

Seasonal variations in fouling diatom communities on the Yantai coast*

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Abstract Fouling diatoms are a main component of biofilm, and play an important role in marine biofouling formation. We investigated seasonal variations in fouling diatom communities that developed on glass slides immersed in seawater, on the Yantai coast, northern Yellow Sea, China, using microscopy and molecular techniques. Studies were conducted during 2012 and 2013 over 3, 7, 14, and 21 days in each season. The abundance of attached diatoms and extracellular polymeric substances increased with exposure time of the slides to seawater. The lowest diatom density appeared in winter and the highest species richness and diversity were found in summer and autumn. Seasonal variation was observed in the structure of fouling diatom communities. Pennate diatoms *Cylindrotheca*, *Nitzschia*, *Navicula*, *Amphora*, *Gomphonema*, and *Licmophora* were the main fouling groups. *Cylindrotheca* sp. dominated in the spring. Under laboratory culture conditions, we found that *Cylindrotheca* grew very fast, which might account for the highest density of this diatom in spring. The lower densities in summer and autumn might result from the emergence of fouling animals and environmental factors. The *Cylindrotheca* sp. was identified as *Cylindrotheca closterium* using 18S rDNA sequencing. The colonization process of fouling diatoms and significant seasonal variation in this study depended on environmental and biological factors. Understanding the basis of fouling diatoms is essential and important for developing new antifouling techniques.

Keyword: biofouling; diatom community; seasonal variation; *Cylindrotheca closterium*

1 INTRODUCTION

Marine biofouling is usually classified as the undesirable accumulation of microorganisms, plants, and animals on artificial surfaces in seawater. Marine biofouling generally consists of three stages. The first stage is rapid accumulation of organic molecules, such as polysaccharides, proteins, proteoglycans, and possibly inorganic compounds on a solid surface, which is called a conditioning film. In the second stage, bacteria and single celled diatoms settle quickly on the surface and secrete extracellular polymeric substances (EPS), and constitute major components of the biofilm. In the third stage, algal spores, barnacle cyprids, and protozoa adhere to the surface and grow to form the fouling communities (Yebera et al., 2004). Some studies have also shown that the fouling process might occur simultaneously or without one of the

three stages (O'Neil and Wilcox, 1971; Mitbavkar and Anil, 2008). Marine biofouling on marine construction surfaces may ruin the structure and increase the costs in operation and management, especially for shipping (hull cleaning processes, petroleum consumption), which hampers ocean economic development and causes economic loss to aquaculture (Schultz et al., 2011; Bloecher et al., 2013).

Biofilm plays an important role as a settlement cue for algal spores and invertebrate larvae and significantly contributes to nutrient turnover in

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aquatic ecosystems (Joint et al., 2002; Ganesan et al., 2010; Sawall et al., 2012; Salta et al., 2013). Diatoms are dominant components of biofilm, especially in the light penetrable portion of sea water. Previous studies have shown that the fouling diatom species and the associated changes in biofilms affect larval growth and the macro-fouling community structure (Lam et al., 2005; Patil and Anil, 2005a). Dahms et al. (2004) reported in detail the influence of biofilm (bacteria and diatoms) on the adhesion of the bryozoan *Bugula neritina*, showing that a biofilm of the bacterial strain *Pseudoalteromonas* sp. caused significantly lower larval settlement, which was closely related to the densities of the strain. However, the biofilms of diatoms *Achnanthes* sp., *Amphora coffeaeformis*, *Amphora tenerrima*, *Nitzschia constricta*, and a 5-day-old natural biofilm significantly enhanced larval settlement success. Therefore, diatoms play very important roles in the settlement of fouling animals. Previous studies have also shown that antifouling coatings that were once effective against most fouling organisms now largely fail to inhibit diatom settlement (Molino and Wetherbee, 2008). Therefore, more and more attention has been paid to the study of fouling diatom community structure.

The settlement of fouling diatoms depends on many factors, such as properties of substratum, geographic location and climate zone (Thiyagarajan, 2010). Based on the development of marine antifouling technology, people initially focused on the diatom community structure of the antifouling coating surface. Zargiel et al. (2011) and Zargiel and Swain (2014) reported that the hull coatings of cargo ships affected the community structure of fouling diatoms, and the main fouling diatom species varied significantly with different coatings and the geographical area. The effects of static and dynamic immersion on the adhesion and settlement of diatoms were different, and the diversity of diatoms on static panels was significantly greater than that on dynamic panels. These results demonstrated that hydrodynamic stress influences the microfouling community. Patil and Anil (2005b) studied the influence of temporal variability on the community structure of diatom biofilm and showed that the structure varied significantly depending on the season, owing to physico-chemical and biological changes in the seawater. Other reports concerned the properties of substrata such as material type, color, and roughness and the impact on diatom communities (Dobretsov et al., 2013). Knowledge of the importance of diatoms in the fouling process has promoted more

studies on the community structure of fouling diatoms. Understanding fouling diatoms is useful in environmental studies, pollution control, and strategies for developing antifouling techniques.

Fouling communities in the Chinese coastal zone have been reported, but most of them are about fouling animals (Huang and Cai, 1984; Yan and Yan, 2003; Cao et al., 2013). Studies on marine fouling diatoms are promising because fouling diatoms are very important components in marine biofouling and community structure determines the settlement of macrofouling animals. Understanding the diatom communities and seasonal variation are very useful in guiding the development of marine antifouling techniques. In this experiment, taking the Yantai coast site as an example, fouling diatom community structures were investigated in different seasons, using hanging glass slides to observe the process of fouling diatom colonization.

2 MATERIAL AND METHOD

2.1 Study area

All the experiments were carried out at Fisherman's Wharf located south of Moon Bay, Yantai (37°30'48"N, 121°26'41"E), northern Yellow Sea, China.

2.2 Experimental design

Yantai Fisheries Research Institute provided the study area and vessels to conduct the experiment during April, July and October of 2012 and January of 2013, representing spring, summer, autumn, and winter in Yantai, China. The study site owned a floating ship on which several submerged 'slide frames' were suspended vertically in the seawater. Each frame held 12 sample glass slides (75 mm×25 mm×1 mm) at 50 cm below water surface. Four frames were immersed in the seawater during each season. At time points of 3, 7, 14, and 21 days, glass slides were immersed and one frame including 12 slides were brought back to the laboratory where the community structure of fouling diatom was analyzed. Eight slides per sample were used for algal community composition analysis. Another four slides were used for scanning electron microscopy (SEM, Hitachi S-4800, Hitachi, Japan) analysis.

2.3 Sample preparation

The samples for visualization of the attached diatoms on each sample slide surfaces were prepared

according to the methods of Zargiel et al. (2011). The glass slides were washed three times with filtered seawater (0.22 μm) to remove any non-attached material and microorganisms and were photographed to determine the formation process of fouling algae with fluorescence microscope (Olympus BX51, Japan). For diatom community composition and abundance analysis, the attached organisms on the glass slides were removed with a nylon brush and the removed organisms fixed in 1 mL solution of 4% formaldehyde with sterile filtered seawater. For further observation and identification, the glass slides were fixed with 4% formaldehyde for 2 h, followed by dehydrating in a graded series aqueous solutions of 25%, 50%, 75%, and 95% ethanol for 20 min. The slides were air dried and kept in a desiccator overnight for SEM examination (Fang et al., 2002; Villa et al., 2009).

2.4 Diatom community analysis

Diatom taxonomic identification was assisted by reference to Jin et al. (1982, 1992), Chen et al. (1996) and Yang and Dong (2006), and was determined under a light microscope (Olympus CX21, Japan) at 40 \times and 100 \times objective. The SEM images can be referred to for further identification. Diatom abundances were used to calculate species richness and the Shannon-Wiener diversity indices.

2.5 Algal cell isolation, identification and culture

Cylindrotheca sp. was isolated from the sample immersed in seawater for 14 days in spring, 2012. This algal strain was identified by morphological and molecular biology approaches. Algal morphology was observed by optical microscopy and SEM according to the above reports. The 18S rDNA sequence region was used for molecular species identification. DNA extraction was performed by the CTAB method (Winnepenninckx et al., 1993), with some modifications. The cultured algal cells in 10 mL (3×10^5 cells/mL) were harvested by centrifugation at 2 570 $\times g$ for 10 min and washed twice with distilled seawater. Cells were resuspended in 0.8 mL CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, CTAB; 100 mmol/L Tris-HCl, pH 8; 20 mmol/L EDTA; 1.4 mol/L NaCl; 0.2% β -mercaptoethanol; 0.1 mg/mL proteinase K) and incubated at 60 $^\circ\text{C}$ for 1 h. After adding 0.8 mL of chloroform/isoamylalcohol (24:1) solution, the extracted solution was gently mixed for 2 min by inverting the tube and centrifuged at 13 000 $\times g$ for

10 min. The supernatant was removed and the pellet was washed twice with 70% ethanol at 4 $^\circ\text{C}$. The DNA pellet was resuspended in sterile H₂O and stored at -20 $^\circ\text{C}$ as template DNA. PCR amplification of the eukaryotic 18S rDNA gene fragment was performed on 20 μL volume containing 3.7 μL mixture (DNA polymerase, dNTP and MgCl₂ buffer), 1 μL of each primer, the forward primer (5'-GCTCGTCTCAAAG-ATTAAGCC-3') and the reverse primer (5'-GTTACG-ACTTCACCTTCCTCT-3'), which yielded a fragment of about 1 700 bp, template DNA 1 μL and 13.3 μL double distilled water. The following PCR protocol was used with two steps: one denaturation step at 94 $^\circ\text{C}$ for 30 s and 30 cycles of 55 $^\circ\text{C}$ for 30 s, followed by 72 $^\circ\text{C}$ for 1 min. PCR products were sequenced by Shanghai Biological Engineering Co. Ltd. The obtained sequences were compared by NCBI database to determine algal species.

Diatoms were cultured with *f/2* medium in a light incubator, at a light intensity of 48 $\mu\text{mol photons}/(\text{m}^2 \cdot \text{s})$ and 20 $^\circ\text{C}$ with a 12 h:12 h light: dark cycle and the growth rate was detected at an optical density of 680 nm with a UV spectrophotometer (Yang et al., 2013).

2.6 Data analysis

All the data shown in the study were the means \pm SD of at least three independent experiments and were evaluated by using one-way analysis of variance (ANOVA) followed by a least significant difference test (LSD), $P < 0.05$ (Origin 7.5 for Windows).

3 RESULT

3.1 The formation process of fouling algae attachment

The fouling algae community that attached to the glass slides from 3 to 21 days is presented in Fig.1. The results from fluorescence microscopy and SEM showed that the abundance of algae and EPS increased with exposure period. Taking the samples from April (SEM) and July (fluorescence microscopy) in 2012 as examples, only a few algal cells were attached to the slide after 3 days of exposure in the seawater. On the 7th day, the numbers of algal cells increased and a small amount of EPS appeared. After 14 days exposure, the abundance of algal cells and EPS continued to increase on the glass slides. After 21 days, fluorescence microscopy showed that the fouling algae increased and the SEM showed that thick and dense EPS were formed around algal cells.

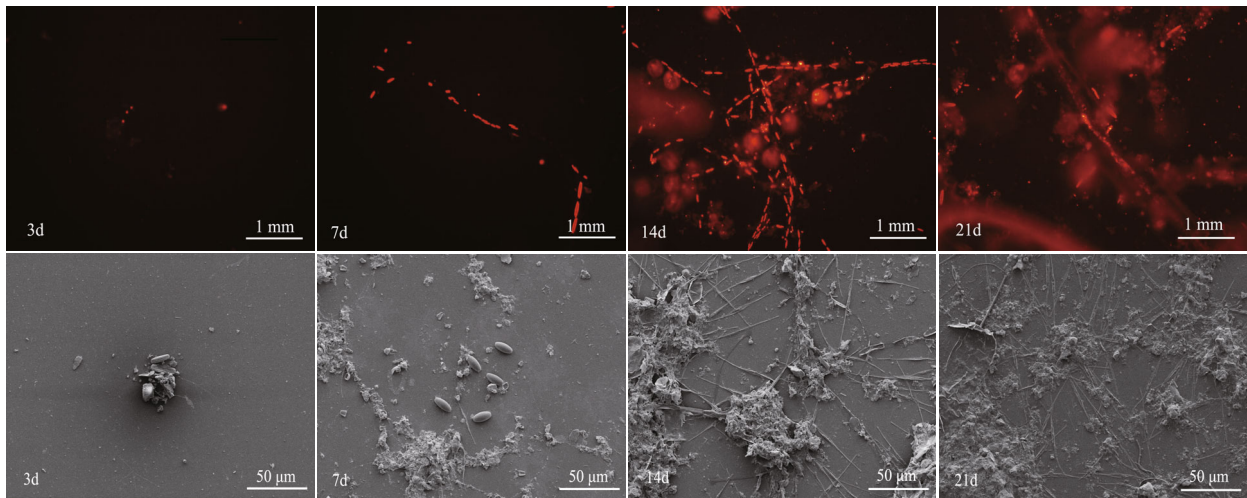


Fig.1 Formation process of marine fouling algae communities

The top row is a series of epifluorescence microscope images (July in 2012) and the bottom row is a series of SEM images (April 2012).

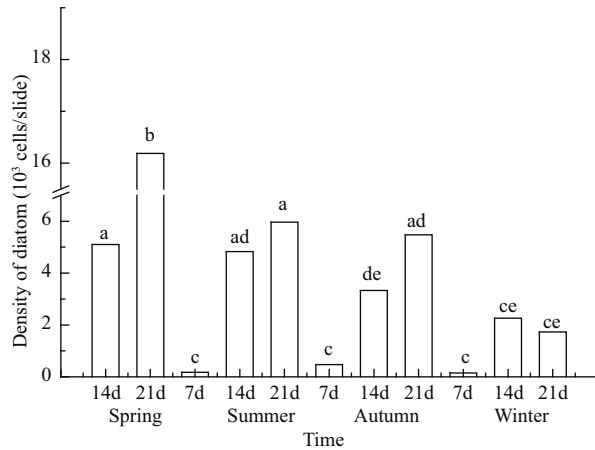


Fig.2 Total density of diatoms developed on glass slides during 21 days of exposure in the experiment

All error bars indicate SD of the three replicates. Letters represent whether there are differences between values ($P < 0.05$).

To further illustrate the formation process of fouling diatom communities on the glass slides, the temporal variation in the fouling diatom density is shown in Fig.2. Overall, diatom density showed an increasing trend with exposure period in every season. The highest diatom density was observed during spring and the density reached 1.62×10^4 cells every slide, which was almost 2.7, 3.0 and 9.4 times that of summer, autumn, and winter after 21 days exposure, respectively. Diatom density was minimal during winter. In summer and autumn, the diatom densities kept at similar levels and showed no significant difference between them at the same exposure time.

3.2 Variations in fouling diatom communities

We calculated differences in the Shannon-Wiener

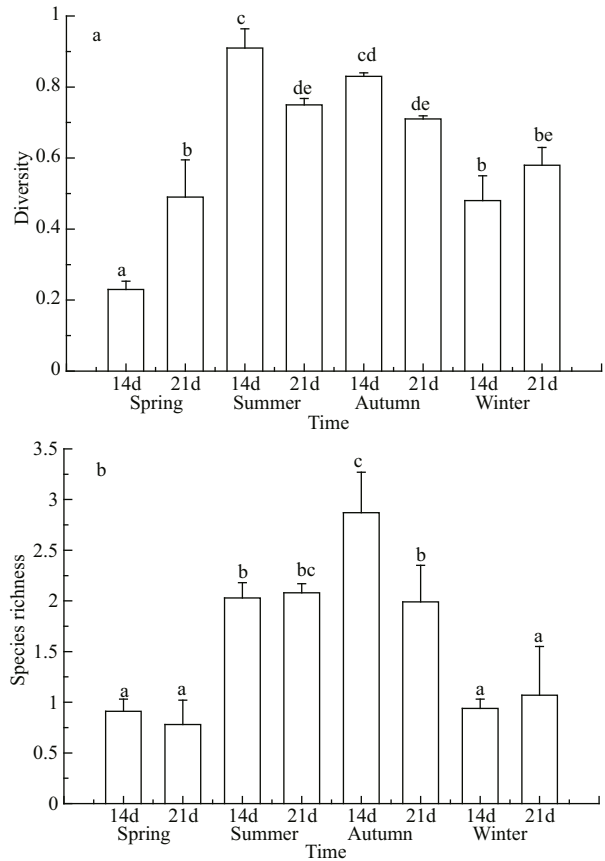


Fig.3 Shannon-Wiener diversity (a) and Margalef's species richness indices (b) of fouling algal communities

All error bars indicated SD of the three replicates. Letters represent whether there are differences between values ($P < 0.05$).

diversity and Margalef's species richness for fouling diatom communities on the glass slides after 14 and 21 days exposure to sea water (Fig.3). Lower diversity and species richness were observed in the spring and

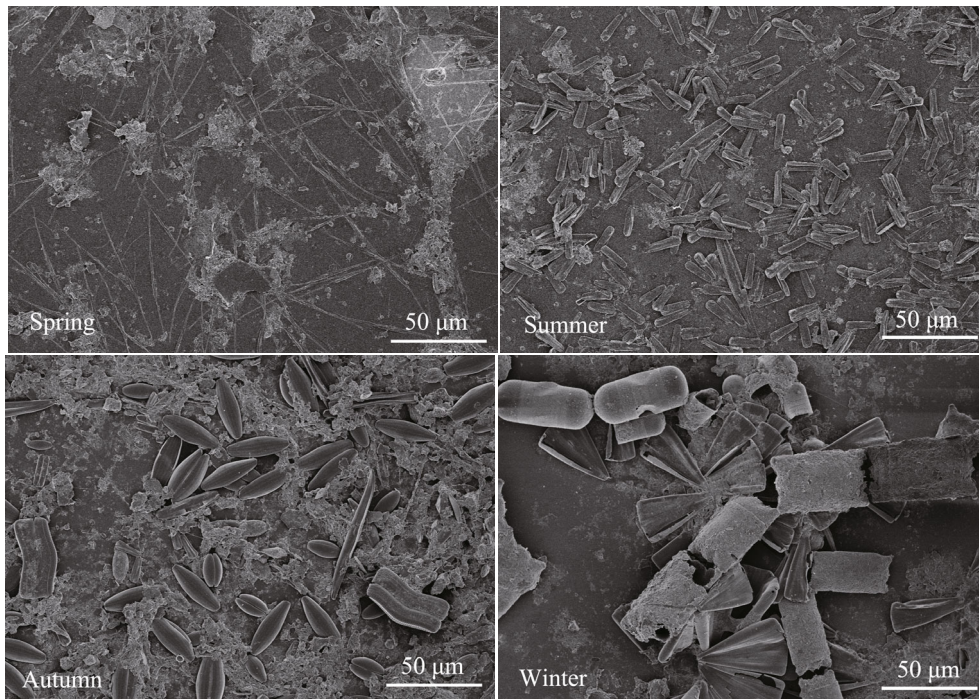


Fig.4 Development of fouling diatom community in each season

In spring, *Cylindrotheca* was widely distributed on the slides. The diatom compositions were almost constant in summer and autumn, and a single species was aggregated on the slides. The species were mainly *Gomphonema*, *Nitzschia*, *Amphora*, *Cocconeis*, *Navicula*, *Achnanthes* in summer and autumn. The main species in winter were *Licmophora* and *Biddulphia*.

winter. In summer and autumn, the indices of species richness and diversity increased. The genera *Cylindrotheca*, *Achnanthes*, *Nitzschia*, *Navicula*, *Cocconeis*, *Licmophora*, *Gomphonema*, and *Amphora* were the principle fouling diatoms on the substrate and different dominant species were found in different seasons. *Cylindrotheca* sp. was widely distributed on the slides, especially in spring. In summer and autumn, the species composition of the attached diatoms was constant and species such as *Gomphonema*, *Amphora*, *Cocconeis*, *Nitzschia*, and *Navicula* were dominant. The abundance of diatoms was very low in winter and the main species were *Licmophora* and *Biddulphia* (Fig.4).

3.3 Biology of the fouling diatom *Cylindrotheca* sp.

Cylindrotheca sp. was isolated from the samples in spring and was the dominant species. After morphological observation (light microscopy and SEM) and 18S rDNA sequence fragment structure identification (GenBank accession number KF698630), the algal strain was identified as *Cylindrotheca closterium*. The diatom was purified and cultured with an f/2 medium in the laboratory and the growth curve was determined (Fig.5a). The

specific growth rate was reached in 0.56/days (Fig.5b), which was higher than that of three other diatoms *P. tricornerutum*, *S. costatum* and *N. closterium* f. *minutissima* under the same culture conditions as in our previous experiment (Yang et al., 2012, 2013).

4 DISCUSSION

Surfaces immersed in seawater were rapidly coated by biofilm, which consisted of bacteria and diatoms. Diatoms were the earliest eukaryotic colonizer during biofouling formation. The attached diatoms played a significant role in the development of the subsequent macrofouling community (Joint et al., 2002; Huang et al., 2007; Qian et al., 2007). Only a few studies have focused on the community of biofilm diatoms in the marine environment (Molino and Wetherbee, 2008; Sweat and Johnson, 2013). Recent reports covered the influence of substrate properties and anti-fouling coatings on the diatom community (Cao et al., 2011; Dobretsov and Thomason, 2011; Zargiel et al., 2011; Dobretsov et al., 2013). In this experiment, a preliminary study of fouling diatom communities was carried out to reveal the formation process, community structure, seasonal variation and dominant species on the Yantai coast, China.

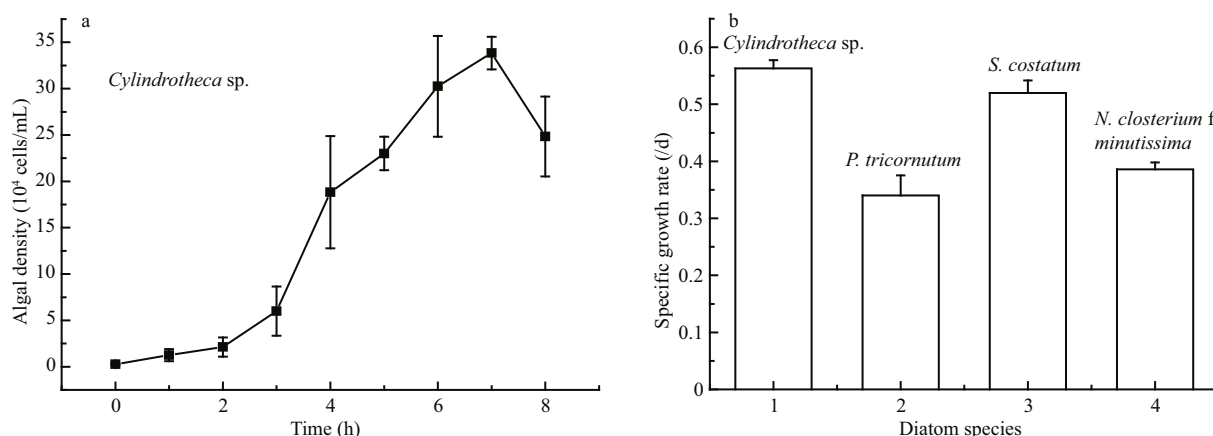


Fig.5 Growth and the specific growth rate of diatoms

a. Growth curve of *Cylandrotheca* sp.; b. specific growth rate of diatoms. 1. *Cylandrotheca* sp.; 2. *Phaeodactylum tricorutum* 3. *Skeletonema costatum* 4. *Nitzschia closterium* f. *minutissima*. The diatoms of *P. tricorutum*, *S. costatum*, and *N. closterium* f. *minutissima* come from our previous reports (Yang et al., 2012, 2013), and these diatoms were cultured in the same conditions in the laboratory.

Our results showed that the diatom community structure differed considerably during different seasons and that pennate diatoms were widespread in the biofilm. Previous literature also suggested that pennate diatoms dominated in diatom fouling communities irrespective of the nature of the substratum and the exposure period. Patil and Anil (2005b) reported that the dominance of pennate diatoms in biofilm had also been found at Dona Paula Bay on the west coast of India and the main species belonged to the genera *Navicula*, *Amphora*, *Nitzschia*, *Pleurosigma*, and *Thalassionema*. The biofilm diatom community structure showed significant seasonal variations, which were attributed to physico-chemical and biological changes in both the water and substratum. In the present study, the density and diversity of diatoms gradually increased over the slide suspension period. Figure 1 shows low numbers of attached diatoms at the initial 3-day sampling. After 2 weeks, both the diatom numbers and EPS significantly increased with the exposure period and complex diatom communities were formed. With the same exposure period, the adhered diatom number varied with the seasons and the dominant species were different in different seasons, which implied that the external environmental factors influenced the diatom community structure, for example temperature, hydrodynamics, organisms in the seawater etc.

According to the Chinese marine environment report, the average sea temperatures in our sample exposure period ranged from 6.9 to 10.9°C in spring, from 23.9 to 25.3°C in summer, from 18.8 to 20.7°C in autumn, and from 2.3 to 7.9°C in winter. The lowest temperature was in winter and the algal density was

also the lowest among the four seasons. The suitable temperature for the growth of diatoms was about 20°C (Cao et al., 2011; Leflaive and Ten-hage, 2011). The temperatures of summer and autumn met the optimal growth of fouling diatoms. However, the algal density in spring was higher than that of summer and autumn. Yantai entered the rainy season in summer and autumn and encountered rainy days, and windy weather in autumn during the sampling period. The wind and rain definitely affected the attachment amount of fouling diatoms. The influence of season on the fouling diatom communities was also reported and the results showed that the maximum biofilm diatom population was found during the calm period (post- and pre-monsoon) rather than during the highly disturbed monsoon period, which suggested that water flow conditions might play an important role in diatom community structure (Patil and Anil, 2005b). Summer and autumn were suitable for the growth of both algae and larger organisms, and many animals feed on algae, which might also affect the algae adhesion amount. *Cylandrotheca* sp. was the dominant diatom and was widely distributed on the slides in spring. In this experiment, *Cylandrotheca* sp. was purified and cultured in the laboratory. It was found that the growth and proliferation rate of this diatom was more rapid than three other common diatoms (Fig.5b) (Yang et al., 2012, 2013). However, both the indices of algal diversity and species richness in summer and autumn were higher than that of spring and winter, which suggested that summer and autumn were more suitable for the growth and reproduction of the algae than the other two seasons.

The pennate diatoms *Cylandrotheca*, *Nitzschia*,

Navicula, *Amphora*, *Achnanthes*, *Gomphonema*, and *Licmophora* were dominant genera in the fouling biofilm on the glass slides in the study area. *Cylindrotheca* sp. dominated on the slides in spring. Dobretsove and Thomason (2011) also reported that the diatom *Cylindrotheca* sp. dominated on non-biocidal coatings in the Marina Bandar al Rowdha (Muscat, Sea of Oman). The genera *Amphora*, *Navicula*, *Nitzschia*, *Licmophora*, and *Cylindrotheca* were dominant in marine biofilms developed on different substrata (Zargiel et al., 2011; Briand et al., 2012). In this study, the dominant algal species were found to be different in different seasons (Fig.4). There were smaller diatoms about 20–50 µm in length as dominant genera in summer and autumn, such as *Gomphonema*, *Amphora*, and *Achnanthes* and these diatoms were commonly distributed in an aggregated state of a single species. The aggregated diatom community may originate from a single algal cell, which adapts to survive in the microenvironment on the slides and begins to divide, until the diatom completely dominates localized areas on the surfaces of the substrate. Molino et al. (2009) concluded that benthic diatoms presented in the plankton could randomly encounter and adhere to a solid surface by observing the spatial distribution of diatoms directly on the surface in situ. The model of diatom colonization on substrate surfaces could be expressed using similar principles to those in an “infection” type epidemiological model. Once attached, the algal cells began division, exponentially increasing in number in a suitable area of the substratum. Subsequently, algal cells might glide across the solid surface, colonize new areas until the whole surface is fully occupied. Therefore, diatoms were found clustered together in a large numbers for some species. The dominant diatoms were significantly different in winter from the other three seasons in this study area and the main genera were *Licmophora* and *Biddulphia* (Fig.4). The results indicated that there was a seasonal change in the diatom community. Similar reports found that the fouling diatom community was significantly influenced by seasonal variations at the mouth of the Zuari estuary, Goa, on the west coast of India. *Navicula delicatula* dominated during the post-monsoon and *Melosira* and *Odontella* were observed during the monsoon. Low diatom abundance was recorded during the pre-monsoon season (Mithavkar and Anil, 2008). Seasonal variations in the diatom community depend on the physical, chemical and biological interactions.

5 CONCLUSION

The present study reported the seasonal variation in fouling diatom community structure on the Yantai coast, Shandong, China for the first time. A significant difference in density and diversity of diatoms on the glass slides was observed during the four seasons. However, the species composition remained almost the same in summer and autumn. Pennate diatoms dominated in the biofilm showed temporal variation. The obvious seasonal differences are related to external environmental factors, which might help determine the formation of subsequent fouling communities.

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