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Interactions between *Karlodinium veneficum* and *Prorocentrum donghaiense* from the East China Sea

Chengxu Zhou^a, Allen R. Place^b, Xiaojun Yan^{a,*}, Jilin Xu^a, Qijun Luo^a, Ernest William^b, and Ying Jiang^a

^aKey Laboratory of Applied Marine Biotechnology (Ningbo University), Ministry of Education, Ningbo, China

^bInstitute of Marine and Environmental Technology, University of Maryland Center for Environmental Science, Baltimore, MD, USA

Abstract

The dinoflagellate *Prorocentrum donghaiense* is a dominant harmful algal bloom (HAB) species on the East China Sea (ECS) coast. The co-occurrence of *Karlodinium veneficum* with *P. donghaiense* is often observed and can later develop into dense blooms. However, the role of *K. veneficum* in *P. donghaiense* population dynamics is unknown. In the current study, three *K. veneficum* (GM1, GM2, and GM3) strains were isolated from the ECS with one (GM1) from a mixed, dense bloom of *P. donghaiense* and other HAB species. All three isolates had identical ITS sequences that were concordant with the species designation. Unique karlotoxin congeners were isolated from one strain (GM2). The sterol compositions of *P. donghaiense* and *K. veneficum* were consistent with sensitivity to karlotoxin in the former and insensitivity in the latter. Additional experimentation showed that: (1) in monocultures, higher growth rate of *P. donghaiense* than *K. veneficum* is observed in nutrient-enriched and nutrient-depleted media. In co-cultures, the growth of *P. donghaiense* is inhibited; (2) feeding on *P. donghaiense* by *K. veneficum* is clearly demonstrated by fluorescent dye tracking; and (3) the isolated karlotoxin is lethal to *P. donghaiense* in a concentration-dependent manner. From these studies we propose that *K. veneficum* may play a negative role in *P. donghaiense* bloom maintenance and that *P. donghaiense* may in turn be a bloom initiator as a prey item for *K. veneficum*.

Keywords

Karlodinium veneficum, *Prorocentrum donghaiense*, Co-culture; Phagotrophy; Karlotoxin; Sterol

1. Introduction

Since the first description of *Karlodinium veneficum* from the East China Sea (ECS; Zhou et al., 2008a), both field (Xu et al., 2012; Dai et al., 2014) and laboratory studies (Wang et al., 2011; Zhou et al., 2011) of this species in China have increased. However, whether this species is endemic or was imported by ballast water is unknown. It is noted that *K.*

*Corresponding author. Tel.: +86 13968328344., yanxiaojun@nbu.edu.cn (X. Yan).

veneficum is consistently detected in the phytoplankton community assemblages and in adjacent aquaculture ponds. In fact, some of the ECS *K. veneficum* isolates are genetically and phenotypically identical to strains of American origin as revealed by ITS sequencing and toxin profiling (unpublished data).

The ECS is well known for its frequent harmful algal blooms (HAB; Zhou et al., 2008b). The particular properties of the ECS contribute oceanographic and hydrographical characteristics that promote bloom forming in regional HAB species (Zhou, 2010). The dominant harmful bloom species in the coastal area of the ECS is *Prorocentrum donghaiense* (Lu and Goebel, 2001; Lu et al., 2002; Zhou et al., 2006; Li et al., 2010). Even though no toxin has been detected in *P. donghaiense*, the dense blooms can span thousands of kilometers and last as long as a month. The blooms can cause large economic losses to aquaculture and have adverse ecological impacts in the ECS (Zhou et al., 2006; Li et al., 2010).

The dinoflagellate *Karlodinium veneficum* has a global distribution with many well-documented harmful effects (Place et al., 2012). In addition to developing into dense single-species blooms (Braarud, 1957; Hall et al., 2008; Glibert and Terlizzi, 1999), it accompanies or co-occurs in the phytoplankton community with other HAB species (Marshall, 1980; Larsen and Moestrup, 1989), as well as showing mixotrophic and phagotrophic abilities (Li et al., 2000). It exhibits species specific toxins, karlotoxins (Van Wagoner et al., 2008; Kempton et al., 2002; Deeds et al., 2002), which play a role in feeding on the prey and deterring the predators (Adolf et al., 2006, 2007; Sheng et al., 2010). Sterol composition of the ambient organisms is critical to whether they will be karlotoxin sensitive (Deeds and Place, 2006).

In the ECS phytoplankton communities, *Karlodinium veneficum* is often detected with *Prorocentrum donghaiense* and its blooms. It has been noticed that *K. veneficum* can even develop into a dense bloom after the *P. donghaiense* bloom declines (Dai et al., 2014). The aim of this study was to assess whether *P. donghaiense* blooms are terminated or impacted by *K. veneficum*. The study focused on the role of *K. veneficum*, as a co-occurring species, in *P. donghaiense* population growth: whether either species influenced population growth in the other; whether *K. veneficum* feeds on *P. donghaiense*; whether the ECS strains produce karlotoxin; and if the toxin affects *P. donghaiense*.

2. Materials and methods

2.1 Algal cultures

The dinoflagellate *Prorocentrum donghaiense* (strain NMBjah045) was isolated from a dense bloom collected in Dongtou, ECS, in June 2005, in which *Karenia mikimotoi* dominated while both *P. donghaiense* and *Karlodinium veneficum* co-occurred (Zhou et al., 2011).

Three *Karlodinium veneficum* strains were isolated from different locations in the ECS at three different times. Strain NMBjah047 (GM1, GenBank code DQ459434, ITS sequence) was isolated from the above-mentioned bloom water in 2005. Strain NMB jah047-1 (GM2)

was isolated from Yushan, Zhejiang province, in 2006. Strain NMB jah047-2 (GM3) was isolated most recently from an aquaculture pond in Zhaoan, Fujian province, in 2011. The ITS sequences of all three strains were identical (unpublished data). All of the monoalgal cultures were maintained in sterilized natural seawater enriched with f/2-Si at the Microalgae Collection in Ningbo University, China.

2.2 Co-culture of *K. veneficum* and *P. donghaiense*

Based on field and mesocosm observations, dinoflagellates frequently bloom after diatoms when the water is stratified (Smayda and Trainer, 2010). The two dinoflagellate species, *Karlodinium veneficum* and *Prorocentrum donghaiense*, bloom in the ECS in similar nutrient poor stratified waters (Dai et al., 2014). To mimic the oligotrophic conditions after a diatom bloom media, filtrates of a *Thalassiosira rotula* culture at the early stationary phase were used as culture media. The diatom *T. rotula* was isolated and purified from Nanji Islands, Zhejiang province, in 2006, and maintained in the Microalgae Collection in Ningbo University. The concentrations of nitrogen and phosphorus in the filtrates were 10.3 ± 2.7 $\mu\text{mol/l}$ nitrate and 3.9 ± 0.9 $\mu\text{mol/l}$ phosphate. Before inoculation, both media were filter sterilized. For nutrient-replete media, filtered natural seawater was enriched to a final nutrient solution that was two-fold that in the f/2-Si formula. The experimental cultures were inoculated by adding 100 ml of the cultures to 100 ml of the media in a 250-ml glass flask. A monoculture of the same initial cell density in each media was set as the control. The initial *K. veneficum* cell density was 0.5×10^4 cells/ml. For *P. donghaiense*, the initial cell density was 5×10^4 cells/ml and 0.5×10^4 cells/ml. The cultures were maintained in an air-conditioned culture room at 20 ± 2 °C, illuminated by cool white fluorescent bulbs $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ illumination on a 12:12 dark:light cycle. Natural seawater from the coastal ECS 25 PSU (practical salinity units) was used. The cell number was determined using a hemocytometer (Sigma-Aldrich, USA) at the same time each day. The cells of these two dinoflagellates in co-culture can be easily distinguished by their shapes.

2.3. Feeding on *P. donghaiense* cells by *K. veneficum* ECS strain

To determine if *Karlodinium veneficum* ECS strain (GM3) feeds on *Prorocentrum donghaiense* in culture, a stationary-phase *P. donghaiense* culture was stained with fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich, USA) according to the methods of Rioboo et al. (2009) and Buhmann et al. (2013) with modifications. A CFSE stock solution was made by dissolving CFSE powder in DMSO (Sigma-Aldrich) to a final concentration of 5 mM. For the feeding experiments, 994 μl of a stationary-phase *P. donghaiense* culture, cell density 6.8×10^5 cells/ml, was incubated with 6 μl CFSE (5 mM) at a final concentration of 20 μM per 6.8×10^5 cells suspended in f/2-Si culture medium for 2 h at 20 °C in the dark. The CFSE-labeled cells were washed with f/2-Si medium three times by centrifugation at 3500 rpm for 15 min and resuspended in f/2-Si culture medium to 2 ml. The CFSE-labeled culture was put in a culture room for 1 day for the cells to recover from the treatments. CFSE-labeled *P. donghaiense* (200 μl) was added to a 48-well plate containing 200 μl stationary-phase *K. veneficum* (GM3) at a cell density of 5.9×10^4 cells/ml. The cell ratio of *K. veneficum* to *P. donghaiense* was ca. 1:5. The treatment was carried out eight times. The plate was returned to the culture room set at 20 °C with illumination of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ on a 12:12 (D:L) cycle.

After 1, 2, 3, and 24 h, the mixed culture from two wells of the 48-well plate were pipetted to two wells of another 48-well plate to be fixed with formaldehyde at a final concentration of 1.5% (v/v). The plate with the fixed cells was stored at 4 °C in darkness. After all of the sampling was done, the fixed samples were observed under an inverted microscope (Nikon Eclipse Ti-U equipped with Nikon Digital Sight DS-Ri1, Japan).

2.4. Karlotoxin isolation

The dinoflagellate *Karlodinium veneficum* (strain GM2) was grown in ESAW medium (Harrison et al., 1980) in 250-ml polycarbonate flasks in a culture room with natural light illumination (no direct sunlight) at 18–20 °C. When the culture had reached the stationary phase at a cell density of 1.63×10^5 cells/ml, 220 ml of the culture was vacuum filtered through a GF/F filter membrane (Whatman, USA). Karlotoxin in the filtrate was applied to a 3-ml packed volume tC-18 solid phase extraction cartridge column (Waters, USA) that had been activated by 100% methanol. The column was then eluted with 20 ml deionized water and 20%, 40%, 60%, and 80% methanol sequentially. The 60% and 80% methanol/water elute was collected and dried by rotary evaporation. The dried fraction was resuspended in 1 ml 20% methanol and stored at 4 °C until analyzed by LC-MS (Bachvaroff et al., 2008).

2.5. The effects of karlotoxin on *P. donghaiense*

The purified karlotoxin (GM2) was stored in 20% methanol in an amber glass bottle at 4 °C before use and was determined gravimetrically to be 240 µg/ml. Exponential phase *Prorocentrum donghaiense* (3.5×10^4 cells/ml) cultured in ESAW (25 PSU) was exposed to 0.048 µg/ml, 0.48 µg/ml, and 4.8 µg/ml purified karlotoxin. The total volume was adjusted to 100 µl with 20% methanol when necessary. Two controls were set up in this experiment. One control was set by adding 100 µl 20% methanol to a *P. donghaiense* culture (Control M). The other control was the original *P. donghaiense* culture (Control) with no added karlotoxin or methanol.

All of the treatments were duplicated over a 24-h period at 20 °C, 50 µmol photon/m² s (L:D = 12:12). After 24 h exposure, cell number and cell size were analyzed in a Coulter™ Multisizer (Beckman, USA). Active chlorophyll a fluorescence was detected with a fluorometer (Turner Designs, USA). One hundred microliters of the cultures were sampled onto a 96-well culture plate, fixed by adding 50 µl 2% glutaraldehyde solution, and stored at 4 °C overnight to let the cells sink to the bottom. The fixed samples were used to analyze the proportion of empty cells by counting 100 cells under a light microscope.

2.6. Sterol analysis on *K. veneficum* and *P. donghaiense*

Dinoflagellates *Karlodinium veneficum* (GM1) and *Prorocentrum donghaiense* were cultured in f/2-Si media, respectively. The cultures were gradually scaled up to a final volume of 4000 ml in 5000 ml glass flasks. All of the cultures were maintained in a culture room at 20 ± 2 °C, 40 µmol m⁻² s⁻¹ illumination on a 12:12 light:dark cycle. The cultures were shaken twice daily. When the cultures had reached the stationary phase with a sufficient number of cells for the sterol analysis, the microalgae were harvested by centrifugation at 4000 rpm, freeze dried, and stored at –20 °C until analysis.

Total lipids were extracted using a modified Bligh-Dyer method (Bligh and Dyer, 1959). Sterol extraction and analysis were performed according to Xu et al. (2008). The lipids containing the sterol TMS ethers were dissolved in hexane and analyzed in a QP2010 GC-MS with an AOC-20 auto-sampler (Shimadzu Co., Japan) fitted with a 30 m $\times$ 0.25 mm $\times$ 0.25 mm SPB-50 column (Supelco Co., USA). The sterol structures were mainly identified by comparing the mass spectra according to the regular mass spectra patterns of sterol TMS derivatives (Miao et al., 2009) and relative retention times, NIST and WILEY library entries, and previously published GC/MS data on sterols (Barrett et al., 1995; Leblond and Chapman, 2002; Volkman et al., 1999; Rampen et al., 2009). Peak areas were used to determine the relative sterol proportions.

2.7. Data analysis

Culture growth rates (rt) were determined by the following equation: $rt = (\ln N_t - \ln N_0)/t$, where N_0 is the initial cell density (cells/ml), and N_t is the cell density at time t . Statistical analyses were conducted in SPSS (v19.0). Significant differences were determined by one-way ANOVA test.

3. Results

3.1. Population growth of *K. veneficum* and *P. donghaiense* in mono-and co-cultures

In enriched natural seawater (Fig. 1A and B), *Karlodinium veneficum* (GM1) monocultures grew to more than 10 times the initial cell density within 12 days. Growth rate in the first 4 days was 0.58 d⁻¹. In the *Prorocentrum donghaiense* monocultures, the growth rate varied depending on the initial cell density. At higher initial cell densities (5×10^4 cells/ml), the growth rate was 0.30 d⁻¹ for the first 4 days, while at the lower initial cell density (0.5×10^4 cells/ml) the growth rate was 0.73 d⁻¹. When the two species were co-cultured, growth of *P. donghaiense* decreased significantly in the presence of *K. veneficum*. After a short period of initial growth for 3 days, *P. donghaiense* biomass declined in both co-cultures at the two initial densities. In contrast, *K. veneficum* population growth in co-culture was not affected. In the K/P = 1/10 co-culture (Fig. 1A), the growth rate in the first 4 days was slightly lower than that in the monoculture control (0.41 d⁻¹ compared with 0.58 d⁻¹). In the K/P = 1/1 co-culture (Fig. 1B), growth was also slightly inhibited in the first 3 days; however, it increased to the same level as that in the monoculture after the 4th day (0.5–0.6 d⁻¹). The growth rate in the monoculture declined from the 8th day, while that in the co-culture was >0.3 d⁻¹ until the end of the experiment. The final maximum *K. veneficum* biomass in the co-culture was significantly higher than that in the monoculture ($P < 0.05$).

It is showed that *Karlodinium veneficum* did not grow in the nutrient-depleted media from the diatom filtrates (Fig. 1C and D). Cell density remained at the initial inoculum density and then plummeted at day 5. However, in the co-cultures, *K. veneficum* survived and grew (Fig. 1C). In the K/P = 1/1 co-culture (Fig. 1D), the growth rate of *K. veneficum* (0.3 d⁻¹ in 12 days) was significantly higher and reached a higher maximum cell number (1.7×10^5 cells/ml) than that in the K/P = 1/10 group ($P < 0.05$). In the *Prorocentrum donghaiense* monoculture, the scenario was basically the same as that in enriched clean natural seawater, except that the growth period was shorter and the population declined quickly after reaching

the maximum cell number (Fig. 1C and D). The *P. donghaiense* growth rates in the diatom filtrates were lower than that in the enriched culture medium. Interestingly, in the diatom filtrates, monoculture *P. donghaiense* with higher initial cell numbers (5×10^4 cells/ml) also had a lower growth rate than that of the culture with lower initial cell numbers (0.5×10^4 cells/ml), 0.23 d^{-1} compared with 0.65 d^{-1} , respectively. The growth of *P. donghaiense* in the co-cultures was also obviously affected by *K. veneficum*. In the K/P = 1/1 co-culture, *P. donghaiense* declined sharply while *K. veneficum* grew quickly from day 4 to 5 (Fig. 1D). In the K/P = 1/10 co-culture (Fig. 1C), where there were relatively lower numbers of *K. veneficum*, the *P. donghaiense* population growth decline phase was delayed compared with that in K/P = 1/1.

3.2. Feeding on *P. donghaiense* by *K. veneficum*

For the first time, the feeding activity of *Karlodinium veneficum* (GM3) in the presence of CFSE-labeled *Prorocentrum donghaiense* cells was clearly observed (Fig. 2). Frequently, *K. veneficum* cell(s) actively attached to *P. donghaiense*, as shown in Fig. 2A. After 1.5 h, *K. veneficum* cells with small CFSE fluorescence particle(s) were observed. At 2 h, obvious feeding activity was observed. The interesting feeding behavior is described as follows: *K. veneficum* appeared to drag *P. donghaiense* around rapidly. However, *K. veneficum* did not completely engulf *P. donghaiense* cells but swam away after feeding for some period, occasionally leaving behind an empty thecal shell of the prey. In the fixed samples, *K. veneficum* cells with different quantities of CFSE-labeled material were found at different time points (Fig. 2E and F). Not all *K. veneficum* cells fed because some were observed with no CFSE fluorescence in the fixed samples (Fig. 2C and D).

3.3. The effect of karlotoxin on *P. donghaiense*

The karlotoxin isolated from *Karlodinium veneficum* (GM2) is a KmTx-2-like molecule. As shown in Fig. 3, both sulfated and unsulfated congeners were present. The UV spectra of the congeners are consistent with the terminal chlorine found in KmTx 2. However, the mass spectra (Fig. 4) indicated that these are unique congeners not previously reported. The toxin cell quota was estimated at 6.6 pg/cell and 2.5 pg/cell for the nonsulfate and sulfate congeners, respectively.

When *Prorocentrum donghaiense* was exposed to the nonsulfate congeners at a concentration of 0.048–4.8 $\mu\text{g/ml}$, significant effects were recorded. After exposure to 4.8 $\mu\text{g/ml}$ for 24 h, the cell number decreased to 58% that of the controls (Fig. 5A), the cell size significantly decreased compared with the other groups (Fig. 5B), the chlorophyll *a* fluorescence declined to 39% that of the control (Fig. 5C), and 75% of the cells were empty thecal shells (Fig. 5D). No negative effects on *P. donghaiense* cells exposed to methanol were observed in this experiment. The chlorophyll fluorescence was sensitive enough to reflect the effect of karlotoxin according to the Turner Designs reads, and it declined to 85% of the control when the cells were exposed to 0.48 $\mu\text{g/ml}$ of the toxin (Fig. 5C), while there was no significant variation from other parameters, such as cell number or cell size. Empty *P. donghaiense* thecae were obvious when the fixed cells were observed under the microscope. The epitheca and hypotheca were either fully attached or slightly separated with

no cellular contents. Empty cells were proportional to the amount of karlotoxin in the culture.

3.4. Sterol profiles of *K. veneficum* and *P. donghaiense*

The sterol analysis results for the two species are shown in Table 1. The dinoflagellate *Karlodinium veneficum* contained (24S)-4 α -methyl-5 α -ergosta-8(14), 22 diene-3 β -ol (gymnodinosterol), 27-nor(24S)-4 α -methyl-5 α -ergosta-8(14), and 22 diene-3 β -ol (brevesterol) as the major sterols, representing more than 70% of the total sterol, together with two unknown C29:2 sterols (12.84% and 3.24%); the minor sterols were 24-ethylcholesta-5-en-3 β -ol (sitosterol), 4 α ,23,24-trimethylcholest-5,22-dien-3 β -ol (dehydrodinosterol) and its isomer (3.73% and 2.82%, respectively), and Choles-5-en-3 β -ol (cholesterol; 3.6%). The results are similar to that described by Place et al. (2004) for other *K. veneficum* strains, in which, gymnodinosterol and brevesterol represented 85% of the sterols. These two sterols are considered to be important players for host immunity against karlotoxin because of the 4 α -methyl and 8(14) ethylenic bond in their sterol backbone.

The dinoflagellate *Prorocentrum donghaiense* contained 4 α ,23,24-trimethyl-5 α -cholest-22-en-3 β -ol (dinosterol) and 24-methyl-5 α -cholest-24(28)-en-3 β -ol as the major sterols, 45.5% and 24.5%, respectively. The minor sterols were 4 α ,23,24-trimethylcholest-5,22-dien-3 β -ol (dehydrodinosterol isomer; 7.12%), 4 α ,24-dimethylcholest-7-en-3 β -ol (1.35%), 24-methylcholest-5,22-dien-3 β -ol (diatomsterol; 4.37%), C28:2 (8.9%), and Choles-5-en-3 β -ol (cholesterol; 1.3%), as well as other unknown sterols.

4. Discussion

The dinoflagellate *Prorocentrum donghaiense* is the most frequently reported blooming alga in the ECS, with a wide distribution throughout the region. The ability to adapt and grow in a wide range of temperatures from 10 °C to 27 °C and salinities from 20 PSU to 40 PSU enables this species to dominate and bloom in the ECS (Xu et al., 2010). After concentrating in the mid-water column, it can then migrate to the surface to develop into blooms with cell densities as high as 10⁸ cells/ml (data from State Oceanic Administration, China). It is usually found that *Karlodinium veneficum* accompanies other blooms, including *P. donghaiense*. The present study shows that laboratory co-culture of *K. veneficum* and *P. donghaiense* results in population decline of the latter under various nutrient and initial ratio conditions. In contrast, *K. veneficum* exhibited enhanced growth in the co-cultures compared with the monocultures under the same nutrient and ratio conditions.

It has been previously observed that *Prorocentrum donghaiense* thrives in eutrophic waters (Zhou et al., 2003). The growth rate of *P. donghaiense* monocultured in f/2-Si medium with an initial cell density of 5 × 10⁴ cells/ml reached 0.3 d⁻¹, which is in the same range reported by Li et al. (2012). The *P. donghaiense* monoculture grew in the oligotrophic diatom filtrate medium. It has been demonstrated that *P. donghaiense* is a mixotrophic dinoflagellate, which can graze on cyanobacteria and cryptophytes (Jeong et al., 2005a,b). Such abilities allow *P. donghaiense* to outgrow other blooming species when nutrients are limited (Li et al., 2009). The *Karlodinium veneficum* monocultures crashed at low nutrient conditions, but survived and steadily increased in the presence of *P. donghaiense* to a

biomass of 1.6×10^4 cells/ml by the end of the experiment. This observation agrees with that of Dai et al. (2014) on the bloom dynamics. It is found that *K. veneficum* initially at low cell densities in *P. donghaiense* blooms can later develop into denser blooms, becoming the dominant species.

Phagotrophy is a prominent feature of *Karlodinium veneficum* and involves diatoms, cryptophytes, and even zooplankton (Li et al., 2000; Place et al., 2012). In this laboratory study, *K. veneficum* (GM3) isolated from the ECS actively fed on *Prorocentrum donghaiense*. *K. veneficum* (GM3) did not engulf *P. donghaiense* as observed when feeding on either *Rhinomonas reticulata* or *Hemiselmis brunnescens* (Place et al., 2012). Instead, CFSE-labeled *P. donghaiense* cell contents were transferred (*i.e.*, myzocytosis) to the *K. veneficum* food vacuole. *K. veneficum* may sip (*via* its peduncle) the cell contents of the larger *P. donghaiense* rather than engulf the whole cell and leave the empty theca in the medium. Hence, phagotrophic activity in *K. veneficum* may have been responsible for the decline in *P. donghaiense* cell density seen in the co-cultures (Fig. 1). This *P. donghaiense* growth inhibition appears to be specific to *K. veneficum*. *P. donghaiense* is not significantly affected by co-culture with *Karenia brevis* (Li et al., 2012). Further studies on the feeding activity of *K. veneficum* on *P. donghaiense* *in situ* are warranted, especially as it relates to bloom dynamics.

The cells of *Prorocentrum donghaiense* are lysed in a dose-dependent manner upon addition of purified karlotoxin from the *Karlodinium veneficum* ECS strain. The prominent karlotoxin toxicity mechanism is through membrane pore formation (Deeds et al., 2014), which disrupts membrane potential. This membrane disruption is consistent with the chlorophyll *a* fluorescence decrease measured in *P. donghaiense* in the presence of karlotoxin. Cell diameter shrank significantly after a 24-h exposure at the highest karlotoxin concentration. Concomitant with exposure intracellular cell content was released, resulting in empty thecae. The mechanism of karlotoxin pore formation is sterol-dependent (Deeds and Place, 2006). In the current study, the absence of gymnodinosterol and brevestanol is clearly evident in *P. donghaiense*, which may explain the toxicity of karlotoxin to this species. Members of the genus *Prorocentrum* basically have broadly similar sterol compositions (dinosterol being dominant), but none have an 8(14) ethylenic bond in their sterol backbone (Volkman et al., 1999).

In the Li et al. (2012) study, *Karlodinium veneficum* inhibited cocultured *P. minimum* population growth rate to about 40% of the monoculture. Ozbay et al. (2014) found that when *P. minimum* was treated with 0.256 µg/ml karlotoxin (KmTx2) for 24 h, it experienced mortality and cell diameter increases. The *P. minimum* is sensitive to karlotoxin but only at high concentrations (Ozbay et al., 2014).

Allelopathic effects between *Prorocentrum donghaiense* and other algae are quite common. Wang et al. (2007) found that *P. donghaiense* growth is inhibited by fresh macroalgae (*Ulva linza*, *Corallina pilulifera*, and *Sargassum thunbergii*) and their culture medium filtrates. The growth of *P. donghaiense* was also negatively affected by *Alexandrium tamarense*. However, the negative potency was not correlated with the content of paralytic shellfish poison (PSP) toxin of *A. tamarense* but with its hemolytic properties (Yang et al., 2010). The *P.*

donghaiense cell membrane may be particularly sensitive to the hemolytic properties of the allelopathic substances.

5. Conclusions

After more than 15 years of intensive study, *Karlodinium veneficum* is now recognized as a globally distributed and toxic harmful alga (Place et al., 2012). Our current work extends this distribution to the East China Sea (ECS). We describe new karlotoxin congeners that are highly toxic to sympatric *Prorocentrum donghaiense* and may allow the ECS *K. veneficum* strain to feed on its sympatric cousin. We also propose that *P. donghaiense* blooms may initiate subsequent *K. veneficum* blooms in the ECS and that *K. veneficum* may in turn terminate *P. donghaiense* blooms.

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Fig. 1.

The population growth of *Karlodinium veneficum* (K) and *Prorocentrum donghaiense* (P) in different media cultured alone and co-cultured at different initial cell densities (K/P). The data are the means of triplicates. Error bars (SD) have been omitted for the sake of clarity.

(A) Enriched clean natural seawater (f/2-Si), inoculating cell number proportion K/P = 1:10.

(B) Enriched clean natural seawater (f/2-Si), inoculating cell number proportion K/P = 1:1.

(C) Oligotrophic media of diatom filtrates, inoculating cell number proportion K/P = 1:10.

(D) Oligotrophic media of diatom filtrates, inoculating cell number proportion K/P = 1:1.

Solid triangles ($\blacktriangle$): *K. veneficum* growth in co-culture. Solid circles ($\bullet$): *P. donghaiense* growth in co-culture. Hollow triangles ($\triangle$): monoculture of *K. veneficum* as control. Hollow circles ($\circ$): monoculture of *P. donghaiense* as control.

Fig. 2.

Feeding on *P. donghaiense* cells by *K. veneficum*. (A) *K. veneficum* (solid arrows) actively attached *P. donghaiense* (hollow arrow). Arrow head indicates an empty *P. donghaiense* theca. (B) CFSE-labeled *P. donghaiense* cell. (C) *K. veneficum* and CFSE-labeled *P. donghaiense*. (D) Same view of (C) observed under fluorescence microscope, showing CFSE-labeled *P. donghaiense* (hollow arrows) and *K. veneficum* (solid arrows) without CFSE fluorescence. (E) *K. veneficum* cell with CFSE-labeled *P. donghaiense* components (arrows). (F) *K. veneficum* cell with CFSE-labeled *P. donghaiense* components.

E or F was assembled images taken by a differential interference contrast (DIC) microscope and a fluorescence microscope.

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Fig. 3.

Reverse phase chromatograms (LiChrosphere 125 mm × 4 mm × 5 μm bead size RP-8) of the 60% (A) and 80% (B) methanol fractions from strain NMB jah047–1 culture filtrates fractionated with a Sep-Pak tC-18 cartridge prior to chromatography. Injected toxin was equivalent to 3.5×10^7 cells. The UV spectra of the peaks (1, 2, and 3) for each fraction were identical and are shown as an inset in Fig. 2B. The 235-nm UV maximum is characteristic of a terminal chlorine (Bachvaroff et al., 2008).

Fig. 4. Mass spectra of the UV peaks (1,2, and 3) shown in Fig. 3. Putative identifications of the different ions (sodiated ions assumed) are shown. The karlotoxins in peak 1 appeared to be the sulfated forms of those in peaks 2 and 3.

Fig. 5.

Variations in *Prorocentrum donghaiense* cells after exposure to different amounts of karlotoxin in 24 h. Error bars are standard deviations. Control M: *P. donghaiense* culture exposed to 20% methanol. Control: Original *P. donghaiense* culture.

Table 1

The sterol contents of *Karlodinium veneficum* and *Prorocentrum donghaiense* (the data shown in parentheses are the percentages of sterols).

Sterols (%)	<i>Prorocentrum donghaiense</i>	<i>Karlodinium veneficum</i>
C23 sterol	C _{23:1} (/4Me) (trace)	
C27 sterol	C _{27:1} (⁵) (1.34)	C _{27:1} (⁵) (3.6)
C 28 sterol	C _{28:2} (^{5,22} /24Me) (diatomsterol) (4.37)	C _{28:2} (^{8(14),22} /24,4-dime,27-nor)NED (24.94)
	C _{28:2} (^{5,24(28)} /24Me) (24.57)	
	C _{28:2} (8.9)	
C29 sterol	C _{29:1} (⁷ /4,24diMe) (1.35)	C _{29:2} (^{8(14),22} /4,24dime)ED (47.68)
		C _{29:2}
		Isomer 1 (12.84%)
		Isomer 2 (3.24%)
		C _{29:1} (⁵ /24ethyl) (1.15)
C30 sterol	C _{30:2} (7.12)	C _{30:2}
	C _{30:1} (²² /4,23,24triMe) (dinosterol) (45.5)	Isomer 1 (3.73%)
		Isomer 2 (2.82%)
Unknown	(5.86)	
	(0.99)	