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Characterization of *Dinophysis acuminata* from the Yellow Sea, China, and its response to different temperatures and *Mesodinium* prey

by

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Abstract

Dinophysis species are distributed worldwide and cause diarrheic shellfish poisoning (DSP). This paper documents the first successful culture of a *Dinophysis* (DAYS01) strain taken off Xiaoping Island, the Yellow Sea, China. The strain was identified as *Dinophysis acuminata* Claparède & Lachmann by morphological and phylogenetic analysis. The effects of temperature and different *Mesodinium* prey on the physiological and toxigenic characteristics of *D. acuminata* DAYS01 were also investigated. The results showed that the toxin concentration (toxin amount per ml of culture) increased with an increase in cell densities. Okadaic acid (OA), dinophysistoxin 1 (DTX1) and pectenotoxin 2 (PTX2) were the major toxin components of DAYS01, with the highest recorded toxin content of 5.66, 0.56 and 192.87 ng ml^{-1} culture medium, respectively. The prey type significantly influenced the growth yield in *Dinophysis* cultures. There was an effect of prey on the total toxin content, suggesting that the origin or strain of ciliate directly impacted the *D. acuminata* blooms and the overall toxin concentration in the system.

Key words: *Dinophysis*, Diarrheic Shellfish Poisoning (DSP), pectenotoxins (PTXs), okadaic acid (OA), dinophysistoxins (DTXs)

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Introduction

Several species of *Dinophysis* are responsible for severe gastrointestinal symptoms termed "Diarrheic Shellfish Poisoning" (DSP), which was first documented in 1978 (Yasumoto et al. 1978). Since their discovery, the toxins from these organisms have received much attention due to the considerable threat to public health and fisheries resources in many parts of the world (Hallegraeff & Lucas 1988; Lee et al. 1989; FAO 2004; Reguera et al. 2014; Trainer et al. 2013). Okadaic acid (OA), dinophysistoxins (DTXs) and/or pectenotoxins (PTXs) are groups of lipophilic compounds, which have been detected and confirmed in many geographical strains of *Dinophysis* (Hackett et al. 2009; Kamiyama & Suzuki 2009; Fux et al. 2011; Nagai et al. 2011; Nielsen et al. 2012; Reguera et al. 2012). Typically, these major toxin compounds of *Dinophysis* species are extracted, qualified and quantified together.

Numerous field studies have demonstrated the widespread distribution of *Dinophysis* spp. However, *Dinophysis* blooms are not necessarily accompanied by DSP events. The reported toxin content of *Dinophysis* species varies temporally, geographically (MacKenzie et al. 2005; Pizarro et al. 2009; Fux et al. 2011) and between and/or within species (Lee et al. 1989; MacKenzie et al. 2005). For instance, investigations of the DSP toxin of field *D. acuminata* cells from Limfjord, Denmark, indicated that the OA content ranged from non-detected to 72 pg cell^{-1} (Jørgensen & Andersen 2007). The OA content of *D. acuminata* and *D.* cf. *ovum*, isolated from Northeast America, and the Gulf of Mexico, reached peaks of 0.9 and 12.6 pg cell^{-1}, respectively, when grown under the same environmental conditions (Tong et al. 2015a). In the past 20 years, lipophilic toxins have been found to be widely distributed in different shellfish species along Chinese coastal waters (Zhou 1999; Li et al. 2012). In May 2011, a seafood-borne intoxication in the East China Sea affected 200 patients and was diagnosed as DSP poisoning (Li et al. 2012). Studies of the ecology and population dynamics of *Dinophysis* in China have just been instigated and will require continuation.

Physiological and toxigenic studies of *Dinophysis* spp. have been systematically conducted since their successful culturing and maintenance in the laboratory (Park et al. 2006). Mixotrophic dinoflagellates of this genus require a unique three-stage food chain for their growth, specifically a cryptophyte (photosynthetic nanoflagellate) – *Mesodinium rubrum* (phototrophic ciliate = *Myrionecta rubra*) – *Dinophysis*. The effects of temperature (Kamiyama et al. 2010; Tong et al. 2010), light intensity (Kim et al. 2008; Tong et al. 2011; Nielsen et al. 2012; Nielsen et al. 2013), prey quantity (Kim et al. 2008) and dissolved inorganic nutrients (Hattenrath & Gobler 2015; Hattenrath et al. 2015; Tong et al. 2015b) on cell growth and toxin production of *Dinophysis* species have been examined. The results indicated that temperature and prey quantity were key factors controlling the cell growth (Kim et al. 2008; Kamiyama et al. 2010), whereas their effects on toxin production varied significantly at different growth phases of the population (Tong et al. 2011).

The unsuccessful culturing of Chinese species of *Dinophysis* made it difficult to characterize the local strains and investigate its physiology and toxigenicity. Thus, in the present study, we attempted to isolate and culture *Dinophysis* spp. from Chinese coastal waters based on the established *Mesodinium rubrum* cultures. Furthermore, the effects of temperature and different prey on the growth and toxin production of a clonal strain of Chinese *Dinophysis* were examined.

Materials and methods

Isolation and maintenance of *Dinophysis* strains

A unialgal culture of *D. acuminata* was established by a single-cell isolation from surface seawater samples collected in Xiaoping Island, the Yellow Sea, China (121.53E 38.83N) in July 2014, when water temperature was 20°C. Samples were sequentially filtered through 45 and 23 µm Nitex sieves. The 45-23 µm size fraction was rinsed gently into a beaker using freshly sterilized seawater and single *D. acuminata* cells were isolated into each well of several 48-well microplates with micropipettes. About 60 *M. rubrum* cells grown on a *Teleaulax amphioxeia* culture were added to each well as prey. Once the abundance of *Dinophysis* exceeded 50 cells per well, the well content was transferred into a 12-well microplate and fed with *M. rubrum* in a predator:prey ratio of about 1:5. Following the three steps of the feeding protocol, *D. acuminata* cultures were successfully established.

Two sets of strains of *M. rubrum* and *Teleaulax amphioxeia* were used and maintained in the laboratory. *M. rubrum* strain JAMR and *T. amphioxeia* strain JATA were isolated from Inokushi Bay (131.89E, 32.79N) in the Oita Prefecture, Japan, in February 2007 as described by Nishitani et al., (2008a); *M. rubrum* strain AND-A0711 and *T. amphioxeia* strain AND-A0710 were isolated from Huelva, Southern Spain in 2007. *Teleaulax amphioxeia* cultures were maintained by inoculating 2 ml of the culture (ca. 150 000 cell ml^{-1}) into 30 ml of f/2-Si medium. *Mesodinium rubrum* cultures were transferred weekly, by mixing 30 ml of stock culture (~ 4 000 cells ml^{-1}) and 1 ml of

T. amphioxeia with 30 ml of f/6-Si medium. *Mesodinium rubrum*, after the complete consumption of the cryptophyte, was used as prey for *Dinophysis*. All the cultures were maintained at 15°C with a 14-h light : 10-h dark cycle.

PCR-amplification and species identification

Subsamples (1.5 ml) of *Dinophysis* cultures were transferred into 2 ml sterile centrifuge tubes and centrifuged for 5 min at 12 000 g. The supernatant was discarded to leave the pellet and ca. 250 µl of supernatant. DNA was then extracted from the pellets using a Yeast DNA Extraction Reagent Kit (Thermo Scientific) following the manufacturer's instructions, and stored at -20°C before PCR amplification. Amplifications were conducted in a T professional PCR cycler with the primers Dinocox1R and Dinocox1F (Lin et al. 2002). The PCR system (20 µl) consisted of primers (0.5 µl of each, 10 µM), up to 20 µl of nuclease-free water, 10 µl of DNA polymerase (2 × Taq Mix, Kangwei) and ~ 200 ng of DNA template. The cycle was programed as follows: denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min, ending with 5 min at 72°C.

Amplified sequences were confirmed using agarose gels (1%) stained with ethidium bromide and a UV transilluminator. After sequencing by Sunny Biotechnology Co. (Shanghai, CHN), sequences were aligned using the CLUSTALW method of the software MEGA6 (Tamura et al. 2013). The phylogenetic relationships among species were determined using the neighbor-joining method.

Morphological characteristics of the *Dinophysis* isolate were described by measurement of length (L) and dorsoventral depth (D) of 25 formalin fixed cells collected during the exponential growth phase (Fig. 1a, see details in Tong et al. 2015a).

Experimental conditions

Growth experiments

We then examined the effect of three temperatures (10, 15 and 20°C) and two strains of *M. rubrum* on the growth and toxin content of the Chinese isolate of *D. acuminata* (DAYS01). To keep the prey biomass/predator ratio consistent, ciliates were measured for size and nutritional quality. Diameters of ciliate cells were measured under a microscope and their volume was calculated (cells were assumed as spheres). Nutrient analysis was conducted on 50 ml of each ciliate culture sample. For particulate carbon and nitrogen analysis, a Flash EA1112 Carbon/Nitrogen Analyzer with a Dynamic Flash Combustion technique was used. Particulate phosphorus of the two ciliate strains was converted to and measured as dissolved orthophosphate (PO_4^{3-}), and analyzed by Lachat QuikChem 8000 at the Woods Hole Oceanographic Institution (Woods Hole, MA). The results showed that the volumes of JAMR (Fig. 1b) and AND-A0711 (Fig. 1c) were about 7939 µm^3 and 2393 µm^3 (mean value, n > 20), respectively, with a ratio of 3:1. The carbon, nitrogen and phosphate ratios of JAMR to AND-A0711 were 2.3, 3.3 and 3.4, respectively. Therefore, the initial cell ratios of *D. acuminata* to *M. rubrum*, JAMR and AND-A0711, were set up as 50:1000 and 50:3000, respectively. After starvation for over 2 weeks, *D. acuminata* and *M. rubrum* cells were inoculated into 48-well microplates, with a total volume of 1 ml of culture medium in each well. Twenty-four parallel samples in each treatment were established in each 48-well microplate. Every 3 days, all contents (cell & medium) in two wells of each treatment (each microplate) were selected and harvested randomly and fixed with 3% (v/v) formalin for cell counting separately. Samples were enumerated using a

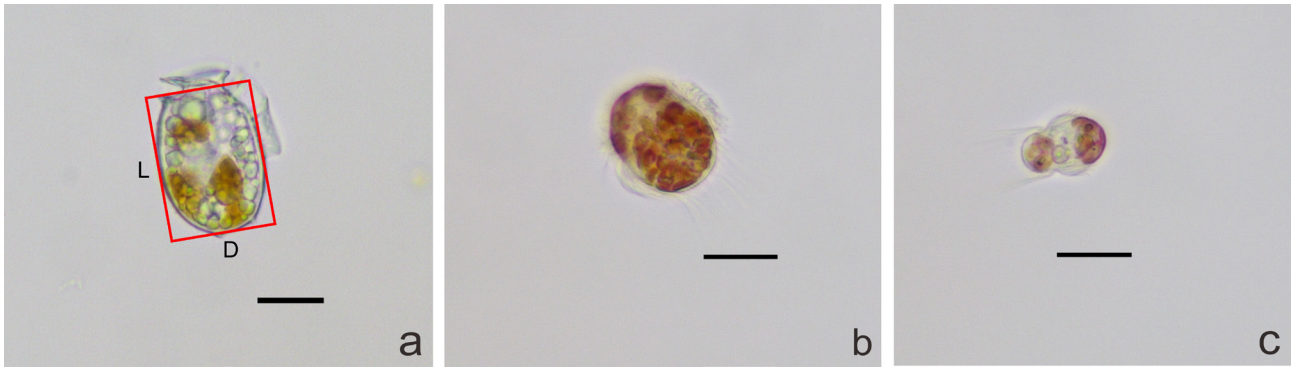

Figure 1

Light micrographs of cultured cells: (a) *Dinophysis acuminata* DAYS01, from Xiaoping Island, the Yellow Sea, China; (b) *Mesodinium rubrum* JAMR, from Japan; (c) *Mesodinium rubrum* AND-A0711, from Southern Spain. Scale bars = 10 µm

Sedgewick-Rafter counting chamber at a magnification of 100 ×. The incubation experiment lasted for 21 days. At the end of the experiment, all cultures remaining in each treatment were mixed in 15-ml centrifuging tubes and kept at -20°C for over 24 h before toxin extraction.

Toxin extraction

Culture samples were processed through the solid phase extraction (SPE) procedure as described by Smith et al. (2012). A SPE column (Oasis HLB 60 mg; Waters, Milford, MA) was previously conditioned with 6 ml methanol and rinsed with 6 ml Milli-Q water. The *Dinophysis* culture samples (cells + medium) were thawed, resuspended and sonicated for 15 min before being loaded onto the cartridge column. The cartridge was then washed with 3 ml Milli-Q water and toxins were ultimately eluted with 1 ml methanol into a HPLC vial. Elutes from the samples were heated at 40°C in a heating block, dried under a stream of N_2 (HP-S016SY), and re-suspended in 1 ml of methanol for toxin analysis.

Toxin analysis

An Ultimate 3000 LC system (ThermoFisher, USA) and AB 4000 triple quadrupole mass spectrometer system (AB SCIEX, USA) coupled with electrospray ionization was used for the toxin analysis. Chromatographic separation was performed using a Waters X-Bridge C18 column (3.0 × 150 mm; 3.5 µm particle size). Toxins OA, DTX1, DTX2, YTX and HOMO-YTX were analyzed in the negative ion mode. In the mobile phase, eluent A was water and eluent B was acetonitrile-water (90:10, v/v), both containing ammonium water (0.05%). PTX2 was analyzed in the positive ion mode. The mobile phase was 100% water for eluent A and acetonitrile-water (95:5, v/v) for eluent B, both containing 2 mM ammonium formate and 0.2% (v/v) formic acid. Toxins were eluted from the column with 90% eluent B at a flow rate of 0.4 ml min^{-1}. The toxin concentration was determined by comparing the peak areas with toxin standards for OA, DTX1, DTX2, YTX, HOMO-YTX and PTX2, which were purchased from the National Research Council, Canada.

Data analysis

The specific growth rate of *D. acuminata* was measured over the exponential phase using the following formula:

$$\mu = \frac{\ln(N_2 / N_1)}{t_2 - t_1}$$

where N_1 and N_2 (cells ml^{-1}) are cell density at time 1 and time 2, respectively, while t_1, t_2 is the sampling time (day) and μ (day^{-1}) is the growth rate calculated at the sampling interval (Guillard 1973).

Effects of temperature and prey on the growth of *Dinophysis* were examined by two-way ANOVA. Alpha was set at 0.05 for all analyses.

Results

Isolation and cultivation

Phytoplankton samples dominated by *Dinophysis* species – *D. acuminata* and *D. caudata* – were collected from coastal waters of Xiaoping Island, the Yellow Sea, and Gouqi Island, the East China Sea. However, only *D. acuminata* from Xiaoping Island was successfully established in cultures. Many attempts to culture *D. caudata* failed. Following the three steps of the feeding protocol, *D. acuminata* (Fig. 1a) was able to feed on the two strains of *M. rubrum*, which were isolated from Japan (JAMR, Fig. 1b) and Southern Spain (AND-A0711, Fig. 1c), and finally maintained in 6-well microplates at 15°C in dim light (~ 100 µmol photons m^{-2} s^{-1}) with a 14-h light : 10-h dark cycle.

Morphological and phylogenetic analyses

Cells isolated from the Yellow Sea (DAYS01) were studied and identified as *D. acuminata* using light microscopy (Fig. 1a) and DNA sequence similarity (Fig. 2). Measurements of *Dinophysis* (strain DAYS01) cells

Table 1

Growth rates and maximum yield in cultures of *Dinophysis acuminata* isolated from the Yellow Sea, China, at three temperatures, when fed on ciliates *Mesodinium rubrum* from Japan (JAMR) and Spain (AND-A0711) (mean ± standard error, n = 2)

Temp. (°C)	JAMR		AND-A0711	
	Growth rate (d^{-1})	Max yield (cells ml^{-1})	Growth rate (d^{-1})	Max yield (cells ml^{-1})
10	0.09 ± 0.03 (Day 1-Day9)*	112 ± 27	0.09 ± 0.01 (Day 1-Day9)	120 ± 3
15	0.21 ± 0.015 (Day 1-Day18)	2483 ± 46	0.10 ± 0.001 (Day 1- Day21)	360 ± 52
20	0.29 ± 0.008 (Day 1-Day18)	7150 ± 270	0.22 ± 0.004 (Day 1-Day15)	1343 ± 123

*Time indicates the exponential growth period of each treatment

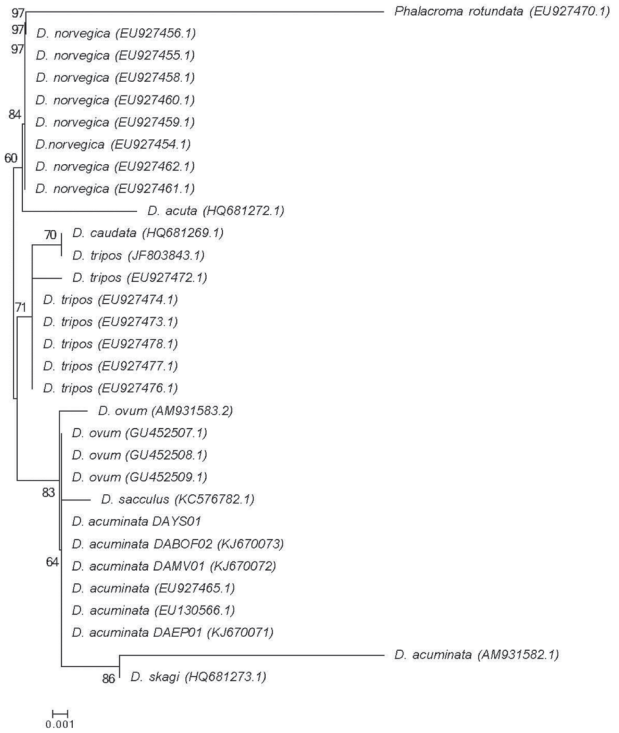

Figure 2

Neighbor-joining phylogenetic tree of dinoflagellates inferred from mitochondrial *cox1*. The corresponding GenBank accession number follows the name of each organism. Numbers at nodes are interior branch test values for 1000 replicates. The scale bar represents the number of substitutions per site.

(n = 25) were 32.5 ± 4.0 µm in length (body length, Fig. 1a) and 24.3 ± 5.1 µm in dorsoventral width (Fig. 1a). Detailed description was presented by Tong et al. (2015a).

DNA sequencing successfully recovered the expected mt *cox1* gene sequence, and was 100% identical to *D. acuminata* DAEP01, DAMV01, and DABOF02 (GenBank Accession No. KJ670071, KJ670072 and KJ670073, respectively) from North America, *D. acuminata* from Passamaquoddy Bay, the Bay of Fundy, Canada (GenBank accession EU927465), and *D. acuminata* from Narragansett Bay, the USA (GenBank accession EU130566), over the aligned region. Our sequence was different at eleven nucleotides compared to the mt *cox1* gene sequence of a Spanish isolate (GenBank accession AM931582). In phylogenetic analyses, our strain was included in a highly supported clade with other *D. acuminata* isolates (Fig. 2).

Physiology

The growth rate of Chinese *D. acuminata* was higher at higher temperature in both feeding treatments (two-way ANOVA, $p < 0.05$, Table 1, Fig. 3a, b). At low temperature (10°C), *D. acuminata* had a slower growth rate (0.09 d^{-1}) during a 9-day incubation than at higher temperatures (15 and 20°C), and stopped growing thereafter in both feeding treatments. Meanwhile, the prey was still present in the mixed culture during the entire incubation at 10°C (Fig. 3a, b). In contrast, at 15 and 20°C, the prey (JAMR and AND-A0711) was fully consumed by day 16 or 18, resulting in better growth of *D. acuminata*. When fed with larger ciliate cells (JAMR), *D. acuminata* had higher growth rates (0.21 d^{-1} and 0.29 d^{-1}) at higher temperatures (15 and 20°C) compared to those fed on small Spanish ciliates, with growth rates of 0.10 d^{-1} and 0.22 d^{-1} at 15 and 20°C, respectively.

Toxin profile and quota

Dinophysis samples were harvested for toxin analysis using LC-MS/MS. OA, DTX1 and PTX2 were the dominant DSP toxins of this Chinese *Dinophysis* isolate (Fig. 4a, b). Toxin data were plotted as total toxin content (e.g. OA amount per ml culture medium, Fig. 3c, d). Samples for toxin analyses were harvested once during the late plateau phase. The content of the remaining wells was combined to ensure enough biomass for toxin analysis. Therefore, no statistical analysis was conducted on toxin content differences in relation to temperature (Fig. 4a, b) and prey types of cultured DAYS01 (Fig. 3c, d). The total toxin concentration (OA, DTX1 and PTX2, per ml) increased as cell densities increased, which resulted from the faster growth of *Dinophysis* at higher temperatures. In the dense culture system (cell density of 7150 ± 270 cells ml^{-1} at 20°C when fed on Japanese ciliates), total OA, DTX1 and PTX2 toxin concentrations reached 5.66, 0.52 and 192.87 ng ml^{-1}, respectively.

Discussion

Isolation and culture of *Dinophysis*

Many *Dinophysis* species have been successfully maintained in the laboratory, using the specific food chain, *Dinophysis–Mesodinium–Teleaulax*, including *D. acuminata* (Park et al. 2006; Tong et al. 2010), *D. fortii* (Nagai et al. 2008), *D. caudata* (Nishitani et al. 2008a), *D. ovum* (Fux et al. 2011; Tong et al. 2015a), *D. acuta* (Jaén et al. 2009), *D. tripos* (Rodríguez et al. 2012),

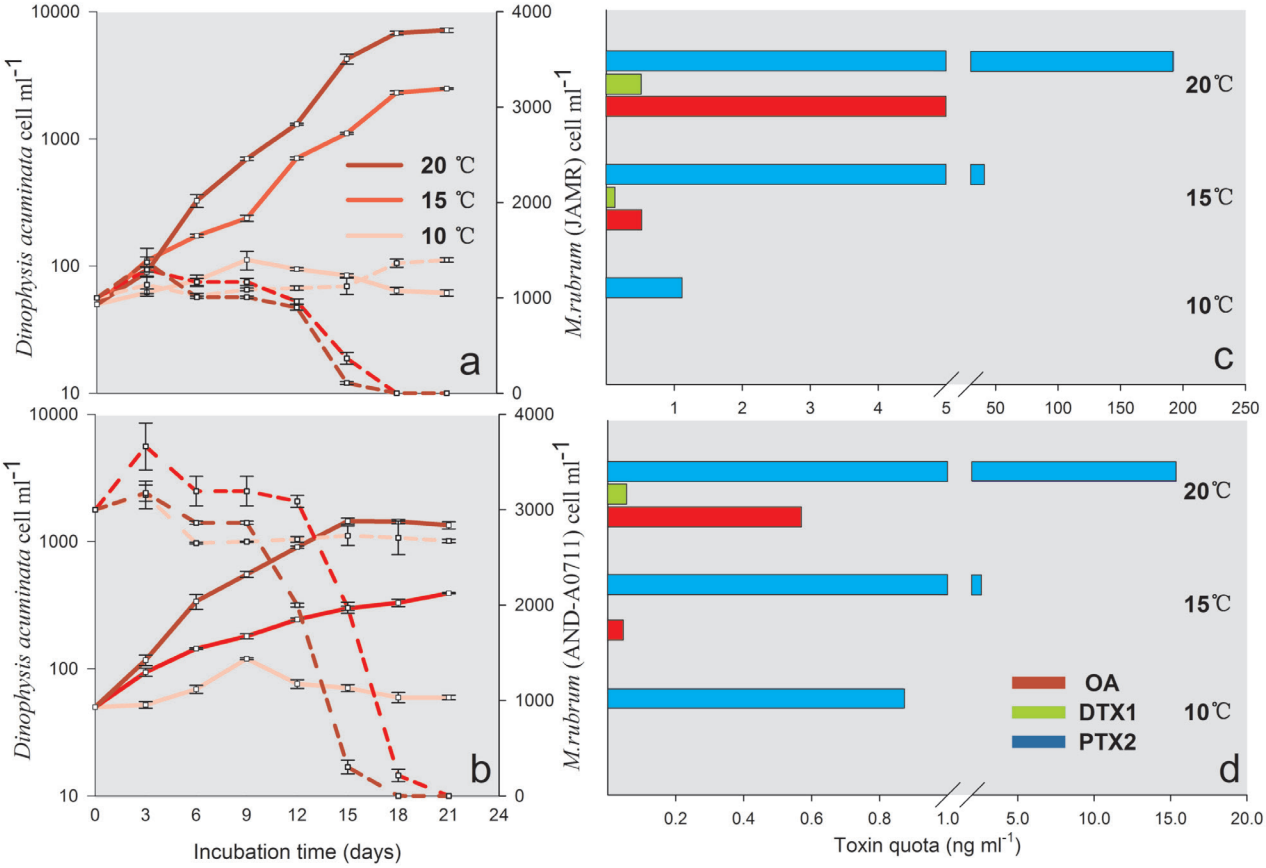

Figure 3

(a, b) Growth responses of *D. acuminata* (solid lines) under multiple temperature (10, 15 and 20°C) and two strains of ciliates (a: JAMR b: AND-A0711, dash lines); (c, d) Toxin profiles of *D. acuminata* (DAYS01) fed with *M. rubrum* from Japan (JAMR) and Spain (AND-A0711) under multiple temperatures (10, 15 and 20°C). Error bars: standard error

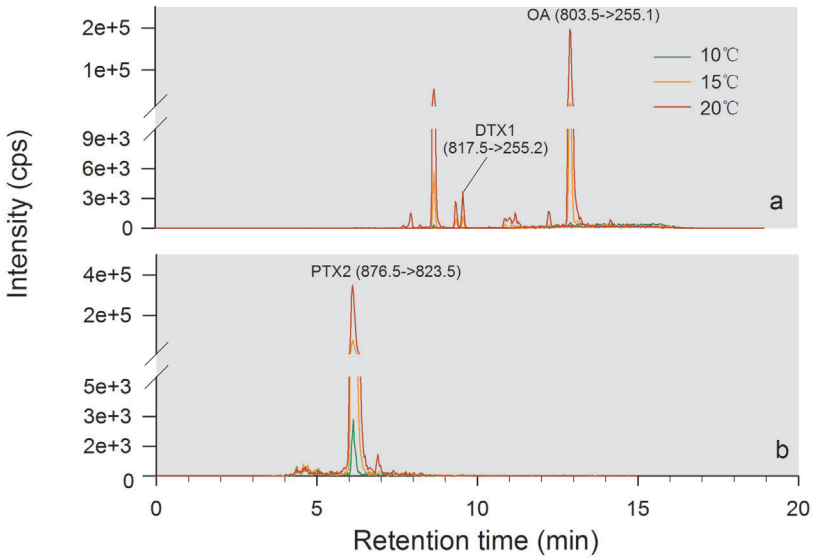

Figure 4

LC-MS/MS chromatograms of OA, DTX1 (a) and PTX2 (b) in *Dinophysis acuminata* (DAYS01) cultures under 10, 15 and 20°C

D. sacculus (Raho et al. 2013) and *D. infundibulus* (Nishitani et al. 2008b). However, although toxic *Dinophysis* species have been reported in China since the late 20th century (Chen 1988; Wang 2007; Li et al. 2012; Jiang et al. 2014; Li et al. 2015), all previous attempts to maintain Chinese isolates of *Dinophysis* in the laboratory were unsuccessful. In this study, we cultured *D. acuminata* from the Yellow Sea (DAYS01) by feeding them with two strains of *M. rubrum*, JAMR and AND-A0711. The feeding activity of DAYS01 was slow during the first day of isolation and increased after several generations (~ 5-day consumption), indicating that DAYS01 specifically selected JAMR or AND-A0711 as its prey. However, the unsuccessful cultivation of *D. caudata* using the same prey was probably due to the poor physiological conditions of *Dinophysis* cells in raw water samples. Nagai et al. (2008) found that their attempts to culture *D. fortii* failed when samples were dominated by small and colorless cells.

Morphology and DNA sequencing of *Dinophysis*

The Chinese isolate DAYS01 was classified as *Dinophysis acuminata* Claparède & Lachmann based on morphological and phylogenetic analysis. Morphologically, DAYS01 cells were identical to *D. acuminata*, with a slightly tapered hypotheca and a left sulcal list supported by three ribs and extending to over halfway down the ventral margin of the hypotheca. When viewed laterally, cells were rounded symmetrically (Tong et al. 2015). In comparison, the DAYS01 isolate (32.5 ± 4.0 µm in length and 24.3 ± 5.1 µm in width) was smaller than the three *D. acuminata* isolates from the northwestern Atlantic (DAEP01, DAMV01 and DABOF02), which were documented as 42.0 ± 2.4, 44.9 ± 2.5 and 44.3 ± 2.3 µm in length, and 27.2 ± 2.3, 30.5 ± 2.0 and 29.2 ± 1.9 µm in dorsoventral width, respectively (Tong et al. 2015a). Molecular analyses based on the *cox1* sequence aligned the Chinese isolate with *D. acuminata* strains from the northwestern Atlantic (GenBank Accession No. KJ670071, KJ670072 and KJ670073), Passamaquoddy Bay, the Bay of Fundy, Canada (Genbank accession EU927465) and Narragansett Bay, the USA (Genbank accession EU130566). Mitochondrial *cox1* allows differentiating *D. acuminata* from *D. ovum* and *D. sacculus* within the "*D. acuminata* complex" (Raho et al. 2008; Raho et al. 2013), but may not be powerful enough to separate all species in this genus. The ITS1-5.8S-ITS2 region proved to be a valuable marker to distinguish *D. acuminata*, *D. acuta*, *D. norvegica* and *D. rotundata* (Edvardsen et al. 2003). It was also a more effective marker than rDNA SSU, LSU, mt *cob*, mt *cox1* and plastid rDNA SSU to discriminate *D. miles* from other *Dinophysis* species (Qiu et al. 2011). Amplified sequences of the LSU D2 and ITS rDNA region of a single *Dinophysis* cell from Qingdao, China, also showed the resolving power to identify *D. acuminata* and *D. rotundata* (Luo 2011). However, the ITS region poorly discriminate between *D. acuminata* and *D. sacculus* due to their identical (Edvardsen et al. 2003) or slightly different ITS sequences (Marín et al. 2001; Guillou et al. 2002). As for species within the "*D. acuminata* complex", mitochondrial *cox*1 has been known so far as a variable marker to discriminate between these morphologically related species (Papaefthimiou et al. 2010; Raho et al. 2013).

Physiology of *D. acuminata*

Temperature response

In the present study, the isolate of *D. acuminata* from the Yellow Sea was characterized by relatively low growth rates when prey was not limited, ranging from 0.09 to 0.29 d^{-1} during the exponential phase. Other cultured *D. acuminata* grew either at a similar level, i.e. the *D. acuminata* isolate from Inokushi Bay, Japan, with growth rates of 0.14 d^{-1} at 10°C and 0.28 d^{-1} at 22°C under a 12-h light : 12-h dark cycle (Kamiyama et al. 2010), or higher, i.e. *D. acuminata* isolates from North America, the USA, with growth rates of 0.23 d^{-1} at 10°C (Tong et al. 2010), 0.37 d^{-1} at 15°C under a 14-h light:10-h dark cycle (Tong et al. 2015a) and from Masan Bay, Korea, with growth rates of 0.91 d^{-1} at 20°C under continuous light (Park et al. 2006). Interestingly, a significantly low growth (0.09 d^{-1}) was observed at 10°C in the present study, when there was still sufficient prey in the mixed culture, indicating that this geographical isolate of *D. acuminata* was able to survive, but not feed and/or divide actively at low temperature. The insufficient growth and predation of *Dinophysis* at 10°C might be a survival strategy of cells exposed to lower temperature, which may be the same response of *Dinophysis* cells to other environmental pressures in natural seawater. As a cosmopolitan species, different strains of *D. acuminata* were revealed to adapt to a wide range of conditions and were capable of growing under temperatures as low as 6°C in North America (Tong et al. 2010) or 8°C in the northeast of Japan (Maestrini 1998). A strain of *D. acuminata* from Inokushi Bay, Japan (32.80°N, 131.90°E) was reported to grow exponentially at a wide range of temperatures (from 10 to 22°C), even though cultures were originally maintained at 18°C (Kamiyama et al. 2010).

Temperature of all the environmental factors may not be identified as the "weighted" factor for

certain physiological and toxigenic characteristics of *Dinophysis* (Alves-de-Souza et al. 2013). However, field studies revealed the succession of *Dinophysis* on a seasonal basis (Alves-de-Souza et al. 2013; Jiang et al. 2014; Fabro et al. 2016). Meanwhile, the positive effect of temperature on the phytoplankton growth has been confirmed by numerous studies. A warmer habitat leads to many ecological variations in an organism, such as frequency of division, active motility and metabolic activities (Wotton 1995). Therefore, it is a rational assumption that the higher temperature in the present experiment (20°C) stimulated the growth of *D. acuminata* by enhancing the encounter rates of the predator and prey, which in turn, activated the phagotrophy of *Dinophysis*.

Prey quantity or biovolume

The type of prey had a significant effect on the population growth and cellular biomass of *D. acuminata*. Both the cell size and biovolume of the two ciliate strains were different. Strains from Japan (JAMR) were three times larger than AND-A0711. Although AND-A0711 was used as prey in three times larger amounts than JAMR, the growth rate and biomass of *D. acuminata* were still significantly higher when fed on JAMR through the entire growth period. It is possible that factors other than biomass, such as pigment or the type of chloroplast, may affect the growth. Park et al. (2010) investigated the fate of "kleptoplasts" in one isolate of *D. caudata* and found that CR-MAL01-type plastids stayed longer than CR-MAL11-type plastids in *D.caudata* cells with the increased starvation time. This indicated that *Dinophysis* treats plastids taken up from different cryptophytes via its ciliate prey *M. rubrum* in different ways. Unfortunately, we could not investigate the effect of the plastid type on the growth of *Dinophysis*, because the plastids of the *T. amphioxeia* strain JATA and the strain AND-A0710 were identical according to their 16S rDNA and *psbA* sequencing. Furthermore, genetic differences in *D. acuminata* may have led to differentiating physiological characteristics within this species. *D. acuminata* from North America (DAMV01) had a growth rate of 0.37 d^{-1} at 15°C when fed on the same prey (Tong et al. 2015b). *Dinophysis* (*D. caudata*, *D. acuta* and *D. tripos*) from Northwest Spain had growth rates of 0.27-0.40 d^{-1} under high light illumination (Rial et al. 2013).

Toxin profile of *Dinophysis*

Environmental factors, such as temperature, light intensity, dissolved inorganic nitrate and phosphate, do not directly affect the toxin profile and the content of *Dinophysis* in batch cultures (Kamiyama & Suzuki 2009; Tong et al. 2011; Nielsen et al. 2013; Hattenrath & Gobler. 2015; Hattenrath et al. 2015; Tong et al. 2015b). Tong et al. (2011) investigated the toxin production of *D. acuminata* under two temperatures (4 and 6°C) and three light intensities (65, 145 and 284 µmol photons m^{-2} s^{-1}), and showed that the toxin content of *Dinophysis* was not significantly altered by changes in these environmental conditions. Kamiyama et al. (2010) found that the cellular PTX2 content was greater at lower temperatures, but no clear differences in OA and DTX1 were observed in relation to temperature. Although relatively higher amounts of OA per ml of culture were observed at higher temperature in both feeding regimes in the present study, the differences were not statistically analyzed. The cellular toxin content was unaffected by irradiance (Tong et al. 2011; Nielsen et al. 2012; Nielsen et al. 2013), but light was required for the growth and toxin production of *D. acuminata* (Tong et al. 2011). Dissolved nitrate and phosphate do not have a direct effect on the toxin production of *D. acuminata*, but these nutrient pools may contribute to prey growth and biomass, thereby indirectly promoting overall toxin concentrations in the *D. acuminata* culture system (Hattenrath & Gobler 2015; Hattenrath et al. 2015; Tong et al. 2015b). Additionally, the cellular toxin content of *Dinophysis* isolates varies greatly due to the variability associated with different growth stages (Tong et al. 2011; Tong et al. 2015a).

Prey availability affected the total amounts of OA, DTX1 and PTX2. In the presence of prey, the number of *D. acuminata* cells increased, resulting in elevated total toxin concentrations (Kamiyama et al. 2010; Nagai et al. 2011; Tong et al. 2011; Tong et al. 2015a). Active toxin production by *Dinophysis* required the presence of ciliate prey (Tong et al. 2015b). In the absence of prey, there were no changes in cellular, dissolved or total OA, DTX1 or PTX2 over one month of incubation (Smith et al. 2012; Tong et al. 2015b).

Prey isolates or prey nutritional quality might be a driver of toxin content in *Dinophysis*. Two nutritionally different ciliates were supplied as prey for *D. acuminata* DAYS01. Although more Spanish ciliates were added to maintain an equivalent biomass/biovolume, the toxin content, especially PTX2, in the two feeding chain systems varied greatly, suggesting that inherent differences in the prey influenced the PTX2 toxin quota of *Dinophysis*. No obvious differences in PTX2 content were observed at 10°C, possibly due to the non-growth of *Dinophysis* at such a low temperature. Therefore, *Dinophysis* cultures at 10°C may have maintained their initial toxin concentration. However,

toxin analysis was not performed for the *Dinophysis* inoculum, which prevented us from testing this assumption.

Intrinsic differences in *Dinophysis* strains may lead to variations in the toxin profile and production. In a batch culture study, OA, DTX1 and PTXs toxin content of northwestern Atlantic *D. acuminata* isolates (DAEP01, DAMV01 and DABOF02) from the northwestern Atlantic was typically 0.01-1.80 pg cell^{-1} of OA or DTX1 in batch culture (Tong et al. 2015a), a value at the lower end of the *D. acuminata* isolates from Japan (0.2-12.2 pg cell^{-1}, Kamiyama & Suzuki 2009; Kamiyama et al. 2010; Nagai et al. 2011) and Brazil (3.2-18.0 pg cell^{-1}, Mafra et al. 2014). Compared to those studies, the extracted toxins were not from isolated cells, but from the culture (including cell and medium) in the present study. Therefore, the OA or DTX1 content of our Chinese *D. acuminata*, nd-0.54 pg cell^{-1}, were overestimated. However, the content of OA or DTX1 was still low. The overestimated PTX2 content of the isolates (9.63-18.49 pg cell^{-1}) in the present study was within the range of quotas reported for many other regions (Nielsen et al. 2012). The relatively low OA and DTX1 toxin content of the Yellow Sea isolates is consistent with scarce harvesting closures in that region due to OA-group toxins, but the potential risk of moderate or high PTX toxin exposure cannot be ignored.

Individual *Dinophysis* strains appear to be able to produce only one type of toxin profile (Reguera et al. 2012). *Dinophysis* cf *acuminata* cells from northern and southern Chile (Blanco et al. 2007; Fux et al. 2011) produced only PTX2; *D.* cf *ovum* cells from Texas contained only OA (Deeds et al. 2010; Fux et al. 2011; Tong et al. 2015a). The toxin PTX2 was the dominant toxic component in *D. acuminata*, *D. norvegica* and *D. infundibulus* from Hokkaido, Japan (Suzuki et al. 2009), and *D. fortii* was reported to produce DTX1 and PTX2 (Kamiyama & Suzuki 2009; Suzuki et al. 2009). The toxin profiles and content of *D. caudata* varied seasonally and geographically. The cellular toxin content was extracted from the isolated cell, and low concentrations of OA were detected in field populations of *D. caudata* from Singapore (0.07-0.14 pg cell^{-1}) (Holmes et al. 1999; Holmes & Teo 2002). Moderate to high values of OA (7.9-56.5 pg cell^{-1}) and DTX1 (7.2-53.9 pg cell^{-1}) were reported in the Philippines, where *D. caudata* populations were seasonally dominant in phytoplankton communities at intervals higher than 1000 cell l^{-1} (Marasigan et al. 2001). *Dinophysis caudata* isolates from Northwest Spain were reported to contain PTX2, ranging from 5 to 130 pg cell^{-1} (Fernandez et al. 2006; Pizarro et al. 2013), and trace amounts of OA and/or DTX2 (Pizarro et al. 2013). A field investigation of *D. caudata* in China (Gouqi Island, the East China Sea) found low PTX2 (0.58 pg cell^{-1}) and trace amounts of OA and DTX1 using LC-MS/MS (Li et al. 2015), suggesting an urgent need to investigate the toxin-producing capacity of local species and to assess the potential risk to local aquaculture industries.

Conclusions

The successful cultivation of the Chinese strain of *Dinophysis acuminata* (DAYS01) provided an opportunity to investigate the physiological and toxigenic characteristics of the local *Dinophysis* isolate. The effects of temperature and the type of prey on the growth and toxin production of local *Dinophysis* species were examined. The results showed that *Dinophysis* culture densities increased at temperatures of 10, 15 and 20°C, and the highest growth rates occurred at the highest temperature. OA, DTX1 and PTX2 were the major toxin components of DAYS01, with the highest recorded toxin content of 0.54, 0.05 and 18.49 pg cell^{-1}, respectively. Toxin concentrations (toxin amount per ml of culture) increased with increasing cell densities. The type of prey significantly influenced the cell growth and toxin content, suggesting that the origin or strain of the ciliate prey directly impacted the *D. acuminata* blooms and the overall toxin concentration in the field.

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