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Source of propagules of the fouling green macroalgae in the Subei Shoal, China

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Abstract

Since 2007, large-scale green tides dominated by *Ulva prolifera* consecutively bloomed in the Yellow Sea and caused great economic losses. The fouling *U. prolifera* on the *Pyropia yezoensis* aquaculture rafts in the Subei Shoal was regarded as the major source of the floating biomass. However, it was still unclear about the seed source of fouling green macroalgae attached on the rafts. In this study, the field surveys and the indoor experiments were conducted to reveal the source of propagules of the fouling green macroalgae on the rafts and to study the anti-fouling material for *P. yezoensis* aquaculture rafts which could possibly be a feasible strategy to control the green tides in the Yellow Sea. The results showed that (1) micro-propagules of several green macroalgal species, including *U. prolifera*, *U. linza*, *U. compressa*, *U. flexuosa*, and *Blidingia* sp. coexisted in the waters and sediments in the Subei Shoal and their proportion remarkably changed over time; (2) the bamboo poles with peeling treatment could significantly reduce the amount of *U. prolifera* micro-propagules attached. This study confirmed that the micro-propagules distributed in the Subei Shoal area were the precursors of the green tides, and provided a feasible method to control the Yellow Sea large-scale green tides at the beginning.

Key words: green tides, source of propagules, *Ulva prolifera*, anti-fouling

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1 Introduction

In the past nine consecutive years, the large-scale green tides dominated by the green macroalgae *Ulva prolifera* occurred in the Yellow Sea and disturbed the coastal cities of Shandong Peninsula (Liu et al., 2013a, b). The local government spent lots of manpower and material resources to deal with this ecological disaster (Ye et al., 2011; Liu et al., 2010, 2013b). The remote sensing data based on Moderate Resolution Imaging Spectroradiometer (MODIS) and the field observations indicated that the *U. prolifera* in the fouling green macroalgal wastes from the *Pyropia yezoensis* aquaculture rafts in the Subei Shoal was the major source of the blooms (Liu et al., 2009; Keesing et al., 2011; Li et al., 2015; Wang et al., 2015).

The Subei Shoal located on the shelf of the southwestern Yellow Sea is composed with a large amount of radial sand ridges. It is exposed at low tide while submerged at high tide (Liu et al., 2010). It is the largest *P. yezoensis* aquaculture base in China, covering an area of over 3.8×10^4 hm^2 (Liu et al., 2013a). The *P. yezoensis* aquaculture raft consists of two bamboo poles, two ropes and one nursery net (Liu et al., 2009, 2010). During the *P. yezoensis* aquaculture period from September to the next May, the green macroalgae colonized the aquaculture rafts, and the fouling biomass as well as the species composition changed (Li et al., 2014; Fan et al., 2015; Wang et al., 2015). At the harvest season (April to May), *U. prolifera* became one of the most dominant species on the rafts, and was proven to be the source of the blooms (Fan et al., 2015; Song et al., 2015b; Wang et al., 2015). However, the propagules source of the rafts-fouling green macroalgae was still unclear. In our previous study, we found that the green macroalgal micro-propagules existed in the waters and sediments in the Subei Shoal area throughout a year and showed remarkable temporal and spatial variations (Song et al., 2015a). We hypothesized that they were the "seed source" of the rafts-fouling green macroalgae and could serve as the precursor of the green tides in summer. However, in that study, we did not conduct the species identification of the micro-propagules and thus could not confirm this hypothesis. In addition, the green macroalgal micro-propagules had strong adaptive capability to harsh environmental conditions (Zhang et al., 2011; Liu et al., 2012). When the *P. yezoensis* aquaculture activities were over, the farm-

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ers recycled the rafts and a large amount of micro-propagules attached on the ropes and bamboo poles (Song et al., 2015a). We were not sure that whether the attached micro-propagules could also serve as the source of the rafts-fouling green macroalgae in the next aquaculture cycle.

In order to control the blooms of the Yellow Sea large-scale green tides, studies reasoned that preventing the adhesion and germination of *U. prolifera* micro-propagules would be an effective method (Zhang et al., 2011). Studies have found that some materials, like rubber and mud, could play well anti-fouling effect (Geng et al., 2015). In addition, according to our continuous field survey data of the rafts-fouling green macroalgae from 2009 to 2015, we found that on the two ends of the bamboo poles (rough surfaces, without bark), the fouling macroalgae were almost all composed by *U. linza*, and few *U. prolifera* were detected in this part. While on the other parts of the poles (smooth surfaces, with bark), the species composition had no significant differences compared with that on the ropes (Song et al., personal observation). Thus, we assumed that the bamboo poles without bark could also prevent the adhesion and germination of *U. prolifera* micro-propagules.

The objectives of this study were (1) to confirm the propagules source of the fouling green macroalgae on the *P. yezoensis* aquaculture rafts, and (2) to clarify whether the bamboo poles without bark could be used as potential "anti-fouling" substances.

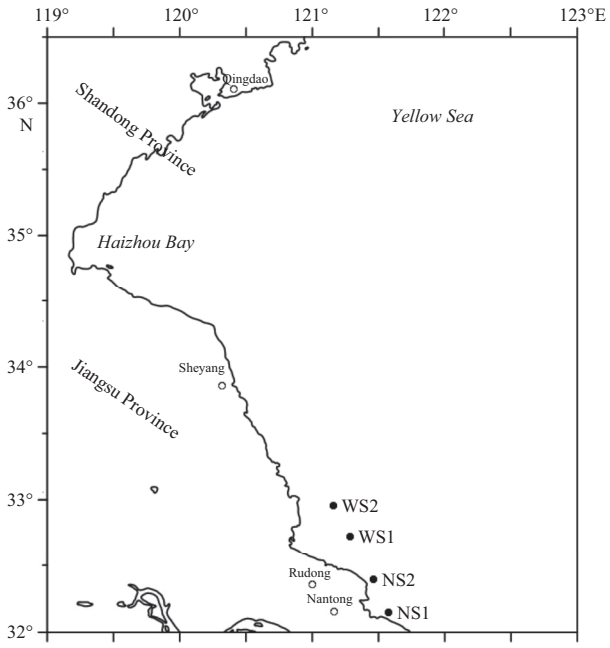

Fig. 1. Map showing survey sites in the Subei Shoal. Sites NS1 and NS2 were located in the Neisha area of the Subei Shoal, and WS1 and WS2 were located in the Waisha area of the Subei Shoal.

2 Materials and methods

2.1 *Selection of sampling locations and sample collection*

The sampling area is located in the coastal area of the Subei Shoal in the southern coastline of Jiangsu Province (Fig. 1). According to the distribution of *P. yezoensis* aquaculture rafts, from south to north, four stations including NS1 (32°08′40″N, 121°35′00″E), NS2 (32°23′52″N, 121°27′40″E), WS1 (32°43′23″N, 121°16′57″E), and WS2 (32°58′08″N, 121°09′20″E) were selected for study (Fig. 1). From March to May 2014, three cruises were carried out (Table 1). Water samples, sediment samples, and rafts-fouling green macroalgae samples were collected at each sampling station every month.

2.2 *Cultivation and quantification of green macroalgae micro-propagules*

The micro-propagules in the water and sediment samples were cultivated and quantified using the method described by Song et al. (2015a). The sea water sample (1 L) was pre-mixed and cultured in a glass beaker with 20 mL PES medium and 1 mL of saturated GeO_2 (n=4). The beakers were maintained at 20°C under 100 μmol photons m^{-2} s^{-1} in artificial climatic chamber (202728-380, Jiangnan Inc., Ningbo, China) and the light regime was 12-h light per day. In order to maintain constant nutrients level, the PES medium was renewed every 5 d. A standard wet weight of 1 g sediment sample was transferred into a 1-L glass beaker containing 1 L sterile seawater and cultured under the same conditions as the seawater samples. After 15 d, the micro-propagules in the waters and sediments developed into approximately 1–3 cm germlings. They could be counted with the naked eyes and the number of the germlings was considered as the quantity of micro-propagules (Liu et al., 2013b). In each cruise, the number of the micro-propagules in the four sampling stations was used to calculate the quantity of the green macroalgal micro-propagules in the Subei Shoal in that month.

2.3 *Separation and identification of the raft-fouling green macroalgae*

The green macroalgal samples collected in each cruise were rinsed with sterile seawater, and then placed on white enamel discs. The thalli were separated and identified according to their morphological characteristics (Blomster et al., 1999; Kraft et al., 2010; Fan et al., 2015). In order to verify the morphological identification results, we have also selected some of the thalli, and conducted molecular identification. Then the surface of the thalli were dried, and weighed using a high-accuracy electronic balance (PL203, METTLER TOLEDO Inc., Zurich, CH). The wet weights of all of the green macroalgal species in the four sampling stations were used to calculate the proportion of their fouling biomass in the *P. yezoensis* aquaculture rafts in the Subei Shoal.

2.4 *Green macroalgal micro-propagules attachment experiments*

In order to ascertain the source of the fouling green macroal-

Table 1. Information on sampling stations and dates

	NS1	NS2	WS1	WS2
Location coordinates	32°08′40″N	32°23′52″N	32°43′23″N	32°58′08″N
	121°35′00″E	121°27′40″E	121°16′57″E	121°09′20″E
Sampling date	March 24	March 26	March 30	March 29
	April 28	April 28	April 29	April 30
	May 19	May 21	May 22	May 23

gae on the *Pyropia* aquaculture rafts and whether the bamboo poles without epidermis could prevent the adhesion of *U. prolifera* micro-propagules, we selected WS1 station and set up several "aquaculture rafts" without the nursery net. The "rafts" were composed by two kind of ropes (Rope A and Rope B) and two kinds of bamboo poles (Pole A and Pole B). Rope A was the new rope with a smooth surface, and Rope B was the old rope used in the previous *Pyropia* aquaculture cycle. Pole A was the common bamboo pole used in the previous aquaculture cycle, and Pole B was the bamboo pole after peeling treatment. This experiment was conducted from April 22 to 28, 2014. Then, the ropes and bamboo poles were collected, and transported to the laboratory for the cultivation and quantification of micro-propagules, using the method described by Song et al. (2015a).

2.5 *DNA extraction, PCR amplification, sequencing, and phylogenetic analysis*

According to the number of the germlings developed from micro-propagules in the seawaters, sediments, ropes and bamboo poles (as indicated in results), the germling samples were selected (Table 2). Based on the morphological characteristics, the five rafts-fouling green macroalgae samples (MTA-1, MTA-2, MTA-3, MTA-4, MTA-5) were classified to be *U. prolifera*, *U. linza*, *U. compressa*, *U. flexuosa* and *Blidingia* sp. respectively. While for the germling samples, we could only use the molecular identification methods (Song et al., 2015b).

The E.Z.N.A.TM HP plant DNA kits (OMEGA Bio-tek Inc., GA, USA) were used to extract the total genomic DNA of the samples. We used the primers pair: ITS-F (5′-TCGTAACAAGGTTTCCGTAGG-3′) and ITS-R (5′-GCTTATTGATATGCTTAAGTTCAGCGGGT-3′) designed by Leskinen and Pamilo (1997) to amplify the sequences of ITS nrDNA (including ITS1, 5.8S, ITS2 regions). While for *U. prolifera* and *U. linza* which could not be separated by ITS sequence polymorphism (Xiao et al., 2013), the 5S rDNA spacer sequences were amplified using the primers pair: *Ulva* 5S-F (5′-GGTTGGGCAGGATTAGTA-3′) and *Ulva* 5S-R (5′-AGGCTTAAGTTGCGAGTT-3′) (Shimada et al., 2008). The PCR products of ITS nrDNA sequences and 5S rDNA spacer sequences were respectively checked by electrophoresis in 1% and 3% agarose gel. The targeted DNA bands were purified using E.Z.N.A. TM Gel Extraction Kits (OMEGA Bio-tek Inc., GA, USA). The purified DNA was sequenced at both directions in Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). Two Neighbor-joining trees (NJ tree) were constructed using Mega 5.10 based on the sequences of the ITS nrDNA and 5S rDNA spacer regions, respectively.

2.6 *Statistics*

The data were analyzed by a two-way analysis variance (ANOVA). The difference among means was analyzed by Duncan's new multiple range test followed an ANOVA with a significance level of $P<0.05$. The tests were performed using the SPSS 17.0 statistical program (SPSS Inc., Chicago, USA).

3 Results

3.1 *Variations in quantity and species composition of the micro-propagules in the Subei Shoal*

The number of green macroalgal micro-propagules distributed in the seawaters and sediments changed obviously during the cruises ($P<0.05$, Fig. 2). In the water column, the number of micro-propagules peaked in the late April ($P<0.05$), while in the sediments samples, the number reached to the maximum in the middle May ($P<0.05$). The green macroalgal micro-propagules distributed in the Subei Shoal area were composed by five green macroalgal species, including *U. prolifera*, *U. linza*, *U. compressa*, *U. flexuosa* and *Blidingia* sp. (Figs 3 and 4). According to this result, the proportion of various micro-propagules during spring 2014 was calculated (Fig. 5). The micro-propagules were dominated by *Ulva* species, and the proportion of various species micro-propagules remarkably changed over time.

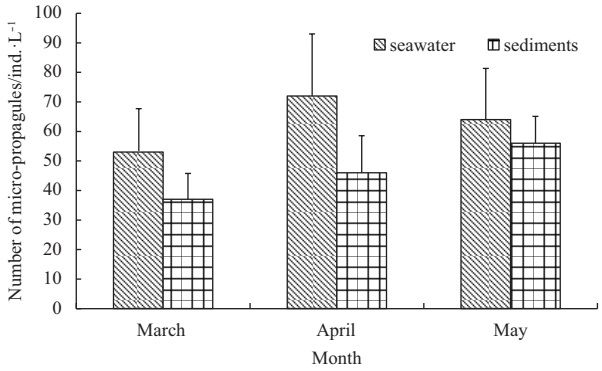

Fig. 2. Germinated number of green macroalgal micro-propagules in the sea water and sediments collected from the Subei Shoal after 15 d of culture. The means and SE are shown (n=16).

3.2 *Species composition of the fouling green macroalgae on the rafts*

The molecular identification results showed that we have ac-

Table 2. Selected rafts-fouling green macroalgae and the germlings developed from the micro-propagules used for phylogenetic analysis

Sample source	Sample
Rafts-fouling green macroalgae	MTA-1, MTA-2, MTA-3, MTA-4, MTA-5
Germlings developed from micro-propagules in the seawaters	MPW3-1, MPW3-2, MPW3-3, MPW3-4, MPW3-5, MPW3-6, MPW3-7, MPW3-8, MPW3-9, MPW3-10, MPW3-11; MPW4-1, MPW4-2, MPW4-3, MPW4-4, MPW4-5, MPW4-6, MPW4-7, MPW4-8, MPW4-9, MPW4-10, MPW4-11, MPW4-12, MPW4-13, MPW4-14, MPW4-15; MPW5-1, MPW5-2, MPW5-3, MPW5-4, MPW5-5, MPW5-6, MPW5-7, MPW5-8, MPW5-9, MPW5-10, MPW5-11, MPW5-12, MPW5-13
Germlings developed from micro-propagules in the sediments	MPS3-1, MPS3-2, MPS3-3, MPS3-4, MPS3-5, MPS3-6, MPS3-7, MPS3-8; MPS4-1, MPS4-2, MPS4-3, MPS4-4, MPS4-5, MPS4-6, MPS4-7, MPS4-8, MPS4-9, MPS4-10; MPS5-1, MPS5-2, MPS5-3, MPS5-4, MPS5-5, MPS5-6, MPS5-7, MPS5-8, MPS5-9, MPS5-10, MPS5-11
Germlings developed from attached micro-propagules in Rope A and Rope B	MPRA-1, MPRA-2, MPRA-3, MPRA-4, MPRA-5, MPRA-6, MPRA-7, MPRA-8, MPRA-9, MPRA-10, MPRA-11, MPRA-12; MPRB-1, MPRB-2, MPRB-3, MPRB-4, MPRB-5, MPRB-6, MPRB-7, MPRB-8, MPRB-9, MPRB-10, MPRB-11, MPRB-12
Germlings developed from attached micro-propagules in Pole A and Pole B	MPPA-1, MPPA-2, MPPA-3, MPPA-4, MPPA-5, MPPA-6, MPPA-7, MPPA-8, MPPA-9, MPPA-10; MPPB-1, MPPB-2, MPPB-3, MPPB-4, MPPB-5, MPPB-6, MPPB-7, MPPB-8, MPPB-9, MPPB-10

Fig. 3. Phylogenetic tree determined by analysis of the ITS nrDNA region including the 5.8S gene of *Ulva* and *Blidingia* species. The numbers at internal nodes were bootstrap values greater than 50% for 1 000 replicates in Neighbor-joining analysis.

curately separated the rafts-fouling green macroalgae according to their morphological characteristics (Figs 3 and 4). As shown in Fig. 6, the species composition and the proportion of the green macroalgal thalli also changed obviously over time. Before the recycling of the *P. yezoensis* aquaculture rafts, the fouling *U. prolifera* became the second dominated species.

3.3 *Quantity of micro-propagules attached on the ropes and bamboo poles*

On the two kinds of ropes, the number of the attached micro-propagules did not showed obvious differences (P>0.05), while in the control group (rope samples without the green macroalgal micro-propagules attachment experiment in the Subei Shoal area), no germlings were detected (Fig. 7). As shown in Figs 3 and 4, the species composition of the attached micro-propagules on these two kinds of ropes was not significantly different either. The quantity of the attached micro-propagules on the two kind of bamboo poles showed the same trend as that on the ropes (Fig. 8). However, the species composition was remarkably different (Fig. 4). The proportion of *U. linza* micro-propagules on Pole B reached to nearly 90%.

4 Discussion

4.1 *Species diversity of green macroalgal micro-propagules in the Subei Shoal*

The results showed that the quantity of the green macroalgal micro-propagules in the coastal area of the Subei Shoal showed obviously temporal variations, which was consistent with our previous study (Song et al., 2015a). In addition, we found that several micro-propagules species coexisted in this area and their proportion remarkably changed. They could serve as the “banks of macroalgae in microscopic forms” in the Subei Shoal (Hoffmann and Santelices, 1991; Santelices et al., 1995), and if at the favorable germination temperature, they could attach to suitable adhesion substrate and develop into the mature thalli (Song et al., 2015b). Some previous studies indicated that the green macroalgal micro-propagules distributed in the Jiangsu coastline were only composed by *Ulva* species (Liu et al., 2012, 2013b). However, our studies showed that *Blidingia* species also existed

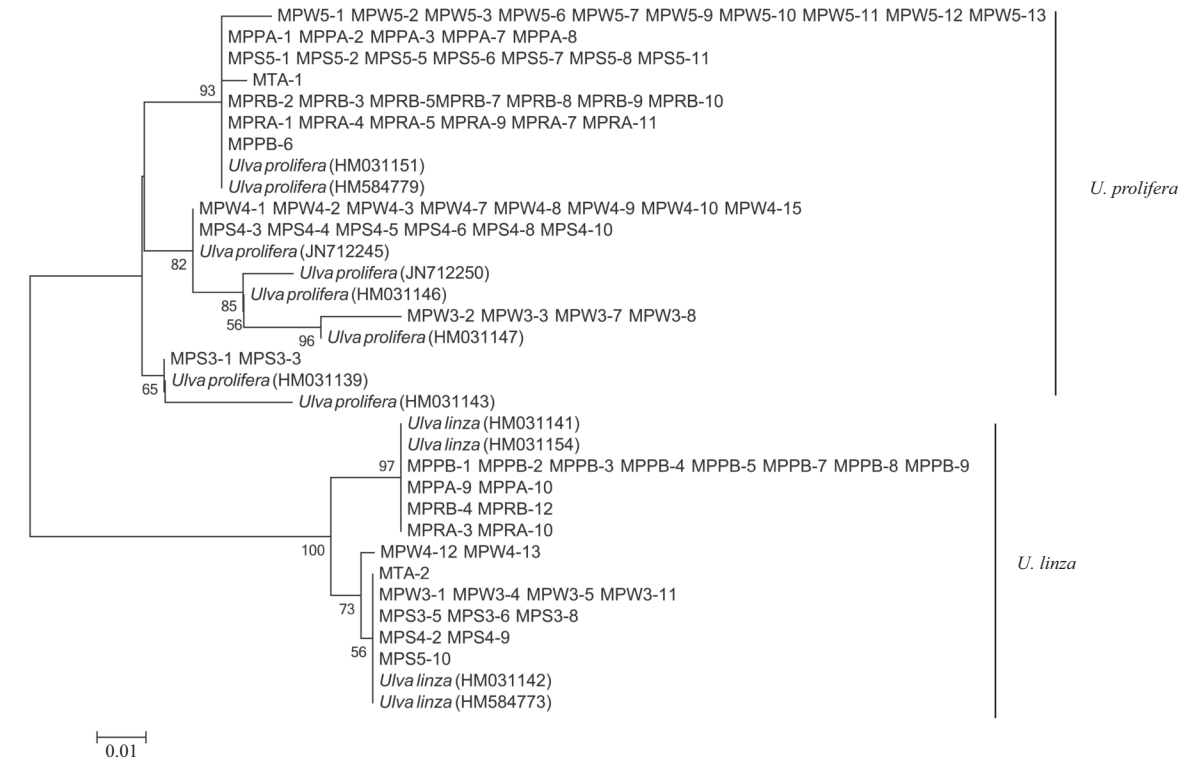

Fig. 4. Neighbor-joining tree determined by the analysis of 5S spacer sequences of *U. linza* and *U. prolifera*. The bootstrapping support values larger than 50% after 1 000 replicates were given aside each node.

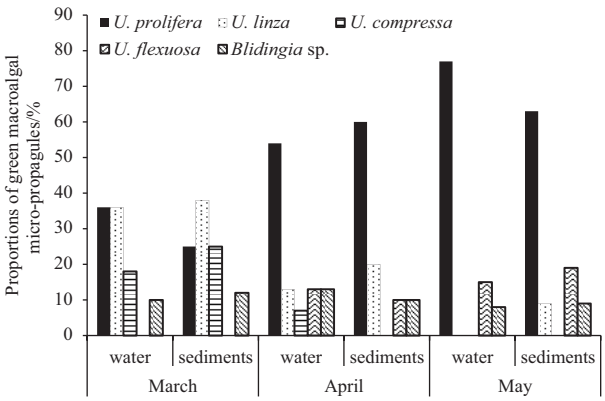

Fig. 5. Proportion of *U. prolifera*, *U. linza*, *U. compressa*, *U. flexuosa* and *Blidingia* sp. in the seawaters and sediments in the Subei Shoal from March to May.

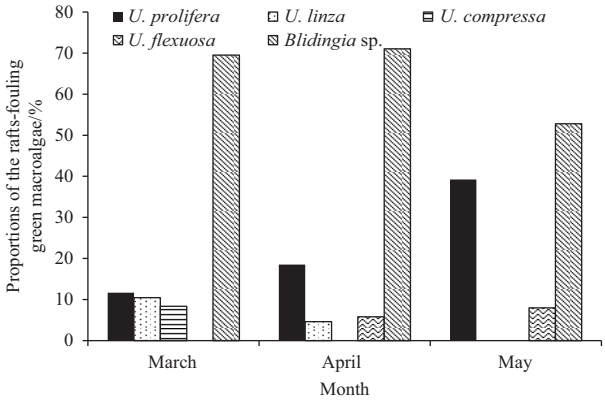

Fig. 6. Changes of the proportion of the rafts-fouling green macroalgal assemblage in the Subei Shoal from March to May.

in the micro-propagules community and could serve as the source of the rafts-fouling green macroalgae (Song et al., 2015b; this study).

4.2 *Propagules source of the rafts-fouling thalli*

In the past, we speculated that the green macroalgal micro-propagules attached on the recycled *P. yezoensis* aquaculture rafts some months ago could also serve as the propagules source of the rafts-fouling green macroalgae because they had strong tolerance capability to harsh environmental conditions (Agrawal, 2009; Liu et al., 2012). While in this study, we found that no green macroalgal germling was detected in the control group, although there were apparent green specks on the surfaces of Rope B and Pole A (Song et al., personal observation). This result could also indicate that the traditional treatments of *P. yezoensis* aquaculture rafts could completely kill the attached green macroalgae and the micro-propagules.

In the treatment group, the quantity and species composition of the attached green macroalgal micro-propagules on Rope A and Rope B did not show remarkable differences. This indicated that these two kinds of materials had no definite selectivity to the attachment and germination of various green macroalgal micro-propagules and could not serve as the materials to prevent the fouling green macroalgae on the rafts (Geng et al., 2015). In addition, we found that the species composition of the micro-propagules on these ropes and in the seawaters and sediments in April were generally same, which suggested that micro-pro-

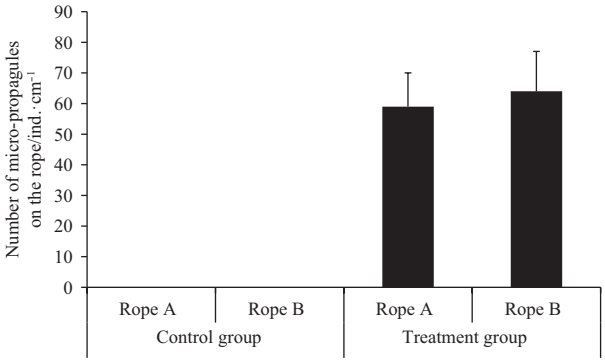

Fig. 7. Germinated quantities of green macroalgal micro-propagules in the two kinds of ropes after micro-propagules attachment experiments in Sta. WS1. Rope A was the new rope with a smooth surface; Rope B was the old rope used in the previous *P. yezoensis* aquaculture cycle. The means and SE are shown (n=4).

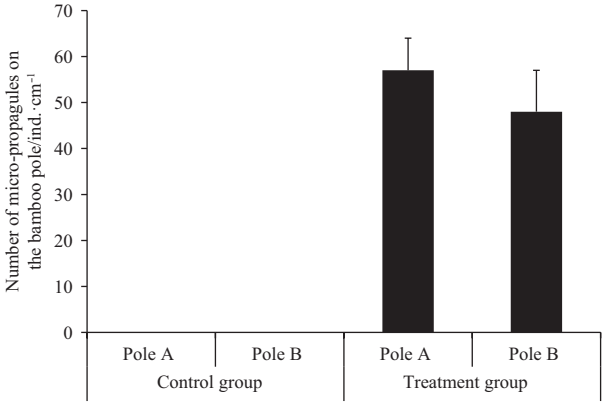

Fig. 8. Germinated quantities of green macroalgal micro-propagules in the two kinds of bamboo poles after micro-propagules attachment experiments in Sta. WS1. Pole A was the common bamboo pole used in the previous *P. yezoensis* aquaculture cycle; Pole B was the bamboo pole after peeling treatment. The means and SE are shown (n=4).

pagules distributed in the seawaters and sediments in the Subei Shoal area were the propagule-source of the rafts-fouling green macroalgae.

The results in this study showed that the biomass as well as the species composition of the rafts-fouling green macroalgae changed obviously in the Subei Shoal from March to May, and further validated the community succession on the rafts (Fan et al., 2015). For the *Blidingia* sp., it needs further indoor and *in-situ* research to explain that why it always occupied the highest fouling biomass while was the lowest proportion in the green macroalgal micro-propagules community. However, in the fouling green macroalgal assemblage and in the micro-propagules community, the species composition was the same, which could also indicate that the propagule-source of the rafts-fouling green macroalgae were the micro-propagules distributed in the seawaters and sediments in the Subei Shoal.

4.3 *Prevention the attachment and germination of U. prolifera micro-propagules*

Geng et al. (2015) found that rubber had a strong inhibitory effect on the attachment and germination of *U. prolifera* micro-propagules, and thus believed that it could serve as ideal potential anti-fouling material for mitigation of green tides. However, the *P. yezoensis* aquaculture activities in the Subei Shoal area have lasted for more than 30 years (Liu et al., 2013a). It will undoubtedly increase the farmers' aquaculture cost if they select new materials to build the aquaculture rafts. Moreover, using "rubber rafts" to conduct the aquaculture activities might also influence the quality of the *P. yezoensis*, and caused more serious losses.

In this study, we found that the bamboo poles after simply peeling treatment could also well inhibit the attachment and germination of *U. prolifera* micro-propagules. In addition, this material had definite selectivity to green macroalgal micro-propagules in various species. Thus, we speculated that if the farmers use this kind of bamboo poles to build *P. yezoensis* aquaculture rafts in the Subei Shoal, there will be large amount of *U. linza* attached on the rafts. The increasing of the fouling biomass of *U. linza* might also affect the biomass of the attached *U. prolifera* (Fan et al., 2015; Song et al., 2015b), and play effective roles for mitigation of green tides. Furthermore, the *U. linza* has high edible and medicinal values (Fleurence, 1999; Wong and Cheung, 2000). They were also cultured as the economic seaweeds in some areas of Zhejiang Province, China (He et al., 2006). Thus, if the farmers selected this kind of bamboo poles, during the *P. yezoensis* aquaculture period, maybe they can also acquire extra benefits.

5 Conclusions

The results showed the green macroalgal micro-propagules in *Ulva* and *Blidingia* genera coexisted in the propagules bank in the Subei Shoal and serve as the source of the rafts-fouling green macroalgae. The bamboo poles after peeling treatment had definite selectivity to the attachment and germination of green macroalgal micro-propagules in various species and could serve as ideal potential anti-fouling material for mitigation of green tides.

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