosynthesis, and the presence or absence of certain toxins provides clear evidence for genetic differences

Table 3. *Gymnodinium catenatum.* Variation in toxin composition (mol%) of *G. catenatum* from Tasmania, isolated from different areas and in different mo and yr. For definitions of toxin abbreviations see "Materials and methods – analysis of toxins". For isolate locations see "Materials and methods – dinoflagellate cultures"

Toxins	Isolate										Mean ± SD
	HU02 Jun 1986	DE01 Jan 1986	DE02 Jan 1986	DE03 Feb 1986	DE04 Feb 1986	DE05 Jan 1987	DE06 Jan 1987	DE07 Jan 1987	DE08 Jun 1987	DE09 Jun 1987	
dcSTX	0.5	4.0	2.3	2.7	0.2	2.1	1.2	0.5	1.7	2.1	1.7± 1.2
GTX1+4	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.0	0.0	0.0± 0.1
GTX2+3	0.1	0.4	0.8	1.2	0.3	0.4	0.0	0.4	0.5	0.3	0.4± 0.3
GTX5	1.6	2.8	3.0	1.3	0.2	7.6	7.0	9.5	2.1	4.1	3.9± 3.1
GTX6	1.7	1.3	1.2	1.9	0.0	0.8	0.5	1.1	0.3	0.8	1.0± 0.6
dcGTX2 +dcGTX3	0.7	0.7	1.2	9.2	0.9	0.3	0.1	1.5	0.8	0.8	1.6± 2.7
C1+C2	27.0	68.0	77.2	71.9	78.3	31.9	20.9	70.0	79.3	71.8	59.6±23.2
C3+C4	68.4	22.7	14.3	11.8	20.1	56.7	70.3	17.1	15.3	20.1	31.7±23.5

between and within populations. The present study has demonstrated that *Gymnodinium catenatum* toxin profiles are a valid biochemical marker to discriminate between strains of this dinoflagellate species, at least when profiles are compiled under carefully defined experimental conditions. The Tasmanian population of *G. catenatum* produces toxins which are clearly distinguishable from those of isolates from Spain and Japan. This seems to disprove the idea of a recent introduction of *G. catenatum* into this area. However, analyses of more isolates, especially those from other areas, are needed to reach a definite conclusion on this subject. In fact, transportation of a large number of viable *Alexandrium* cysts via ship's ballast water has been confirmed (Hallegraeff and Bolch 1991), and *G. catenatum* cysts have been detected in ships entering Australian waters (Hallegraeff and Bolch 1992). A concurrent study on the genetics of *G. catenatum* populations using enzyme electrophoresis and DNA sequencing is also underway.

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