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Seasonal variability of *Protoceratium reticulatum* and yessotoxins in Japanese scallop *Patinopecten yessoensis* in northern Yellow Sea of China

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ABSTRACT

This paper reports a toxic strain of *Protoceratium reticulatum*, its morphology, phylogeny, yessotoxins (YTXs) production and abundance in northern Yellow Sea of China from 2011 to 2015 was investigated. YTXs in hepatopancreas and edible parts of bottom sowing cultured Japanese scallop *Patinopecten yessoensis* in this sea area were determined weekly for 5 years. Other potential producers of YTXs, *Gonyaulax spinifera* and *Lingulodinium polyedrum*, were also investigated. Results revealed that *Protoceratium reticulatum* strain from the northern Yellow Sea belongs to a geographically widely distributed species. Motile cells of *Protoceratium reticulatum* contribute to YTXs in Japanese scallop, and *G. spinifera* may also be a potential contributor. Resting cysts of *Protoceratium reticulatum*, *G. spinifera*, and *L. polyedrum* in sediments were possibly important origins of YTXs in scallop cultured at sea bottom. YTXs in scallop decreased from 2011 to 2015, most toxins were concentrated in hepatopancreas, while a small portion in edible parts which was safe for consumption the whole year around.

1. Introduction

Accumulation of phytotoxins in bivalve shellfish is harmful to human health and aquaculture industry. Yessotoxins (YTXs) are polyether toxins and were first isolated from digestive glands of the Japanese scallop *Patinopecten yessoensis* (Murata et al., 1987). Dinoflagellates *Protoceratium reticulatum* (syn.: *Gonyaulax grindleyi*) (Satake et al., 1997), *Lingulodinium polyedrum* (syn.: *Gonyaulax polyedra* Stein) (Paz et al., 2004), and *Gonyaulax spinifera* (Rhodes et al., 2006; Riccardi et al., 2009) may produce YTXs, which can damage cardiac muscles, liver, pancreas and neuronal tissues in mice (Sala-Pérez et al., 2016). YTXs are considered as potential risk for human health with a lethal dose between 80 and 750 mg/kg (Paz et al., 2008). European Union established 1 mg kg^{-1} (EC 853/2004) as maximum level permitted in shellfish, this value was amended to 3.75 mg kg^{-1} recently (EC 786/2013). YTXs were also monitored in shellfishes from Japan, Norway, Chile, New Zealand, Italy, and Spain (Paz et al., 2013).

The toxic dinoflagellate *Protoceratium reticulatum* is widely distributed in coastal waters around the world and was reported in Argentina, Arctic, Brazil, Canada, Chile, Japan, Italy, Mexico, New Zealand, Norway, Russia, South Africa, Spain, UK, and USA (Röder et al., 2012; Paz et al., 2013; Akselman et al., 2015; Sala-Pérez et al., 2016). *Protoceratium reticulatum* generates more than 100 YTX analogues, and its toxin profiles differ among origin of strains from New Zealand, Japan, Norway, Italy, UK, Canada, Spain, and USA (Paz et al., 2013). YTX is the major toxin in *Protoceratium reticulatum*, and only homoYTX was found to be the main toxin in two strains from Japan and Spain (Konishi et al., 2004; Paz et al., 2007). YTXs produced in cultured *Protoceratium reticulatum* measured 0.9–79 pg YTX $cell^{-1}$, 1.5 pg YTX $cell^{-1}$ in *L. polyedrum*, and 200 pg YTX $cell^{-1}$ in *G. spinifera* (Röder et al., 2012). Quantity of YTXs secreted by *Protoceratium reticulatum* were reported to be influenced by temperature, light, growth phase, and nutritional conditions (Guerrini et al., 2007; Mitrovic et al., 2005; Paz et al., 2006, 2013).

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YTXs were detected in bivalves of *Patinopecten yessoensis*, *Chlamys ferreri*, *Argopectens irrardias*, *Crassostrea gigas*, and *Mytilus edulis* in coastal waters of Yellow Sea in China (Gao et al., 2010, 2012; Chen et al., 2014). This study aimed to isolate *Protoceratium reticulatum* strain from northern Yellow Sea in China and describe its taxonomical and toxicological features. By counting dinoflagellates of *Protoceratium reticulatum*, *L. polyedrum*, and *G. spinifera* motile cells in water column and resting cysts in sediment and detecting YTXs in scallop *Patinopecten yessoensis* cultured at sea bottom every month all year, this study attempted to determine the relationship between toxic dinoflagellate biomass and toxin contents in scallops.

2. Materials and methods

2.1. Study area

Zhangzi Island (122.73310 E, 39.04078 N) is located in northern Yellow Sea of China on the path of Yellow Sea Warm Current (Fig. 1). This island comprises an area measuring 2200 km^2 and with a maximum depth of 45 m. In this area, the Yellow Sea Warm Current is enhanced in winter and weakened in summer. Annual cycle is characterized by cold mixing period in winter, followed by stratification period during spring and summer (Bao et al., 2009). Seawater temperature measures 22.5 °C at the surface and 12.8 °C at the bottom in July, which is the largest thermocline for an entire year. The maximum mean column temperature (19.6 ± 3.1 °C) occurs in August, whereas the lowest (1.3 ± 1.3 °C) can be observed in February (Zhang et al., 2016). Japanese scallop *Patinopecten yessoensis* cultivation began in 1982 and became a dominant maricultural species since 1998. Sampling stations for Japanese scallop are located in cultural areas around this island.

2.2. Isolation of Protoceratium reticulatum

Seawater samples for *Protoceratium reticulatum* isolation were collected from coastal waters of Station No. 15 on April 2010 and placed in an 800 mL plastic bottle. Single motile cells of *Protoceratium reticulatum* were isolated with capillary pipette and washed four times with filtered seawater under a microscope (Eclipse E100, Nikon, Japan). Then, cells were transferred to individual wells of tissue culture plates (Costar 96 well, Corning, USA) containing 200 μL of sterile native seawater at a salinity of 33 from the collected site and cultured in an illumination incubator (HPG 4000G, Ruihua Instrument & Equipment Co., Ltd., Wuhan, China) at 15 °C under artificial light under an irradiance of 100–125 μmol photons $m^{-2}\cdot s^{-1}$ on a 12:12 h light/dark regimen. Parafilm was used to prevent evaporation in culture medium. After four weeks, uni-algal isolates were transferred to 24-well tissue culture plates (Costar 24 well, Corning, USA), L1-Si medium (Guillard and Ryther, 1962) was used. Two months later, only one strain, named ZZD01, survived, and cultures were maintained and grown in 100 mL Erlenmeyer flasks under the same conditions as described above.

2.3. Morphological identification of Protoceratium reticulatum

2.3.1. Light microscopy

Motile cells *Protoceratium reticulatum* were examined under a microscope (Eclipse 80i, Nikon, Japan) equipped with microphoto system (DS-Ri2, Nikon, Japan), and light micrographs were obtained. For identifying thecal plates, samples were stained by 1% fluorescent brightener 28 (Sigma-Aldrich, USA) to discern plates in motile cells following the method of Fritz and Triemer (1985).

2.3.2. Scanning electron microscopy (SEM)

To clarify classification suggested by light microscopy, thecal plate analysis was combined with SEM for definitive identification. To remove thick membrane and impurities on surface of algae cells, 5% Trition X-100 (Sigma-Aldrich, USA) was added and maintained in ultrasonic water bath for 10 min. Then, cells were primarily fixed by 4% glutaraldehyde (EM grade, SPI-CHEM, USA) in 0.1 M sodium cacodylate buffer (pH = 7.5) to one volume of culture for 1 h at 4 °C. After pelleting and washing in filtered seawater for five times and as described above, samples were fixed again by 1% osmic acid (enzyme reagent, Beijing Zhongjingkeyi Technology Co., Ltd., China) for 40 min at 4 °C. Cells were dehydrated in gradient ethanol/water solutions of 20%, 30%, 50%, 70%, 80%, 90%, and 100% twice for 10 min each and washed thrice with 90% and 100% ethanol/water dehydration solution. Then, cells were dried under a critical point dryer (HCPD-15-500, Jeol, China), collected on a cover

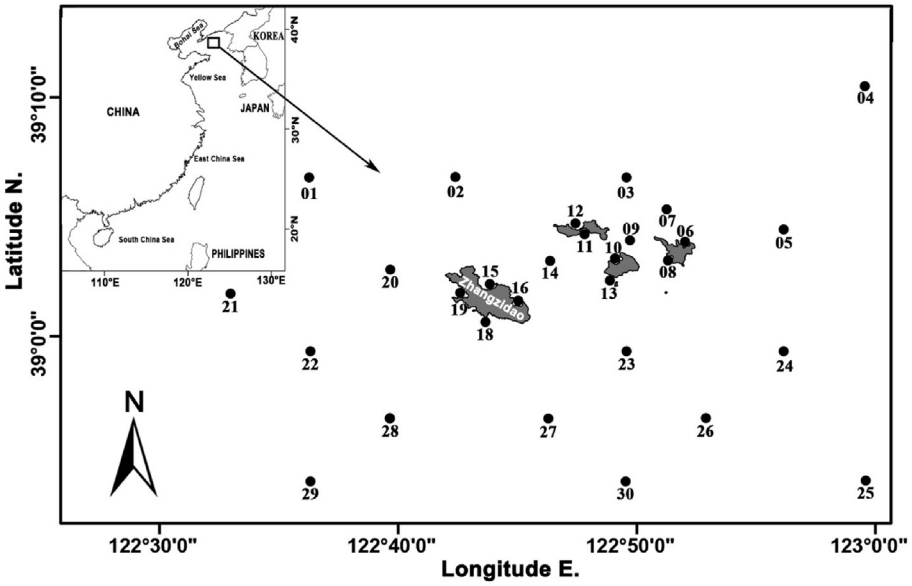

Fig. 1. Geography of northern Yellow Sea area and the investigated station (●) in the study, where the clonal strain of *Protoceratium reticulatum* was isolated at station 15.