Spatiotemporal Distribution of the Harmful Dinoflagellate Stoeckeria algicida in Liaodong Bay (Postprint)

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Abstract

Stoeckeria algicida is a dinoflagellate belonging to the family Thoracosphaeraceae, possessing ichthyotoxic capabilities through invading fish cells, which can lead to mass fish mortalities, while also killing other marine microalgae. Due to its minute size and the difficulties associated with morphological identification, research on this species has been limited, with virtually no reports from Chinese waters. In recent years, advances in high-throughput sequencing technology have significantly advanced research on the identification of micro- and nano-phytoplankton. To investigate the presence and distribution of Stoeckeria algicida in the Liaodong Bay waters of China, the 18S rDNA V4 region was targeted as the molecular marker. Combined with high-throughput sequencing technology, a specific primer pair V4 (F/R) for micro- and nano-phytoplankton identification was designed, and subsequently employed to assess the diversity of micro- and nano-phytoplankton in Liaodong Bay seawater across the four seasons of 2014. The results revealed that Stoeckeria algicida was detected in all seasons except spring, with temperature identified as the primary factor influencing its reproduction. Although the species did not exhibit pronounced dominance in the overall environmental samples, its density was notably elevated during summer (reaching a maximum of 2.753$\times$10^{3}$ cells/L), with high-concentration zones primarily distributed along the eastern and western coasts of Liaodong Bay, indicating a relatively high disaster risk that requires adequate attention from relevant authorities. This study represents the first report of Stoeckeria algicida in Chinese waters, and considering its severe detrimental impacts, enhanced monitoring and regulatory measures are imperative.

Full Text

Preamble

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Title: Distribution of the Toxic Dinoflagellate *Stoeckeria algicida* in Liaodong Bay

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Abstract

Stoeckeria algicida belongs to the family Thoracosphaeraceae within the dinoflagellates. This species can invade fish cells, causing mass fish mortality, and also kills other marine microalgae. First discovered in Masan Bay, Korea, in 2004, its morphology and ecology have been documented, identifying it as an invasive microalga. Its vegetative and biflagellate cells are oval, measuring 7.3–15.9 m (mean 11.6 m) and 2.7–12.2 m (mean 7.3 m), respectively. Based on morphological and genealogical analyses, previous studies suggested it represents a new species in a new genus. Reported grazing coefficients of up to 0.142 min^{-1} indicate *S. algicida* can significantly impact *Heterosigma akashiwo* populations. Morphological identification of *S. algicida* is difficult due to its small size, which has delayed research progress, and studies in Chinese waters have been extremely limited. Recently, high-throughput next-generation sequencing (NGS) technology has greatly advanced research on nano- and picophytoplankton identification.

To determine whether *S. algicida* exists in China' s Liaodong Bay and characterize its distribution, we targeted the 18S rDNA V4 region to design a nano- and picophytoplankton identification primer pair V4(F/R), employing NGS technology to investigate nano- and picophytoplankton diversity in Liaodong Bay waters. The results revealed that *S. algicida* was present in all seasons except spring. Temperature was a significant factor affecting its reproduction. Although *S. algicida* was not dominant in the phytoplankton community, its density was higher in summer (2.753×10^4 cells/L), with high-value areas mainly distributed along the eastern and western coasts of Liaodong Bay where disaster risk is high and should warrant serious attention. This study represents the first report of *S. algicida* in Chinese waters. While no red tide events caused by *S. algicida* have been recorded, preventive measures should be implemented rather than reactive mitigation. Furthermore, monitoring and management of

toxic microalgae in aquaculture areas should be enhanced, and an early warning system should be established to avoid disasters in fisheries ecosystems.

Keywords: *Stoeckeria algicida*; Thoracosphaeraceae; harmful microalgae; nano-phytoplankton; high-throughput sequencing; Liaodong Bay

Introduction

The impacts of invasive marine organisms on ecosystem stability and economic losses have attracted widespread attention from scientists and the public. Since the early 21st century, ballast water discharge from shipping vessels has led to extensive spread of exotic marine plankton, repeatedly causing toxic and harmful red tides that have inflicted substantial damage on coastal aquaculture, biological resource exploitation, seafood quality, ecological environments, and human health [1-2]. Ballast water represents the most important pathway for marine biological invasion, and its damage to the marine environment has been identified by the Global Environment Facility as one of four major threats to oceans, alongside land-based marine pollution, alteration of marine habitats, and biological resource exploitation. The International Maritime Organization, United Nations Development Programme, and Global Environment Facility have jointly proposed the Global Ballast Water Control and Management Convention, and research on ballast water treatment technologies has been conducted.

Due to detection technology limitations, previous research primarily focused on micro- and small phytoplankton (20–200 m). However, nano- and picophytoplankton (2–20 m and 0.22–2 m, respectively) exhibit considerable diversity in marine environments and represent the main causative agents of brown tides. Because nano-sized algae are extremely small and difficult to identify morphologically, research on them has been relatively slow. With the rapid development of molecular biology, studies on nano-phytoplankton diversity have advanced quickly. Researchers have investigated molecular diversity of nano-phytoplankton in major ocean basins including the Pacific [3], Mediterranean [4], and Indian Oceans [5], with relevant gene libraries continuously being updated and expanded. In China, molecular diversity studies of nano-phytoplankton have gradually been conducted in the South China Sea [6], Bohai Sea [7], and North Yellow Sea [8].

Marine ecological disasters occur frequently, with brown tides caused by nano-phytoplankton frequently breaking out in the Bohai Sea, causing significant harm to shellfish aquaculture [9]. The reported causative species of brown tides in China is *Aureococcus anophagefferens*, but recent research indicates this is not the only nano-phytoplankton species that can trigger brown tides [7]. In recent years, high-throughput sequencing technology has greatly facilitated efficient detection research on nano-phytoplankton [10-12], offering advantages including simplicity, rapid processing, high sequencing throughput, low error rates, and

cost-effectiveness, providing new approaches for efficient nano-phytoplankton detection.

Stoeckeria algicida, belonging to the family Thoracosphaeraceae, can invade fish cells causing mass mortality and also kills other marine microalgae. First discovered in Masan Bay, Gyeongnam, Korea in 2004, no reports of this species exist in Chinese waters [13]. As China' s northernmost semi-enclosed inland sea with frequent shipping activities and severe eutrophication, Liaodong Bay faces high risk of invasive harmful microalgae. This study employs high-throughput sequencing platforms combined with bioinformatic methods, targeting the 18S rDNA V4 region to investigate nano- and picophytoplankton diversity in Liaodong Bay waters. Early detection of *S. algicida* diffusion signals is crucial for understanding its ecological distribution status.

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1. Sample Collection

To investigate the distribution and diffusion of the invasive harmful microalga *S. algicida*, we established a grid-based sampling design in Liaodong Bay. Surface seawater was collected and filtered through 10 m pore-size membranes to remove large and small zooplankton. Micro- and picophytoplankton were collected on 1.2 m pore-size membranes. Finally, membranes were transferred to 1.5 mL tubes for preservation.

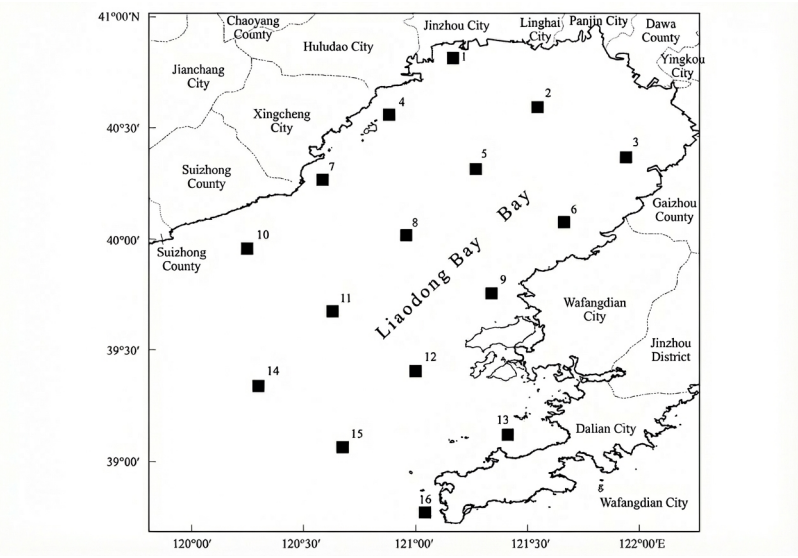

Figure 1: Figure 1

Sampling station locations

2. Analysis Methods

2.1 Genomic DNA Extraction

Macro-genomic DNA from micro- and picophytoplankton was extracted using the CTAB method. The 0.22 m filter membrane was cut into pieces in a 1.5 mL centrifuge tube, to which 500 L CTAB buffer (2% CTAB; 100 mmol/L Tris-Cl pH 8.0; 1.4 mmol/L NaCl; 10 mmol/L EDTA) was added. After incubation, the liquid was transferred to a new centrifuge tube for phenol-chloroform extraction. The DNA pellet was washed with ethanol and resuspended to obtain microalgal macro-genomic DNA. Concentration and purity were determined using 1% agarose gel electrophoresis and UV spectrophotometry, then stored at -20°C.

2.2 Primer Design and PCR Amplification

This study employed self-developed primers for nano- and picophytoplankton 18S rDNA V4 region amplification. The upstream primer was 5 -GATCCCCHWACTTTCGTTCTTGA-3 and downstream primer was 5 -GCGGTAAATTCCAGCTCCAAATA-3 . Primers with appropriate adapters were synthesized by Shanghai Sangon Biotech. PCR reactions were performed in 50 L volumes containing 5 L PCR Buffer, 8 L dNTP Mixture, 2 L DNA template, 2.5 U Taq polymerase, and 2 L each of upstream and downstream primers (10 mol/L). Amplification was conducted on a PE 9700 thermocycler with initial denaturation at 94°C for 3 min, followed by cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were detected using 1% agarose gel electrophoresis.

2.3 High-Throughput Sequencing

Qualified PCR products were sent to Novogene Bioinformatics Technology for library construction using the NEB Next Ultra Library Prep Kit for Illumina (New England Biolabs). Constructed libraries underwent Qubit quantification and quality control before sequencing on the HiSeq2500 PE250 platform.

3. Data Analysis

Raw sequencing data were assembled using FLASH software. The UPARSE quality control pipeline processed assembled sequences through trimming and filtering to obtain valid data. Operational Taxonomic Units (OTUs) were clustered and classified using the RDP Classifier. Community structure statistical analysis was performed using QIIME. Species annotation was conducted at various taxonomic levels.

To calculate *S. algicida* density, all phytoplankton species in concurrent water samples were microscopically examined, and *Chaetoceros* species with the highest occurrence frequency at each station were selected as the reference density [14]. The calculation formulas were as follows:

- *S. algicida* density at a station (NS) = (NC × nS)/nC
  - NC: density of all *Chaetoceros* species in the water sample at the station (cells/L)
  - nS: OTU number of *S. algicida* at the station
  - nC: OTU number of all *Chaetoceros* species at the station
- Seasonal proportion of *S. algicida* = (ni/N) × 100%
  - ni: density of species i (cells/L)
  - N: total density of the statistical unit (cells/L)
- Species dominance (Y) = (ni/N) × fi
  - fi: frequency of species i occurrence in samples

Dominance indicates the advantage degree of a particular species within the nano- and picophytoplankton community.

Results

1. Molecular Primer Optimization

To enhance detection efficiency of nano- and picophytoplankton, primers were screened and optimized. Based on currently known nano-phytoplankton species and historical survey results, we designed specific molecular identification primers suitable for the Yellow Sea and Bohai Sea regions. For the 18S rDNA V4 region, we designed primer pair V4(F/R) (5 -GATCCCCHWACTTTCGTTCTTGA-3 , 5 -GCGGTAAATTCCAGCTCCAAATA-3). For comparison, we also selected universal primer pair V9(F/R) [15] (5 -CCCCTGCCHTTTGTACACAC-3 , 5 -CCTTCYGCAGGTTACCTAC-3) and C4(F/R) (5 -CCAGCASCYCGCGGTAATTC-3 , 5 -ACTTTCGTTCTTGATYRA-3).

Primer design involved downloading 18S rDNA V4 region sequences from databases, performing multiple sequence alignments using MEGA4, locating corresponding 18S rDNA positions, and evaluating primer parameters using DNAman and Oligo Calc software. The V4(F/R) fragment length was 179–450 bp. Results showed V4(F/R) had lower amplification specificity for choanoflagellates compared to V9(F/R), but higher specificity for Rhodophyta and Haptophyceae compared to C4(F/R). To compare primer performance, we amplified environmental samples from Liaodong Bay at stations 8, 11, and 12 using V4(F/R), V9(F/R), and C4(F/R). Sequencing on Illumina HiSeq2500 PE250 revealed that V4(F/R) identified more nano- and picophytoplankton species (68, 73, 92 species) compared to V9(F/R) (37, 46, 44) and C4(F/R) (33, 59, 81), while maintaining higher specificity for eukaryotic algae. Therefore,

V4(F/R) was selected for subsequent analyses.

2. Species Identification of *Stoeckeria algicida*

High-throughput sequencing yielded partial 18S rDNA sequences of *S. algicida*. BLAST analysis against NCBI showed 100% similarity with *Stoeckeria algicida* 18S rDNA (AJ841809.1), with the corresponding gene position at 595–969 bp (375 bp length). A phylogenetic tree was constructed using the neighbor-joining method with selected dinoflagellate 18S rDNA sequences. The tree showed that the *S. algicida* sequence obtained (HG005133) clustered with the reference *S. algicida* 18S V4 sequence with 100% bootstrap support, confirming the species identification.

[FIGURE:2] *Stoeckeria algicida* phylogenetic tree

3. Density Distribution of *Stoeckeria algicida*

Stoeckeria algicida was not detected at any station in Liaodong Bay during spring. Summer density ranged from 0.023–2.753 $\times$ 10^4 cells/L, with high-value areas primarily distributed along the eastern and western coasts of the bay (stations 1, 4, 7, 9, 10, 12, 13, 16). The maximum density (2.753 $\times$ 10^4 cells/L) occurred at station 9, while the minimum (0.023 $\times$ 10^4 cells/L) was at station 6. Autumn density ranged from 0.011–2.488 $\times$ 10^4 cells/L, with high-value areas in the northwestern bay. The maximum (2.488 $\times$ 10^4 cells/L) occurred at station 12, minimum (0.011 $\times$ 10^4 cells/L) at station 6. Winter density ranged from 0.002–0.073 $\times$ 10^4 cells/L, with relatively uniform distribution across stations and low quantities overall.

4. Dominance

Summer dominance of *S. algicida* ranged from 0.03–0.41%, with stations 6, 9, 11–16 exceeding 0.2% and maximum dominance of 0.28% at station 9. Autumn dominance ranged from 0.03–0.54%, with stations 7, 9, 11–16 exceeding 0.2% and maximum dominance of 0.22% at station 12. Winter dominance ranged from 0.02–0.34%, with stations 6, 8, 9, 15 exceeding 0.2% and maximum dominance of 0.18% at station 9.

[FIGURE:3] Relative *Stoeckeria algicida* density in different seasons
[FIGURE:4] *Stoeckeria algicida* dominance in different seasons

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Discussion

Current research on *Stoeckeria algicida* remains limited, with most findings originating from the Red Tide Research Center at Seoul National University' s Institute of Marine Sciences. First discovered in 2004 in Masan Bay, Korea, this new Thoracosphaeraceae species caused two large-scale red tides, resulting

in billions of Korean Won in economic losses to fishermen. The species name combines "algicida" (Latin for "killing algae") with the discoverer Stoecker' s name. Jeong et al. described the heterotrophic dinoflagellate' s morphological characteristics and obtained ribosomal small subunit DNA sequences from cultured cells. The oval cells measure 7.3–15.9 m (mean 11.6 m) and 2.7–12.2 m (mean 7.3 m). Sequence comparisons showed *S. algicida* ribosomal small subunit DNA (GenBank AJ841809) is most closely related to *Pfiesteria piscicida* and a dinoflagellate from Shepherd' s Crook, yet occupies a distinct branch. Based on morphological and phylogenetic analyses, *S. algicida* was identified as a new species in a new genus [16].

To investigate its impact on other red tide organisms, Jeong et al. monitored red tides in Masan Bay during July 2004, tracking density changes of *Heterosigma akashiwo* and *S. algicida*. Laboratory studies examined *S. algicida* predation on *H. akashiwo*. The results showed *S. algicida* attaches via a stalk-like structure, then uses filaments to capture prey. When *H. akashiwo* density reached approximately 3,500 cells/mL, *S. algicida* maximum density occurred 1–2 days later (5,840–9,920 cells/mL vs. 58,400–99,200 cells/mL for *H. akashiwo*). *S. algicida* specific growth rate increased rapidly with *H. akashiwo* density, reaching a maximum of 1.63/d. Maximum ingestion and clearance rates were 0.142 cells/min (1.9 ng C/grazer·mL or 19 cells/grazer·mL) and 3.7 L/h, respectively, when *H. akashiwo* threshold concentration was 0.75 ng C/mL (7.5 cells/grazer·mL). These results demonstrate *S. algicida* predation significantly impacts *H. akashiwo* populations [17].

Studies indicate *S. algicida* produces both lipid-soluble and water-soluble toxins. *Pfiesteria* toxins can rapidly kill brackish water fish, causing skin ulceration. Lipid-soluble components cause skin damage, while water-soluble components cause neurological damage. Water-soluble toxins are glycosides containing copper and iron elements, while lipid-soluble components include plasticizers [18].

From 2013–2014, large numbers of dead fish were observed near Dalian Changxing Island, with *S. algicida* densities approaching red tide warning criteria. Our seasonal survey in Liaodong Bay showed *S. algicida* was not detected in spring, but was present in other seasons, with density dropping sharply in winter, indicating temperature is a major factor affecting its reproduction. Although not dominant in the overall phytoplankton community, summer densities reached 2.753×10^4 cells/L, with high-risk areas along the eastern and western coasts. Classified as an exotic species in Korea, *S. algicida* is reported for the first time in Chinese waters. Despite no recorded red tide events, its potential consequences are severe, necessitating enhanced monitoring and management.

Based on current distribution patterns in Liaodong Bay, *S. algicida* has become naturalized. Future efforts should: (1) accelerate research on its toxin production mechanisms; (2) supplement invasive microalgae databases with nano- and picophytoplankton population and distribution data; (3) intensify monitoring of toxic microalgae in aquaculture areas and port waters; and (4) establish rapid early warning systems for shellfish toxins to reduce harm to fisheries ecosystems

[20-23].

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