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Source and profile of paralytic shellfish poisoning toxins in shellfish in Daya Bay, South China Sea

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1

Abstract

2 Changes in cell density and cyst flux of *Alexandrium tamarense*, paralytic shellfish poisoning
3 (PSP) toxin contents in shellfishes, and environmental parameters were measured in two
4 stations in Daya Bay, South China Sea from March 2005 to July 2006. Vegetative cells of *A.*
5 *tamarense* occurred sporadically; however, they presented abundantly during the winter
6 months. Meanwhile, cyst flux reached its maximum level just following the peak abundance
7 of motile cells. The PSP contents in shellfish were generally low, but higher in winter with the
8 maximum of 14 015 μg STX equiv./kg. The majority of toxins were found in digestive glands,
9 with a maximum of 66 227 μg STX equiv./kg. There were significant positive relationships
10 between toxin level and vegetative cell density and cyst flux. This indicates that vegetative
11 cells and cysts of *Alexandrium* significantly influenced PSP level, and could be an important
12 source of PSP toxins in shellfish during winter.

13

14 Key words: Paralytic shellfish poisoning (PSP) toxins; *Alexandrium tamarense*; Cyst;
15 Phytoplankton; Algal toxins; Toxicity; South China Sea

16

17 1. Introduction

18 Paralytic shellfish poisoning (PSP) toxins are biotoxins produced by
19 micro-phytoplankton that can accumulate in filter-feeding shellfish (Shumway, 1990;
20 Hallegraeff, 1995). Subsequent ingestion of contaminated shellfish by humans can result in
21 serious illness or even death. The incidence of human poisoning has increased markedly since
22 the early 1970s (Hallegraeff, 1995). Species within the dinoflagellate genus *Alexandrium*,
23 such as *A. tamarense* and *A. catenella*, have been known as important producers of PSP toxins.
24 Resting cysts of these toxic dinoflagellates have been shown to contain toxins (Bravo et al.,
25 1998; Persson et al., 2006) and sometimes have been reported to contain six (Oshima et al.,
26 1992) to ten folds (Yentsch and Mague, 1979) more toxin than vegetative cells. Dinoflagellate
27 cysts are one of the food sources of bivalves (Tsujino and Uchida, 2004; Persson et al., 2006).
28 Many studies have suggested that consumption of cysts may be the direct cause of toxicity in
29 bivalves, especially during the winter months (Shumway et al., 1988; Schwinghamer et al.,
30 1994; Anderson, 1997; Bravo et al., 1998).

31 Daya Bay is a site of recurrent PSP episodes (Anderson et al., 1996; Lin et al., 1999) with
32 the highest PSP toxin levels among the Chinese coastal areas (Lin et al., 1999; Jiang et al.,
33 2000a). Human mortalities and hospitalizations have occurred from time to time in nearby
34 areas following consumption of toxic mussels. Five persons were poisoned due to
35 consumption of the contaminated shellfish in 1989 (Jian et al., 1991), and three persons died
36 after shellfish consumption in 1993 (Lin et al., 1999). Bivalves collected from this sea area
37 had PSP toxin concentrations exceeding the regulatory limit of 800 µg STX equiv./kg, and a
38 maximum concentration of 10 000 µg STX equiv./kg was reported (Jiang et al., 2000a; Yang
39 et al., 2005). The highest contents of PSP toxins were detected during the winter months
40 (Jiang et al., 2000a). Despite the long-known occurrence of PSP toxins, PSP causative species,
41 such as *Alexandrium* spp. and *Gymnodinium catenatum*, were seldom recorded in
42 phytoplankton survey (Zhou et al., 1998; Liu et al., 2006). However, abundant *Alexandrium*
43 cyst were recorded in surface sediments from this area, and it was suggested that high PSP
44 toxin levels in bivalves during the winter months were probably due to ingestion of cysts of

45 *Alexandrium* (Wang et al., 2004). Therefore, we set up sediment traps at two stations of Daya
46 Bay, bred shellfish synchronously at the same areas, and conducted phytoplankton surveys.
47 For phytoplankton community and its relationships with environmental parameters, see Wang
48 et al. (2009). In this study, we analyzed the annual data of *Alexandrium* vegetative cell and its
49 cyst, and the PSP concentrations in shellfish. The purpose is to investigate the seasonal
50 variation in the population size of *Alexandrium* (cell density and cyst flux) and their
51 relationship with PSP levels in shellfish.

52

53 **2 Materials and Methods**

54 *2.1 Site description*

55 Daya Bay is located in the southeast part of the South China Sea (22°30′-22°50′N,
56 114°30′-114°50′E). It is a semi-enclosed shallow embayment with depth ranging from 6 m to
57 15 m. It covers an area of 650 km² and is separated into several small sub-basins. The bay has
58 a mild and wet subtropical climate with an annual mean air temperature of 22 °C. The coldest
59 months are January and February, with a monthly mean air temperature of about 15 °C, and
60 the hottest months are July and August, with a monthly mean air temperature around 28 °C.
61 Salinity usually lies between 22 and 33 with little fluctuation except during the typhoon
62 season from July to September. The Daya Bay Nuclear Power Station (DNPS), the first
63 nuclear power station in China, has been operating since February 1994, and it discharges
64 cooling water at the rate of 2.9×10^7 m³/year (Liu et al., 2006).

65 Sampling stations (Fig. 1) were located in two cultural areas of the bay. St. 1 was located
66 at 22°33′25.7″N, 114°30′54.1″E, where *Pteria* spp. were bred in suspended cages. St. 2 was at
67 a fish cage area (22°33′51.7″N, 114°31′20.2″E), which was closer to the shore and close to the
68 residential area.

69 *2.2 Nutrients and hydrographic parameters*

70 Temperature (T), Salinity (S) and dissolved oxygen (DO) were recorded *in situ* with an
71 YSI meter (YSI 85, YSI Incorporated, USA) once every three days. Transparency and
72 nutrients were measured every two weeks. Transparency was measured with a Secchi disk.

73 Water samples for nutrient determination were collected in the subsurface water column (0.5
74 m), immediately filtered through glass fibre filters (Whatman GF/F, porosity=0.7 μm), and
75 placed in a freezer. Nutrients, including dissolved inorganic nitrogen (DIN, the sum of
76 ammonium, nitrite and nitrate), dissolved inorganic phosphorus (DIP, orthophosphate) and
77 dissolved silicon (DSi, silicate), were analysed following standard methods of State Oceanic
78 China (SOC, 1991) within 24 hours.

79 2.3 Phytoplankton sampling and observation

80 Water samples of 1000 mL were collected in the subsurface water column once every
81 three days for phytoplankton observation and fixed immediately with neutralized formalin to
82 a final concentration of 4%. The fixed water samples were finally concentrated to 20 mL by
83 sedimentation. Phytoplankton species were counted from aliquots of 0.5-1.0 mL sub-samples
84 using a microscope (Leica DMIRB) at magnifications of 100x to 600x. The phytoplankton
85 abundance was presented as cell numbers per millilitre (cells/mL).

86 The thecal plate morphology of *Alexandrium* cells was examined using phase contrast and
87 UV epifluorescence microscopy after calcofluor staining (Fritz and Triemer, 1985) under a
88 Leica DMIRB microscope. The thecal plate tabulation and the shapes of plates and apical
89 pore complex proved that the dominant *Alexandrium* species was *A. tamarense*.

90 2.4 Sediment collection and cyst observation

91 The sediment trap was set to collect settling materials from March 24, 2005 to June 10,
92 2006. The trap consisted of a cylindrical PVC collector (height 35cm, diameter 7cm,
93 height/diameter ratio 5), flexible tube holder, subsurface buoy, surface buoy marker, and
94 anchor. The trap was moored at 1.0 m from the sea bottom (depth at the deployment location
95 was about 6 m).

96 Trap samples were collected every two weeks. The settled material was suspended in
97 2000 mL seawater, and the suspension was divided into two 1000 mL aliquots, one of which
98 was fixed with 4% buffered formalin and subsequently concentrated to 50 mL by
99 sedimentation.

100 Sediment samples were gently sonicated for 30 seconds. The sonicated material was then

101 sieved through 125 µm and 20 µm metallic screens to remove larger particles and detritus, and
102 the slurry remaining on the 20 µm screen was washed into a beaker and collected into a
103 plastic tube to a final sample volume of 10 mL with filtered seawater. For observation, 0.1 to
104 0.5 mL aliquots of sample were placed on a 1 mL counting chamber and diluted with
105 appropriate distilled water.

106 Observation was under an inverted light microscope (Leica DMIRB). Only living cysts
107 that had not germinated were counted. At least three observations were conducted for each
108 sample until 200 dinoflagellate cysts were observed. Mean daily cyst flux (cysts/cm² d) was
109 calculated. Four seasons during the survey were defined as: spring, March to May; summer,
110 June to August; autumn, September to November; and winter, December to next February.

111 *2.5 Shellfish breeding and PSP analysis*

112 *2.5.1 Shellfish breeding and sampling*

113 At the beginning of the survey on March 24, 2005, 600 scallops (*Chlamys nobilis*) were
114 bought from the local shellfish farm. Half of the scallops were placed in lantern nets and hung
115 from long lines 3 m below the surface in each of the two stations. Green mussels (*Perna*
116 *viridis*) were collected from fishing crossrails and ropes and shellfish cages. About 10 scallops
117 (mean shell length of 9.48±0.44cm) and 20 green mussels (mean shell length of 9.19±0.95cm)
118 were collected in each station for PSP toxin determination every two weeks. The shells were
119 dissected immediately after being washed with a brush and rinsed with DI water. Whole soft
120 tissues were removed from the shells and washed with DI water gently. Digestive glands were
121 carefully removed from the rest of tissues. Both the digestive glands and other tissues were
122 weighed and placed in plastic bags separately and stored in a freezer. During the
123 transportation to the laboratory (within 4 h), the tissue samples were stored at -20 °C.

124 *2.5.2 Mouse bioassay*

125 As the toxicities of most samples were below detection level, the digestive glands were
126 treated and analysed first in order to screen out any contaminated samples. If the digestive
127 glands were toxic to test mice, the remaining tissues of the same sample then treated and
128 assayed. Sample treatment and mouse bioassay were performed according to the standard

129 AOAC procedure (Association of Official Analytical Chemists, AOAC, 1990) with the
130 exception that samples sizes were smaller (about 20 g digestive gland homogenate, equal
131 volume of 0.1N HCl and digestive gland homogenate). Mouse bioassays were conducted
132 using ICR strains, 19–21 g male mice. The mice (3-6 mice per extract) were observed for 1 h,
133 and the mean survival time was used to calculate the toxicity. When the extracts were too
134 toxic, it was necessary to dilute the shellfish extract until the survival time was between 5 and
135 7 min. Bioassay results were calculated using a computer-based version of Sommer's table
136 and correction table for mouse weight (Sommer and Meyer, 1937). The mouse bioassay was
137 standardized using pure saxitoxin purchased from the National Research Council of Canada,
138 Institute for Marine Biosciences (Halifax, Canada). The toxicity of the sample equals to the
139 sum of toxicities of the digestive glands and the other tissues per kg total tissue (μg STX
140 equiv./kg), and the toxicity of the digestive gland was expressed as μg STX equiv. per kg
141 digestive gland.

142 2.5.3 HPLC analyses

143 Four digestive gland samples with high toxicity in mouse bioassay were selected for
144 HPLC analysis (Table 1). Each sample was divided into two sub-samples. One sub-sample
145 was extracted in 0.1 N HCl according to the AOAC extraction procedure as described in 2.5.2,
146 and another was extracted in 1% (v/v) acetic acid solution according to Lawrence et al. (2004).
147 Toxin contents and profiles were determined using the post-column oxidation method
148 (Oshima, 1995) modified by Anderson et al. (1996). The HPLC system used was an Agilent
149 Series 1100, fitted with an autosampler and fluorescence detector (Agilent, USA). Three
150 separate isocratic elutions were employed to separate different sets of PSP toxins at a flow
151 rate of 0.8 mL/min: (1) 2 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0
152 with acetic acid for C toxins; (2) 2 mM 1- heptanesulphonic acid in 10 mM ammonium
153 phosphate buffer (pH 7.1) for GTX and dcGTX group; and (3) 2 mM 1-heptanesulphonic acid
154 in 30 mM ammonium phosphate buffer (pH 7.1): acetonitrile (100:5) for the STX group.
155 Toxin standards, GTX1-5, neoSTX, STX, dcSTX, dcGTX2/3 and C1, C2, were purchased
156 from the National Research Council of Canada, Institute for Marine Biosciences (Halifax,

157 Canada). The standards solutions were diluted with 0.03 N acetic acid as required. PSP toxin
158 profiles were calculated from the percentages of individual toxin concentrations of the total
159 PSP toxin concentrations for each sample (mol %). The relative toxicities ($\mu\text{g STX equiv./kg}$)
160 were calculated based on the specific toxicities given by Oshima (1995).

161

162 **3 Results**

163 *3.1 Environmental conditions*

164 Water temperature ranged from 14.0 to 32.8 °C, with the lowest recorded from December
165 to early March and the highest from July to November. Salinity was relatively stable with an
166 annual mean value of 29.3, except for the rainy season from April to May and from July to
167 September.

168 Nutrient concentrations showed similar seasonal changes at the two stations.
169 Concentrations of DIN ranged from 2.04 $\mu\text{mol/L}$ to 14.94 $\mu\text{mol/L}$, and showed an annual
170 cycle characterized by higher values during spring and summer and lower values during late
171 autumn and winter. Concentrations of DIP remained consistently low, and ranged from 0.044
172 $\mu\text{mol/L}$ to 1.67 $\mu\text{mol/L}$. DSi concentrations were remarkably high during the flood season
173 between July and September, and low in winter and the early spring dry season. Annual mean
174 values in DIN and DIP showed no significant differences between the two stations; however,
175 DSi level was obviously higher at St. 1 than St. 2, with mean concentrations of 17.8 $\mu\text{mol/L}$
176 and 12.2 $\mu\text{mol/L}$, respectively.

177 *3.2 Abundance of A. tamarensis vegetative cells*

178 The vegetative cells of *A. tamarensis* occurred sporadically in the water column and
179 showed a regular seasonal cycle. Vegetative cells only occurred during spring (March to May)
180 and winter (November to February) (Fig. 2). The abundance during spring was low, ranging
181 from 0.2 to 1.4 cells/mL. The vegetative cells were not detected during summer and autumn
182 (Fig. 2) when water temperature exceeded 25 °C. Cell numbers increased at the end of
183 November with decreasing water temperature and peaked in January and February, when
184 water temperatures were around 20 °C. Cell numbers were markedly higher in St. 1 than in St.

185 2, and the maximum cell number of 603 cells/mL was observed in St. 1 on February 19, 2006.
186 Maximum number coincided with low water temperature and low nutrient concentrations
187 during the winter months.

188 The phytoplankton assemblages were dominated by diatoms, which constituted 93.2% of
189 total phytoplankton population. Dinoflagellates occurred in low numbers with a mean
190 abundance of 9.4 cells/mL, and represented only 1.2% of total abundance. The abundance of
191 total phytoplankton was 3 920 cells/mL when the maximum cell number of *A. tamarensis*
192 occurred. The most dominant species was *Skeletonema costatum* at that time, with a cell
193 density of 3 230 cells/mL and proportion of 82.5%. *A. tamarensis* accounted for 98.3% of
194 dinoflagellate, but only 15.3% of total phytoplankton.

195 3.3 Cyst flux of *A. tamarensis*

196 Cysts of *A. tamarensis* occurred frequently in the sediment traps, but in low numbers
197 (mostly <5 cysts/cm² d). Cysts appeared to have a seasonal cycle similar to that of the
198 vegetative population and occurred more abundantly during spring and winter (Fig. 2, Fig. 3).
199 High cyst fluxes were observed approximately two weeks to one month after the vegetative
200 cell peaks. For example at St. 1, there was a significant increase in cyst flux in January 2006
201 just following vegetative cell peaks in December and January, and the maximum flux of 42.6
202 cysts/cm² d was observed on February 11-26, 2006, right after the maximum number of
203 vegetative cells. Meanwhile, a strong positive correlation existed between cyst flux and the
204 cumulative number of vegetative cell (Cysts = $6.64 \times 10^{-5} \times \text{Cells} + 0.77$, $r = 0.995$, $n = 27$,
205 $p < 0.0001$).

206 3.4 PSP toxins in shellfish

207 The extracts of shellfish tissues in the present study killed mice with typical symptoms
208 associated with PSP toxins. Seasonal variations in toxin concentration are illustrated in Fig. 4.
209 The PSP toxins in the two stations were generally below the regulatory limit, except for the
210 winter months. The concentrations of PSP toxins were lower in spring and early summer, and
211 PSP toxins were not detected between August and October. Toxin concentrations increased
212 significantly in January, and a maximum level of 14 015 µg STX equiv./kg was recorded in *P.*

213 *viridis* samples collected from St. 1 on January 3, 2006 (Fig. 4). The majority of toxins were
214 detected in digestive glands, with the maximum of 66 227 μg STX equiv./kg in digestive
215 glands from *C. nobilis* collected at St. 1 on January 3, 2006 (Fig. 5). Toxins in other tissues
216 were very low to undetectable except for five samples collected in January 2006, with a
217 maximum level of 392 μg STX equiv./kg. The results showed that the hepatopancreatic
218 tissues from both *P. viridis* and *C. nobilis* were unsafe for human consumption during the
219 winter months, while the tail muscle would be safe.

220 Toxin levels in digestive glands from *C. nobilis* (mean value of 3 373 μg STX equiv./kg)
221 were significantly higher than those from *P. viridis* (mean value of 1 531 μg STX equiv./kg).
222 However, the contribution of digestive gland to total tissues is lower in *C. nobilis* than in *P.*
223 *viridis*. Therefore, toxin concentrations in total tissues of the two shellfish were similar, with
224 mean values of 399 and 394 μg STX equiv./kg in *C. nobilis* and *P. viridis*, respectively.

225 The sharp increase in concentrations of PSP toxins in January was coincident with the
226 occurrence of *Alexandrium* population (both vegetative cells and cysts), with peak
227 concentrations or abundances between January and March. PSP concentrations were clearly
228 higher in St. 1 than in St. 2, and the averages in digestive glands and total tissues were 3 796
229 and 632 μg STX equiv./kg in St. 1 and 1 107 and 161 μg STX equiv./kg in St. 2, respectively.

230 3.5 PSP toxin profiles

231 Fig. 6 illustrates the toxin profiles in four digestive gland samples treated by HCl and
232 acetic acid, respectively. Of the 12 components analyzed in this study, GTX5 and dcGTX2
233 were not detected in any of the samples, and only trace amounts of decarbamoyl toxins were
234 detected with a mean proportion of 2.2%. The PSP toxin profiles in HCl extracts were rich in
235 carbamate toxins (mean 63.7% of the total) and dominated by GTX2 (mean 35.1%), GTX3
236 (mean 26.2%) and GTX4 (mean 15.7%). C toxins accounted for a medium amount of the total
237 (around 0-12% for C1, 1-7% for C2). In addition to these components, a trace amount of
238 decarbamoyl toxins (mean 3.3%) was detected. In contrast, in acetic acid extracts, large
239 amounts of C toxins were detected instead, and C1 (around 38-68% of the total, mean 53.6%)
240 was the most abundant. On the other hand, concentrations of GTX toxins dropped

241 significantly and accounted for 4-31% of the total, with an average of 17.8%. The relative
242 concentrations of STX toxins (including STX, dcSTX and neoSTX) in acetic acid extracts
243 (mean 22.1%) were similar to those in HCl extracts (mean 18.4%); however, percentages of
244 STX increased from 6.7% in HCl extracts to 13.9% in acetic acid extracts on average.

245 Though samples collected from St. 1 on 3 January 2006 were most toxic during study
246 period (Fig. 4, 5), the toxin profiles (sample 1 and 1') showed less toxic GTX2 and C1 (Fig.
247 6). Other less toxic samples (samples 2-4, and 2'-4') had higher proportions of more toxic
248 components such as STX, neoSTX and GTX4. However, toxin concentrations on a molar
249 basis ($\mu\text{mol/kg}$) in sample 1 and 1' were 5-8-fold greater than those in other samples (Table
250 1).

251 Toxin concentrations on a molar basis ($\mu\text{mol/kg}$) were comparable between any two
252 parallel samples treated by HCl or acetic acid (Table 1). The toxicities in HCl extracts
253 approximately coincided with those analyzed by mouse assay. However, samples treated by
254 acetic acid showed only one-third to two-thirds of those treated by HCl, especially in samples
255 with rich contents of C1 toxin (Table. 1, Fig. 6).

256

257 **4 Discussion**

258 High abundance of *A. tamarensis* vegetative cells was observed in this study, with a
259 maximum number of 603 cells/mL. In previous phytoplankton studies, vegetative cells of
260 *Alexandrium* were seldom observed in the water column during seasonal routine observation
261 due to the long interval of sampling (Liu et al., 2006). However, peak abundance of *A.*
262 *tamarensis* was captured in this survey by highly frequent sampling (once every three days).

263 *Alexandrium tamarensis* showed a high dispersal of temperature/cell density couples (Fig.
264 7). It never occurred at temperatures greater than 30 °C, and peak abundances occurred when
265 temperature lies between 16 and 20 °C. *A. tamarensis* is a stenohaline alga (ca. 15-25 °C) with
266 optimal growth at 17-22 °C (Yan et al., 2002). The blooms of *Alexandrium* usually occurred
267 in temperatures between 13 and 18 °C (Giacobbe et al., 1996; Gracéc et al., 2004). After the
268 operation of DNPS, the annual mean water temperature increased by 0.4-1.1 °C (Tang et al.,

269 2003), and the variation in temperature was more significant in winter months, increasing
270 from 16.04 °C to 18.03 °C (Wang et al., 2008), which seem to favor the growth of
271 *Alexandrium*. Meanwhile during this period, nutrient loadings had increased significantly in
272 the bay (Wang et al., 2008), facilitating the rapid growth of phytoplankton, especially the
273 small diatoms with high growth rates (Liu et al., 2006; Wang et al., 2009). The phytoplankton
274 abundances peaked in spring and summer during the early 1990s (Zhou et al., 1998), but
275 frequent winter peaks have been observed since the late 1990s (Liu et al., 2006; Wang et al.,
276 2009). Generally, Daya Bay is characterized by high levels and rapid compensation of
277 nutrients, however, temporary shortage of nutrients occurs when the diatom bloom exhausts
278 the nutrients, particularly during the dry low-nutrient winter season (Wang et al., 2009).
279 Dinoflagellates such as *Alexandrium* are more competitive in nutrient depletion conditions
280 due to its higher nutrient affinities and capability to utilize organic nutrients (Collos et al.,
281 2007). As a result of higher winter temperatures and increased nutrient loading, *Alexandrium*
282 has occurred more frequently and abundantly during winter months, which contributes to the
283 high PSP concentrations in shellfish.

284 Cyst fluxes were recorded during summer and autumn (June to October), when no
285 vegetative cells of *A. tamarense* were observed in the water column. The absence of
286 vegetative cells in the subsurface water column during the observation does not necessarily
287 indicate their absence in the entire water column. More likely, the water sample collection did
288 not catch *A. tamarense* cell patches due to random sampling. In addition, this period is also
289 the typhoon season in South China Sea. Strong storms may cause a high resuspension rate,
290 which could result in redeposition of bottom cysts into sediment traps.

291 Similar to the total phytoplankton abundance, cell density and cyst flux of *A. tamarense*
292 were clearly higher in St. 1, located in a shellfishery area, than those in St. 2, located in a fish
293 cage area. This resulted in higher PSP levels in shellfish collected from St. 1. The low primary
294 production in the fishery area was observed in another study in this area as well (Huang et al.,
295 2005). Meanwhile, higher concentrations of *Alexandrium* cyst were recorded in surface
296 sediments from the shellfishery area in the same sea area (Wang et al., 2004). This

297 phenomenon may be interpreted as compensatory growth of phytoplankton against predatory
298 pressure by shellfish.

299 Daya Bay has been reported to have the highest PSP concentrations among the Chinese
300 coastal areas (Lin et al., 1999; Yang et al., 2005). Jiang et al. (2000a) found that the highest
301 concentration of PSP toxins occurred in January during their survey conducted between 1997
302 and 1999. However, *Alexandrium* was seldom observed in previous phytoplankton surveys
303 (Liu et al., 2006). Our results showed that high cell numbers and cyst fluxes of *Alexandrium*
304 coincided with high PSP levels in shellfish. In addition, content of PSP toxins showed a
305 strong positive relationship with the accumulative abundances of vegetative cells and cysts
306 [$\ln(\text{PSP}) = 0.41 \times \ln(\text{Cells}) + 2.34$, $r = 0.647$, $n = 27$, $p = 0.002$; $\ln(\text{PSP}) = 0.37 \times \text{Cysts} + 5.22$,
307 $r = 0.373$, $n = 27$, $p = 0.04$]. These results suggest that vegetative cells and cysts of
308 *Alexandrium* influenced significantly the PSP levels, and could have contributed to high PSP
309 levels in shellfish during winter.

310 However, toxin levels were not always consistent with concentrations of vegetative cells
311 and cysts. The inconsistency of PSP contamination in shellfish and concentration of
312 *Alexandrium* cells and cysts might be explained by the variability of *Alexandrium* toxicity,
313 which depends on environmental conditions, among species and strains (Anderson, 1990).
314 Toxicities in *Alexandrium* vegetative cells and cysts seem to be species and strain specific.
315 The average toxicity of *A. tamarense* vegetative cells is approximately 16 pg STX equiv./cell
316 (Persson et al., 2006). Oshima et al. (1992) reported as much as 269 pg STX equiv./cyst in *A.*
317 *tamarense*, and Bricelj and Shumway (1998) reported 325 pg STX equiv./cell of an
318 *Alexandrium* sp. Beppu et al. (2008) measured the toxicities of six strains of *Alexandrium* from
319 the coastal water of Hiroshima Prefecture, Japan, and found that variation in the toxin level
320 was varied about 30 fold among strains (from 3.7 to 111.5×10^{-6} MU/cell), or 10 fold even
321 between *A. tamarense* strains isolated from the same sea area in the same month of different
322 years.

323 The toxin profiles of HCl treated samples were similar to those reported by Jiang et al.
324 (2000b) in the same sea area, in which GTX toxins were found to be the main component.

325 GTX toxins were dominant in contaminated shellfish as determined by the AOAC HCl
326 extraction method in other geographic regions (Lassus et al., 1989; Ichimi et al., 2001;
327 Abouabdellah et al., 2008). Toxin profiles of acetic acid extracts showed good similarity with
328 those of *Alexandrium* strains isolated from the nearby sea areas (Jiang et al., 2000b; Wang et
329 al., 2006), which were predominated by C1/C2 toxins and had only trace amounts of STX
330 toxins. The results suggested PSP toxins in filter-shellfish detected in this study were possibly
331 produced by a local strain. It is well known that the AOAC extraction procedure used in the
332 sample preparation converts the sulphocarbamoyl toxins (C1–4, B1, B2) into corresponding
333 carbamates (Laycock et al., 1995; Anderson et al., 1996), i.e., C3,4 to GTX1,4 and C1,2 to
334 GTX2,3 (Turrell et al., 2007). Our results also showed evident toxin conversion through the
335 HCl procedure, as displayed by the remarkable increase of GTX2, 3 and sharp decrease of C1,
336 and meanwhile a comparatively stable overall toxin concentrations on molar basis (mol/kg).
337 The carbamates are considerably more toxic than the corresponding sulphocarbamoyls, and,
338 consequently, changes in toxin profile after HCl treatment will increase the toxicity of the
339 shellfish extract (Kao, 1993). In our study, toxicities of HCl extracts were 1.5-3.5-fold higher
340 than those of corresponding acetic acid extracts. Despite the increase toxicity through HCl
341 procedure, we suggest using HCl extracts in the mouse bioassay. This procedure better
342 protects human health by considering the potential conversion of C toxins into more
343 poisonous GTX toxins in human body.

344

345 **5 Conclusion**

346 Our one-year observation found that high PSP toxin levels in shellfish coincided with
347 peak abundances of cell and cyst of *A. tamarense* during winter months, though the toxin
348 levels were generally below the regulatory limit for most seasons of the year. The significant,
349 positive relationship between toxin level, and vegetative cell and cyst abundance reveals
350 possible toxin source from *Alexandrium* thriving during winter months. More specifically, the
351 majority of the toxins were accumulated in the digestive glands.

352

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475 **Tables**

476 Table 1 PSP toxin contents in digestive gland samples by HPLC analysis and mouse bioassay
 477 using the AOAC HCl procedure and acetic acid extraction procedure, respectively
 478

| | 1 | 1' | 2 | 2' | 3 | 3' | 4 | 4' |
|---------------------------------|--------|--------|-------|-------|-------|-------|-------|-------|
| Toxin concentration by | | | | | | | | |
| HPLC | 191.5 | 173.4 | 18.6 | 15.3 | 23.5 | 18.6 | 22.8 | 24.4 |
| ($\mu\text{mol/kg}$) | | | | | | | | |
| Toxicity by HPLC | | | | | | | | |
| analysis | 41 584 | 11 993 | 4 477 | 1 842 | 5 377 | 3 424 | 6 135 | 2 832 |
| ($\mu\text{g STX equiv./kg}$) | | | | | | | | |
| Toxicity by mouse | | | | | | | | |
| bioassay | 51 565 | - | 6 636 | - | 6 780 | - | 5 551 | - |
| ($\mu\text{g STX equiv./kg}$) | | | | | | | | |

479 1 and 1': *P. viridis* samples from St. 1 on January 3, 2006, 2 and 2': *P. viridis* samples from St.
 480 1 on January 22, 2006, 3 and 3': *P. viridis* samples from St. 1 on February 26, 2006, 4 and 4':
 481 *C. nobilis* samples from St. 1 on March 16, 2006. Samples 1-4 were treated by HCl, samples
 482 1'-4' were treated by acetic acid. "-" indicates without bioassay data.

483 **Figure captions**

484 Fig. 1 Sampling stations in Daya Bay, South China Sea. Also shown is the location of the
485 Daya Bay Nuclear Power Station (DNPS). St. 1 was located at a shellfish cultural area, and St.
486 2 was at a fish cage area close to a residential area.

487 Fig. 2 Seasonal changes in cell density of *Alexandrium tamarense*

488 Fig. 3 Seasonal changes in cyst flux of *Alexandrium tamarense*

489 Fig. 4 Concentrations of PSP toxins in shellfish

490 Fig. 5 Concentrations of PSP toxins in digestive glands of shellfish

491 Fig. 6 PSP toxin profiles in digestive glands from shellfish samples. 1 and 1': *P. viridis*
492 samples from St. 1 on January 3, 2006, 2 and 2': *P. viridis* samples from St. 1 on January 22,
493 2006, 3 and 3': *P. viridis* samples from St. 1 on February 26, 2006, 4 and 4': *C. nobilis*
494 samples from St. 1 on March 16, 2006. Samples 1-4 were treated by HCl, samples 1'-4' were
495 treated by acetic acid.

496 Fig. 7 Relationship between cell density of *Alexandrium tamarense* and water temperature.

Title: Source and profile of paralytic shellfish poisoning toxins in shellfish in Daya Bay, South China Sea

Highlights

> Annual survey on *Alexandrium* population and PSP was conducted. > *Alexandrium* cells and cysts occurred abundantly during the winter months. > PSP contents in shellfish were generally low, but high in winter. > *Alexandrium* thriving during winter months could be possible toxin source in shellfish.

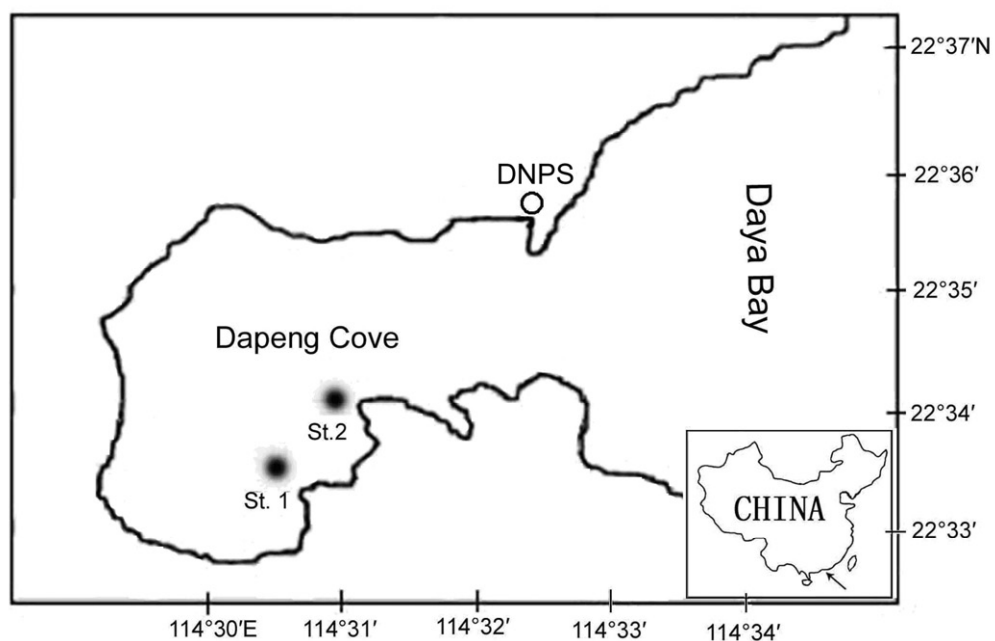


Fig. 1 Sampling stations in Daya Bay, South China Sea. Also shown is the location of the Daya Bay Nuclear Power Station (DNPS). St. 1 was located at a shellfish cultural area, and St. 2 was at a fish cage area close to a residential area.

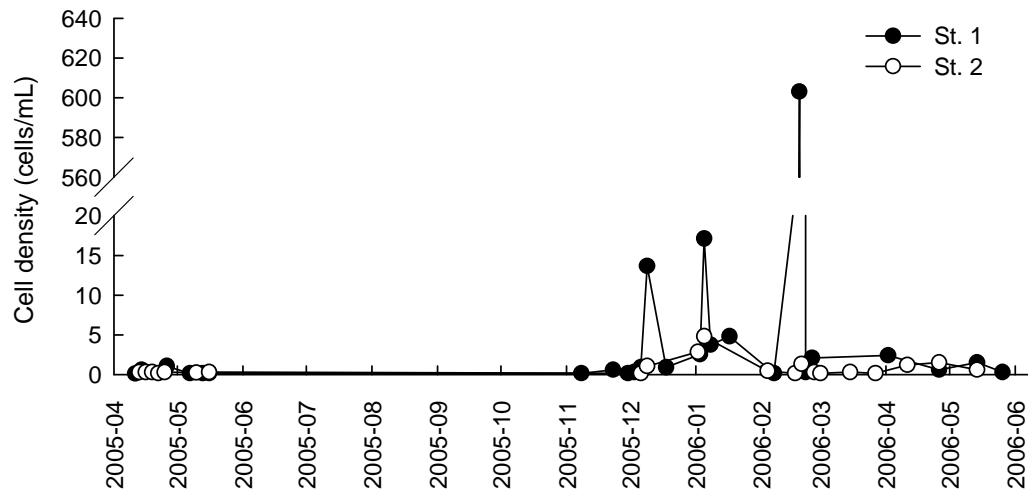


Fig. 2 Seasonal changes in cell density of *Alexandrium tamarensis*

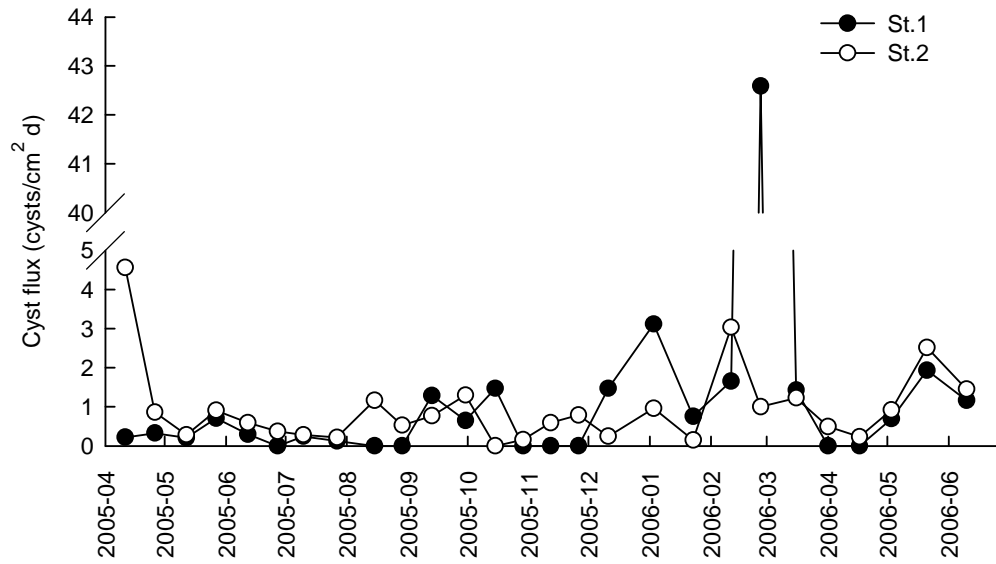


Fig. 3 Seasonal changes in cyst flux of *Alexandrium tamarense*

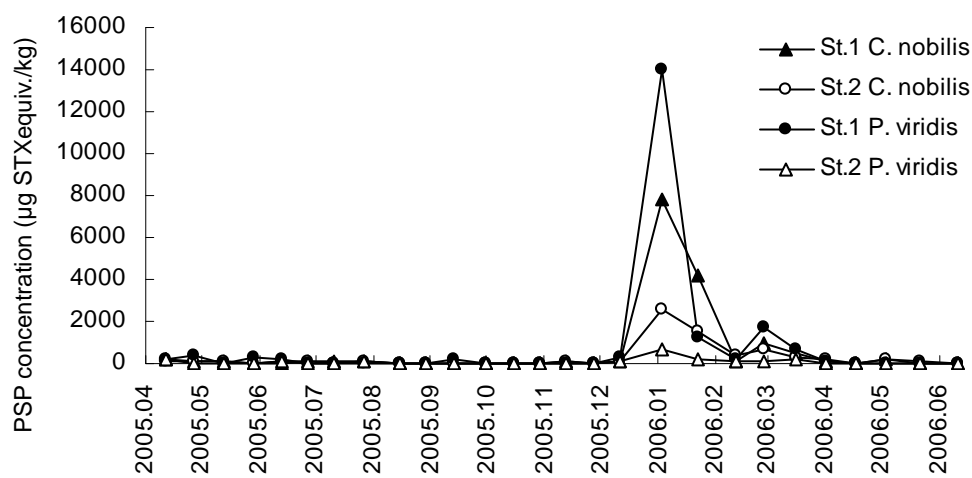


Fig. 4 Concentrations of PSP toxins in shellfish

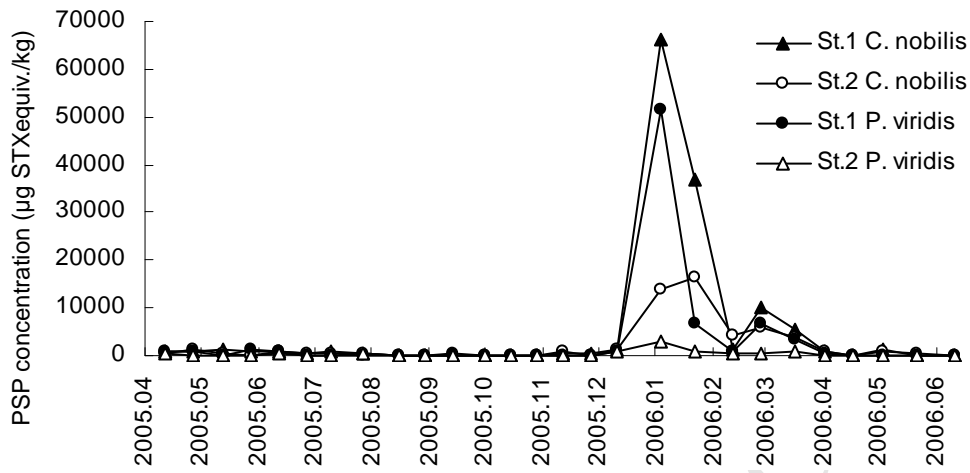


Fig. 5 Concentrations of PSP toxins in digestive glands of shellfish

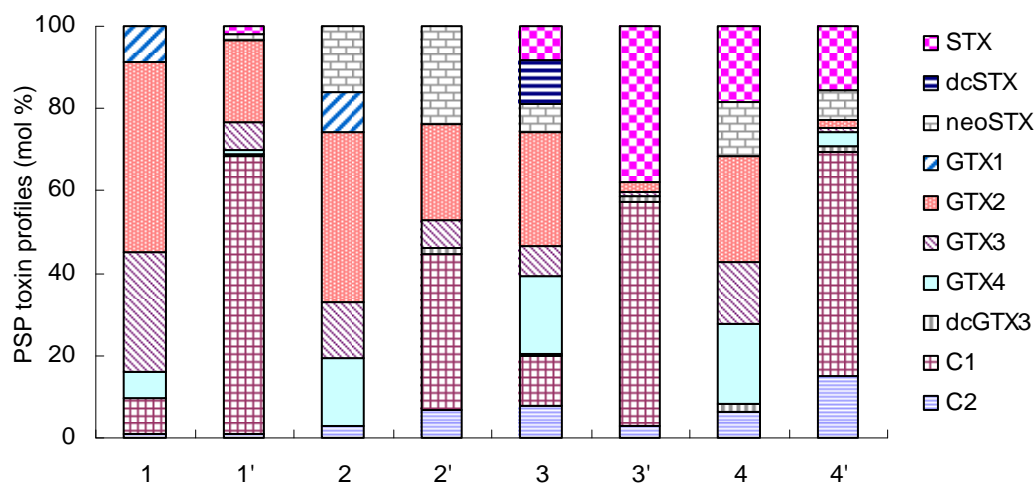


Fig. 6 PSP toxin profiles in digestive glands from shellfish samples. 1 and 1': *P. viridis* samples from St. 1 on January 3, 2006, 2 and 2': *P. viridis* samples from St. 1 on January 22, 2006, 3 and 3': *P. viridis* samples from St. 1 on February 26, 2006, 4 and 4': *C. nobilis* samples from St. 1 on March 16, 2006. Samples 1-4 were treated by HCl, samples 1'-4' were treated by acetic acid.

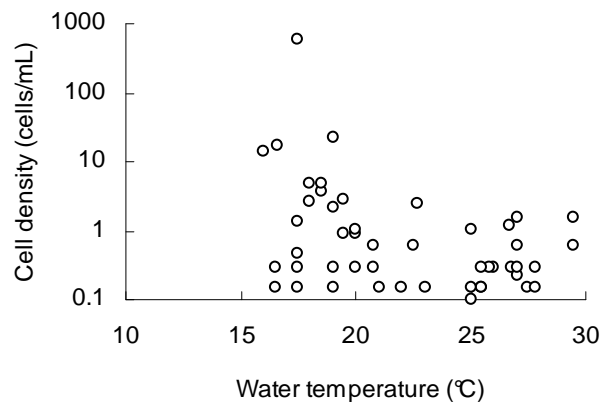


Fig. 7 Relationship between cell density of *Alexandrium tamarense* and water temperature.