Marine Environmental Research 83 (2013) 38–47

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Understanding the recurrent large-scale green tide in the Yellow Sea: Temporal and spatial correlations between multiple geographical, aquacultural and biological factors

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ARTICLE INFO

Article history:
Received 19 August 2012
Received in revised form
15 October 2012
Accepted 21 October 2012

Keywords:
Ulva prolifera
Algal blooms
Sediment
Eutrophication
Radial shoals
Coastal animal aquaculture ponds

ABSTRACT

The coast of Jiangsu Province in China – where *Ulva prolifera* has always been firstly spotted before developing into green tides – is uniquely characterized by a huge intertidal radial mudflat. Results showed that: (1) propagules of *U. prolifera* have been consistently present in seawater and sediments of this mudflat and varied with locations and seasons; (2) over 50,000 tons of fermented chicken manure have been applied annually from March to May in coastal animal aquaculture ponds and thereafter the waste water has been discharged into the radial mudflat intensifying eutrophication; and (3) free-floating *U. prolifera* could be stranded in any floating infrastructures in coastal waters including large scale *Porphyra* farming rafts. For a truly integrated management of the coastal zone, reduction in nutrient inputs, and control of the effluents of the coastal pond systems, are needed to control eutrophication and prevent green tides in the future.

1. Introduction

In the Yellow Sea, large-scale green tides have broken out for six consecutive years from 2007 to 2012 (Jiang et al., 2008; Leliaert et al., 2009; Liu et al., 2012a). Intensive field investigations have revealed that the causative free-floating green alga, *Ulva prolifera*, is distributed broadly in various niches along Jiangsu Province coast – including *Porphyra* rafts (Liu et al., 2009, 2010a; Keesing et al., 2011), coastal animal aquaculture ponds (CAAPs) (Pang et al., 2010), and intertidal seawater and sediments in its microscopic propagule form (Liu et al., 2010d, 2012a). Satellite data based on Moderate Resolution Imaging Spectroradiometer (MODIS) from NASA data distribution system indicated that the south part of Jiangsu Province coast from Sheyang to Rudong was the initial region where the floating green-tide algae were always firstly spotted every year (Hu et al., 2010).

The coast of Jiangsu Province is uniquely characterized with radial sand ridges on the shelf of the southwestern Yellow Sea, expanding over 200 km from Sheyang estuary to Changjiang estuary and 90 km from shore to open sea. This exposed large mudflat covers nearly 22,740 km^2, forming a strikingly unique extended intertidal mudflat zone in the world (Li, 2011). These radial ridges constitute an ideal environment for the growth of the commercially important red seaweed, *Porphyra yezoensis* (nori) (Fig. 1). Cultivation of nori at a scale of roughly 21,000 ha has been located here. Annually, 126,000 tons of fresh nori biomass is harvested (Pang et al., 2010). On the shore, multiple species of marine animals – including juvenile freshwater crabs (larval stage in seawater), clams and shrimps – have been farmed in CAAPs. Among the farmed animals, rotifers, a crucial live feed for juvenile freshwater crabs, are farmed by use of the called “natural ecological mud-pond method” (NEMPM) (Xu and Zhang, 2009). This method was developed from 2001 onwards firstly in Panjin City of Liaoning Province in the north of China, and rapidly spread to Jiangsu Province coast thereafter. Today, rotifer ponds cover roughly 1,400 ha from Sheyang to Rudong regions along the Jiangsu Province coast (Fig. 1).

Regarding the original source(s) of the floating *Ulva* biomass, Liu et al. (2009, 2010a) hypothesized and later reiterated that they were from the nori nets and pointed out that the expansion of nori

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Abbreviations: CAAPs, coastal animal aquaculture ponds; FCM, fermented chicken manure; MODIS, Moderate Resolution Imaging Spectroradiometer; NEMPM, natural ecological mud-pond method.

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http://dx.doi.org/10.1016/j.marenvres.2012.10.007

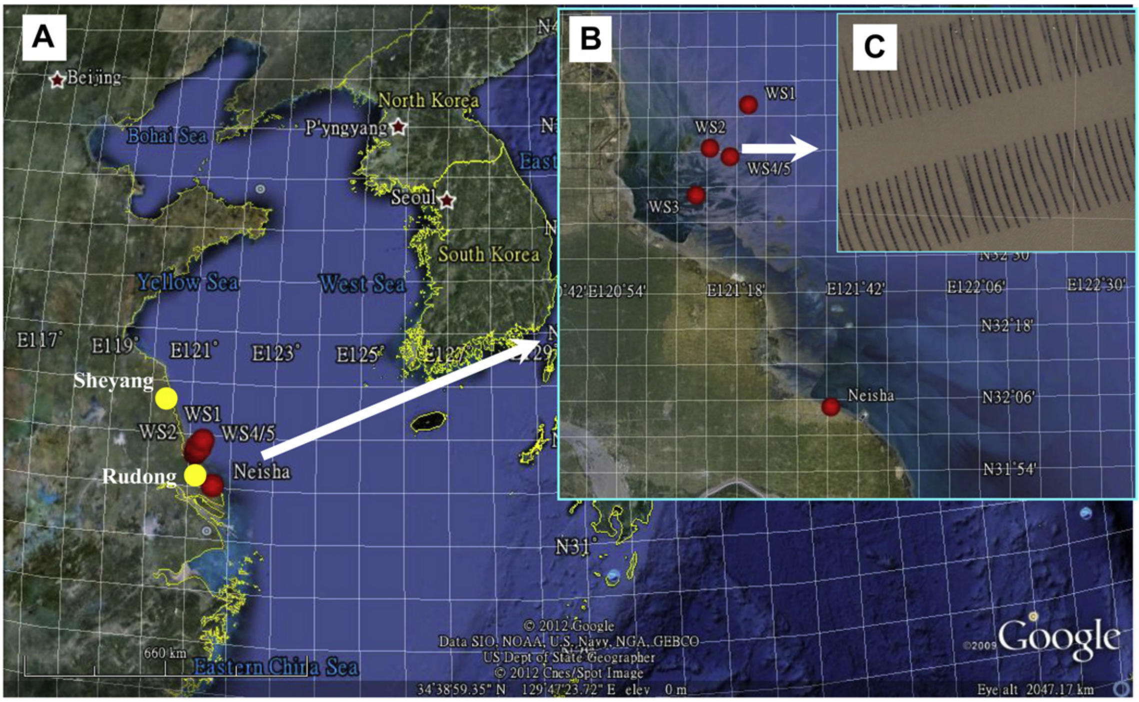

Fig. 1. (A and B) Maps of sampling locations in Jiangsu Province. (C) Close-up of *Porphyra* aquaculture rafts on the sandy shoals. The maps were obtained from the software Google Earth.

aquaculture along Jiangsu Province coast led to the occurrence of the green tides in the Yellow Sea. Our previous field sampling and identification revealed that the microscopic propagules of *U. prolifera* in the sediments and coastal waters of Jiangsu province's coast formed an overwintering "seed stocks". They constituted the precursor of the green tides in the spring in the Yellow Sea (Liu et al., 2010d, 2012a; Zhang et al., 2010, 2011). It is a well-known and widely accepted fact that blooming of green tides is closely related with eutrophication (Conley et al., 2009). However, it still remains a mystery why the *Ulva* blooms occurred along Jiangsu coast, and not along other eutrophicated coast of the south Yellow Sea given the fact that *U. prolifera* widely exists in the coastal marine environments. Furthermore, what are the local special/unique conditions that make it occurring continuously since 2007?

The objectives of this investigation were (1) to understand how and where the green tide algae initially started along the Jiangsu coast, and (2) to analyze the timing and spatial correlations of their occurrence with the particularly special geographical, aquacultural and biological factors of this area by employing field on-site net-installing experiments, laboratory cultures and molecular identifications.

2. Materials and methods

2.1. Selection of sampling locations

Waisha (WS), which represents the large area of mudflat, is periodically exposed during daily tidal events (Fig. 1A and B). A large part of the exposed sandy shoals in this mudflat are now used to install nori nets from November to May, which can easily be identified by satellite images (Fig. 1C). Four locations (WS1, WS2, WS3 and WS4/5) were selected in the main area of the radial shoal where the floating *Ulva* biomass was found each year. They represent the principal region of nori aquaculture in this area. Five cruises (WS1–WS5) were conducted from January 2011 to April 2012 (Table 1). A prerequisite for a shoal to be selected as a "land" to grow nori is that it must be fully exposed during low tides such that the nori on the nets could be exposed to air for several hours each

Table 1
Number of *Ulva* microscopic propagules in seawater and sediment samples collected from different locations in the Jiangsu Province radial shoals (Fig. 1), after 3 or 4 weeks of aerated culture in nutrient-enriched seawater at 18° C under 100 μmol photons m^{-2} s^{-1} in a 12 h light per day light–dark regime. Means and SE are shown ($n = 4$).

Location	Geographical coordinate	Collecting date	Surface water temperature (°C)	*Ulva* propagules in seawater (Individuals L^{-1}, ±SE)	*Ulva* propagules in sediment (Individuals g^{-1}, ±SE)
WS1	32°56′1″N, 121°20′29″E	7–8 Jan. 2011	2	16 ± 3^{a}	12 ± 5^{A}
WS2	32°40′43″N, 121°8′57″E	17–18 Mar. 2011	8	31 ± 5^{b}	26 ± 9^{B}
WS3	32°48′41″N, 121°14′41″E	27–28 Apr. 2011	16	37 ± 13^{bc}	–[a]
WS4/5	32°47′11″N, 121°16′47″E	13–14 Mar. 2012	5.5	28 ± 7^{b}	22 ± 4^{B}
WS4/5	32°47′11″N, 121°16′47″E	24–25 Apr. 2012	15.5	45 ± 10^{bc}	31 ± 7^{B}
Neisha	32°4′49″N, 121°36′51″E	13 Mar. 2012	6	56 ± 11^{c}	35 ± 11^{BC}
Neisha	32°4′49″N, 121°36′51″E	24 Apr. 2012	16	120 ± 21^{d}	52 ± 10^{C}

Values with statistically significant differences ($P < 0.05$) are indicated by different letters.

[a] Sediment sample was not collected.

day. Exposure to air is required during nori farming for killing epiphytic algae including green ones.

2.2. Quantification of the Ulva propagules in the water and sediments

Ulva propagules in the water and sediments are invisible to the naked eye. To know their abundance at each of the locations, water and sediment samples were taken and transported to the laboratory in cool boxes within 48 h after collection. *Ulva* propagules in seawater were quantified using the method described by Liu et al. (2010b,c). About 250 mL of seawater samples in the plastic wide-necked bottles were enriched with nutrients to the level of 200 μM NH_4^+ and 20 μM PO_4^{3-} ($n = 4$). About 125 μL saturated GeO_2 solution was added to inhibit the growth of diatoms (Shea and Chopin, 2007). The bottles were maintained at 20° C at an irradiance of 100 μmol photons m^{-2} s^{-1} in GXZ-260C temperature-controlled incubator (Ningbo Jiangnan, China). After 4-week culture, the number of green algal germlings was determined. Quantification of the *Ulva* propagules in sediments was described previously by Liu et al. (2012a). A standard wet weight of 1 g sediment from each location was added in 1 L glass beaker filled with 1 L nutrient-enriched sterile seawaters (200 μM NH_4^+ and 20 μM PO_4^{3-}, $n = 4$). The sediment fluid was thoroughly stirred and then about 500 μL saturated GeO_2 solution was added. The beakers were maintained at 20° C at an irradiance of 100 μmol photons m^{-2} s^{-1} in incubators. Irradiance was determined using a 4π QSL-2100 (Biospherical Instruments Inc., USA). Light regime was 12-h light per day. *Ulva* thalli growing up from propagules were examined microscopically according to their morphological characteristics. Seventeen samples from sediment and twenty-one samples from seawater were selected for DNA extraction.

2.3. Net mounting experiment in the mudflat

To better understand the occurrence and species composition on nori nets which were hypothetically thought to be the original sources of the floating biomass (Liu et al., 2009), we selected two locations, WS4/5 and Neisha, to setup unseeded nori nets (without conchospores of *P. yezoensis*) on 14 November 2011 and 13–14 March 2012, respectively. Naisha and WS4/5 were about 1 and 35 km off the coast, respectively. Both are within the main nori farming area. At each location, three nets (2 × 3 m) were mounted. On 24–25 April 2012, the nets were harvested from the field and transported to the laboratory within 48 h. The green algae were isolated from the nets and total biomass was quantified. The dominant alga on the nets was determined according to morphological and molecular data (Liu et al., 2010b,d).

2.4. DNA extraction, sequence amplification and sequencing

Approximately 100 mg uni-algal samples were grounded to fine powder in liquid nitrogen for DNA extraction with the CTAB method (Liu et al., 2010b) or the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The nuclear encoded internal transcribed spacer DNA (ITS) region including the 5.8S rDNA was amplified using primers published by Leskinen and Pamilo (1997). To further distinguish samples in the *Ulva linza-procera-prolifera* (LPP) clade, the 5S rDNA spacer region was amplified using the primers according to Shimada et al. (2008). Polymerase chain reaction (PCR) amplification used the Tag PCR Master Mix (Tiangen Biotech, Beijing, China). Amplification products were separated by 1.0% agarose gel electrophoresis. Fragments of ITS region and the shortest DNA fragments of 5S rDNA spacer region were excised from the gels and purified using a DNA Gel Extraction Kit (Bio Basic Inc., Canada). Sequencing reactions were performed using ABI 3730 XL automated sequencers (Shanghai Biosune Biotechnology Co., Ltd., China).

2.5. Phylogenetic analysis

To elucidate the relationship among the green algal samples, a total of 116 were included in the phylogenetic analyses: 83 samples collected from four different locations in the radial mudflat during the five cruises, 28 collected during the green tide events of the previous five years and 5 from CAAPs (Tables 2 and 3). Sequence datasets of the 116 samples and other data from GenBank were aligned using Clustal W (Thompson et al., 1994). Neighbor-Joining (NJ) analyses were performed with Mega 5.05 (Tamura et al., 2007). *Blidingia minima* was selected as an out-group taxa for analysis of ITS dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

2.6. Effect of fermented chicken manure (FCM) on the growth of U. prolifera

For testing the effect of FCM-enriched seawater on growth performance of *U. prolifera*, 0.1, 0.5 and 1.0 g of fresh FCM purchased from a rotifer farmer at Rudong were added to 1 L sterile seawater, respectively, and mixed up thoroughly. These FCM media were prepared to simulate the aquaculture waste water from the rotifer ponds. PES medium was used as a positive control, and pure seawater as a negative control. A standard algal weight of 0.5 g FW was added into separate glass beakers filled with 1 L of PES, FCM-enriched seawater or pure seawater, respectively. The cultures were run at 18 (±1) °C at the irradiance of 100 μmol photons m^{-2} s^{-1} in a 12 h light per day light–dark regime for 24 days without renewal of the culture medium. The algal biomass was determined at eight day's interval. Relative growth rate (RGR) was calculated as $RGR = (\ln W_2 - \ln W_1)/t$, in which W_1 is the initial fresh biomass and W_2 the fresh biomass of day t. Each treatment was performed in four replicates.

2.7. Floating experiment under high irradiance

To understand how the filamentous *U. prolifera* biomass floats and sinks in the sea under illumination, healthy algal samples of *U. prolifera* were collected from Qingdao coasts in July 2011 when they appeared in the sea at large scale. The algal biomass was pre-cultured in 80 L indoor tanks (82 × 40 × 30 cm) at ambient room temperature (20° C) at low irradiance (ca. 10 μmol photons m^{-2} s^{-1}) in a 12 h light per day light–dark regime. Under such environmental conditions, algal thalli sank to the bottom of the tanks. Over time, the condition of the thalli was evaluated by measuring the Fv/Fm value. Fv/Fm was determined using a Portable Chlorophyll Fluorometer (Mini PAM, Walz, Germany) with the method of Fleming et al. (2007). Before measurement, samples were dark adapted for 20 min. Optimal chlorophyll fluorescence quantum yield was calculated according to the following equation: $Fv/Fm = (Fm - Fo)/Fm$, where Fo and Fm refer to the minimal fluorescence and the maximal fluorescence from dark adapted samples, respectively, and Fv is the difference between Fm and Fo.

Table 2
Green algal samples collected from the last 5 year green tides and coastal animal aquaculture ponds (CAAPs) for phylogenetic analysis.

Sample no.	Collection location	Location coordinate	Collection date	Source
2008-1, 2008-2, 2008-3	Qingdao	36°2′55″–36°5′28″N, 120°18′32″–120°21′50″E	28 Jun. 2008	Free-floating, bloom-forming alga
2009-1, 2009-2, 2009-3, 2009-4, 2009-5, 2009-6, 2009-7, 2009-8			19–21 Jul. 2009	
2010-1, 2010-2, 2010-3, 2010-4, 2010-5			8–9 Jul. 2010	
2011-1, 2011-2, 2011-3, 2011-4, 2011-5, 2011-6, 2011-7, 2011-8			7 Jul. 2011	
2012-1, 2012-2, 2012-3, 2012-4			18 Jun. 2012	
AAP-1, AAP-2, AAP-3	Lianyungang	34°35′3″N, 119°34′39″E	11 May 2009	Coastal animal aquaculture ponds (CAAPs)
AAP-4, AAP-5	Xiangshui	34°6′14″N, 120°19′26″E	19 Apr. 2009	

Algal samples in different health conditions (reflected by their Fv/Fm) were used to conduct the floating experiment. A standard algal weight of 15 g FW with different Fv/Fm values were added into 2 L glass beakers filled with 2 L of sterile seawater at 20° C. Algal biomass sank on the bottom of the beakers was supplied with a strong irradiance of 800 μmol photons m^{-2} s^{-1} from sun-light studio (S-150 L, Shenao, China). The time needed by the algal biomass to completely float on the water surface was recorded. Five groups were designed and each treatment was performed in triplicates.

2.8. Statistics

Data were analyzed by one-way analysis of variance (ANOVA). The difference among means was analyzed by Duncan's new multiple range test following by significant ANOVA at $P < 0.05$. All values cited in this study were obtained from fully independent samples. Tests were performed using the SPSS 13.0 statistical program (SPSS Inc., Chicago, USA).

3. Results

3.1. Quantification of the Ulva microscopic propagules

Ulva microscopic propagules have been consistently present in seawater and sediments of the investigated region in the radial shoals. From January to late April, the number of *Ulva* microscopic propagules in both seawater and sediments increased along with average seawater temperature from 2° C to 16° C every year ($P < 0.05$), indicating that average abundance of propagules in seawater and sediments was seasonally dependent.

3.2. Phylogenetic analysis

Alignments of ITS dataset from 116 *Ulva* samples and additional taxa from GenBank included about 560 bp. The phylogenetic analyses based on the above two datasets showed that all the 116 samples fell into four clades with high bootstrap values (99–100%): the *U. linza-procera-prolifera* (LPP) clade (87 samples), the *Ulva flexuosa* clade (21 samples), the *Ulva compressa* clade (2 samples) and the *Blidingia* sp. clade (6 samples) (Fig. 2). The unrooted phylogenetic analyses of the 5S rDNA spacer region dataset (about 388 bp) revealed that the 87 samples in the LPP clade fell into two clades with high bootstrap values: 64 samples in the *U. prolifera* clade and 23 in the *U. linza* clade (Fig. 3). The *U. prolifera* clade contained the samples from the green tides of the previous five years, the seawater and sediments, the CAAPs and the *P. yezoensis* rafts. The 64 samples in the *U. prolifera* clade could be further divided into three sub-clades: sub-clade A with 369 bp in length of 5S rDNA spacer region (38 samples), sub-clade B with 328 bp (21 samples) and sub-clade C with 379 bp (5 samples). Twenty-eight samples from the floating algae in the green tide events in the Yellow Sea distributed in all the three sub-clades of *U. prolifera*.

Ulva microscopic propagules of *U. prolifera* were always detected in the seawater and sediments in the investigated areas during the five cruises, based on molecular data (Table 4). The green algae on the nori rafts varied in different locations and seasons. In 2011, *U. prolifera* was not found on the nori rafts in three investigated locations (WS1, WS2, and WS3), but was detected at location WS4/5 in 2012.

3.3. Biomass, species composition of the green algae on the unseeded nori nets and field observation at two sites from the winter of 2011 to the spring of 2012

In the near shore location of Neisha and offshore location of Waisha, the nets installed on November 14, 2011, when the seawater temperature was 15° C, had a few green algae attached on them over the winter, three months after being in place (Fig. 4A1 and B1). In the middle of March, green algae attached on the nets increased at both sites (Fig. 4A2 and B2). On the harvest dates of April 24–25, 2012, significantly different biomass of *U. linza* and

Table 3
Sampling information and green algal samples from different sources of Waisha area in the Jiangsu radial shoals during the five cruises.

Cruise no.	Sample no. and source			
	Porphyra rafts (R)	Free-floating (F)	Sediment (M)	Seawater (W)
WS1	WS1-1 R, WS1-2 R, WS1-3 R, WS1-4 R, WS1-5 R, WS1-6 R, WS1-7 R, WS1-8 R, WS1-9 R, WS1-10 R	Not found	WS1-11 M, WS1-12 M, WS1-13 M, WS1-14 M, WS1-15 M	WS1-16 W, WS1-17 W, WS1-18 W, WS1-19 W, WS1-20 W, WS1-21 W
WS2	WS2-1 R, WS2-2 R, WS2-3 R, WS2-4 R, WS2-5 R, WS2-6 R	Not found	WS2-7 M, WS2-8 M, WS2-9 M, WS2-10 M, WS2-11 M, WS2-12 M	WS2-13 W, WS2-14 W, WS2-15 W, WS2-16 W, WS2-17 W
WS3	WS3-1 R, WS3-2 R, WS3-3 R, WS3-4 R, WS3-5 R, WS3-6 R, WS3-7 R, WS3-8 R, WS3-9 R, WS3-10 R, WS3-11 R, WS3-12 R	WS3-13 F, WS3-14 F	No collection	WS3-15 W, WS3-16 W, WS3-17 W, WS3-18 W
WS4	WS4-1 R, WS4-2 R, WS4-3 R, WS4-4 R, WS4-5 R, WS4-6 R	Not found	WS4-7 M, WS4-8 M, WS4-9 M, WS4-10 M	WS4-11 W, WS4-12 W
WS5	WS5-1 R, WS5-2 R, WS5-3 R, WS5-4 R, WS5-5 R, WS5-6 R	WS5-7 F, WS5-8 F, WS5-9 F	WS5-10 M, WS5-11 M	WS5-12 W, WS5-13 W, WS5-14 W, WS5-15 W

Fig. 2. Phylogenetic tree constructed from analysis of the ITS rDNA region including the 5.8S gene. The tree was rooted with *Blidingia minima*. The numbers at internal nodes were bootstrap values greater than 50% for 1000 replicates in Neighbor-Joining (NJ) analysis. Branch lengths are proportional to the amount of sequence change, which is indicated by the scale bar below the tree. Geometric drawings represented sample sources: free-floating (●), *Porphyra* aquaculture rafts (▲), coastal animal aquaculture ponds (CAAPs) (○), sediments (■) and seawater (□).

U. prolifera were obtained with the former dominating the Neisha's nets and the latter the Waisha's nets ($P < 0.05$, Fig. 4A3 and B3).

The nets newly installed on March 13–14, 2012, at both locations were uniformly dominated by *U. prolifera* with significantly different biomass when harvested on April 24–25, 2012 (Table 5). It is interesting to point out that in the offshore location WS4/5 the nets installed on the two dates were uniformly dominated by *U. prolifera* with similar biomass when harvested ($P > 0.05$). Field observation at WS4/5 on April 25, 2012, revealed that the filamentous *U. prolifera*, in different sizes, was present either floating in the water, or stranded on the bottom of the mudflat or twisted on nori rafts or any substrates encountered during drifting (Fig. 5A–C).

3.4. *Effect of fermented chicken manure (FCM) on the growth of U. prolifera*

Compared with the control, addition of FCM significantly increased the growth of *U. prolifera* during the 24-day's culture ($P < 0.05$. Fig. 6). There was no significant difference in biomass and RGR between enrichment by 500 mg FCM and the PES medium

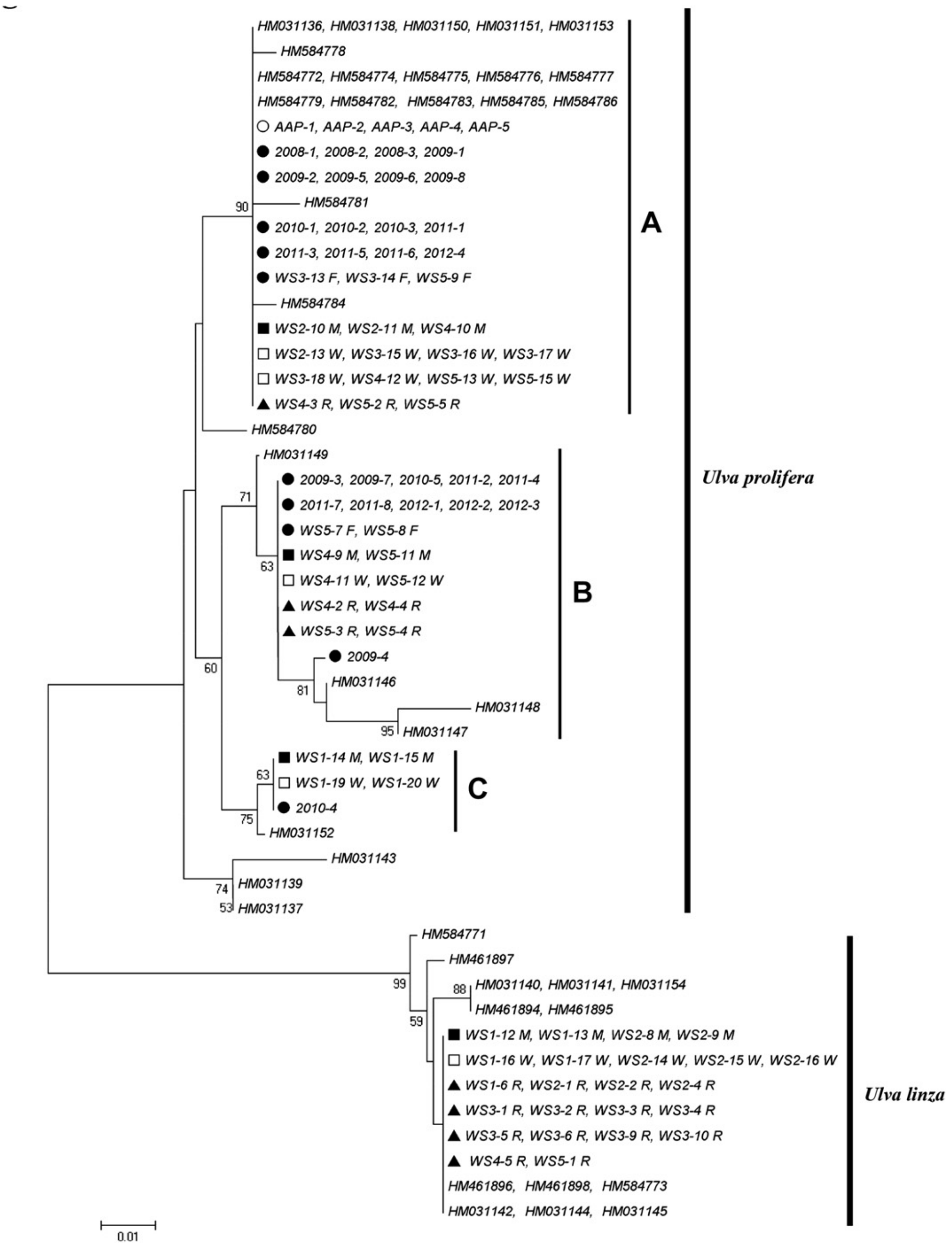

Fig. 3. Unrooted phylogenetic tree constructed from analysis of the 5S rDNA spacer sequences of the *Ulva linza-procera-prolifera* (LPP) clade. The numbers at internal nodes were bootstrap values greater than 50% for 1000 replicates in Neighbor-Joining (NJ) analysis. Branch lengths are proportional to the amount of sequence change, which is indicated by the scale bar below the tree. Geometric drawings represented sample sources: free-floating (●), *Porphyra* aquaculture rafts (▲), coastal animal aquaculture ponds (CAAPs) (○), sediments (■) and seawater (□).

Table 4
Presence (+) or absence (−) based on the molecular data of *Ulva prolifera* at different locations of Waisha in the Jiangsu Province radial shoals during the five cruises in 2011–2012.

Cruise no.	*Porphyra* rafts (R)	Free-floating (F)	Sediment (M)	Seawater (W)
WS1	−	−	+	+
WS2	−	−	+	+
WS3	−	+	+	+
WS4	+	−	+	+
WS5	+	+	+	+

($P > 0.05$). During the first 8-day, the highest RGR was detected in the 500 mg FCM group (23.03% d^{-1}), while in the second 8-day, the 1000 mg FCM group had the highest growth rate (12.34 ± 1.83% d^{-1}).

3.5. Floating experiment under high irradiance

Under the irradiance of 800 μmol photons m^{-2} s^{-1}, the time required by the branched *U. prolifera* to float on the water surface was strongly associated with the algal health condition (Fig. 7).

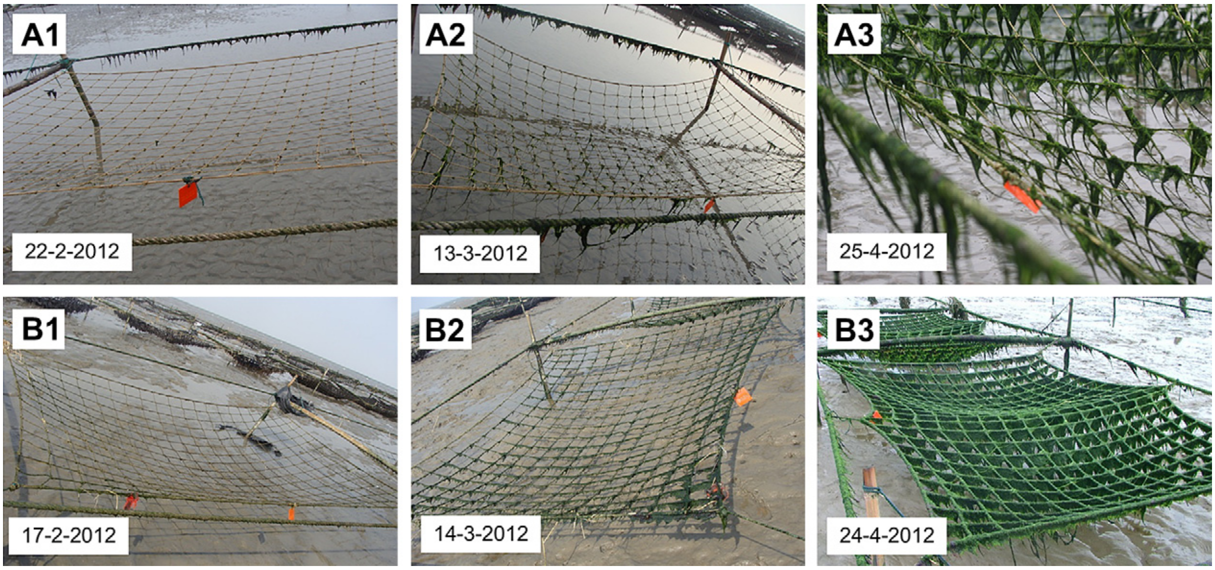

Fig. 4. The green algal biomass on the unseeded nets installed at Waisha (A1–A3) and on the unseeded nets installed at Neisha (B1–B3).

When algal Fv/Fm values were around 0.674, it took 52 min to achieve flotation. Decrease in algal Fv/Fm significantly led to a delay of floating ($P < 0.05$). Algal biomass needed 6 h and 20 min to float on the surface when Fv/Fm values were around 0.185.

4. Discussion

4.1. *The large-scale radial mudflat along Jiangsu coast and the propagules of U. prolifera in the water and sediments*

Results obtained in this investigation indicated that the recurrent dominant *U. prolifera* in the Yellow Sea is spatially tightly related with the 20,000 km^2 radial mudflat along Jiangsu's coast. This unique radial mudflat is exposed to air, forming a large-scale muddy or sandy substratum composed of suspending particles with sizes ranging from 0.007 to 0.015 mm (Huang et al., 2011). Swift local water currents and the large tidal amplitude work together to make this large intertidal zone a more than 90 km sandy/muddy plateau on which the filamentous green alga *Ulva* spp. could sparsely attach and grow during the daily low water periods. This giant mudflat functions as a large "incubator" in which *Ulva* seedlings are nurtured in the spring when other factors (nutrients, irradiance, etc.) are becoming optimal. In this investigation, the microscopic propagules of the bloom-forming alga *U. prolifera* were consistently detected in the coastal water and sediments at different seasons. Propagules of *Ulva* spp in sediments and coastal waters are believed to construct an overwintering "seed stock" which serves as the precursor of the recurrent green tides in the Yellow Sea and other waters (Liu et al., 2012a; Schories and Reise, 1993; Schories, 1995). This investigation revealed that the *Ulva* microscopic propagules were seasonally dependent on the radial mudflat. *Ulva prolifera* has been shown to reproduce itself with the mechanism of spontaneous, partial sporulation under relatively higher irradiance, thus dispersing enough spores or gametes into the surrounding waters (Liu et al., 2010d). In 2009, a broader-scale investigation in the Yellow Sea showed that the abundance of *Ulva* propagules in the surface water was in a range from 10 to 100 individuals L^{-1} (Liu et al., 2010c,d). The production and release of *U. prolifera* propagules (spores and gametes) is thought to play an important role in enlarging the algal biomass during the outbreak stage of the green tides.

4.2. *Seasonally discharged land-derived effluents intensify the eutrophication of the coastal waters leading to the recurrent floating green-tides along the Jiangsu Province coast*

Eutrophication of seawater has been widely believed to be the most important cause of harmful algal bloom events worldwide (Heisler et al., 2008; Conley et al., 2009). In the Yellow Sea, no evidence of large-scale floating green algae was found before 2006, indicating that the nutrient delivery reached the threshold level for promoting the development of the green tides from 2007 onwards. Recent reports suggested that— (1) the flux of NH_4–N and phosphorus in principal rivers (e.g. Guan river and Sheyang river) of Jiangsu Province increased during the period from May to August, for example, from 718.84 tons NH_4–N in 2004 to 5807.01 tons NH_4–N in 2007 (Ma et al., 2010), and (2) the expansion of animal aquaculture industry along the Jiangsu Province coast discharged high levels of nutrients into the adjacent coastal waters of the radial mudflat in the Yellow Sea (Pang et al., 2010).

Among those factors, it is noteworthy to point out that the intensification of land-based aquaculture, especially the application

Table 5
Biomass and species composition of green algae on the nets without inoculation of *Porphyra yezoensis* in Jiangsu radial shoals. Means and SE are shown ($n = 3$).

Location	Geographical coordinate	Setup date	Collection date	Green algal biomass (g net^{-1}, ±SE)	Species composition of green algae (%: biomass in fresh weight)
WS4/5	32°47′11″N, 121°16′47″E	14 Nov. 2011	25 Apr. 2012	4360.7 ± 769.8^a	90% *U. prolifera*; others: *U. linza*, *U. flexuosa*, *Blidingia* sp.
		13 Mar. 2012		4101.3 ± 1354.5^a	90% *U. prolifera*; others: *U. linza*, *U. flexuosa*
Neisha	32°4′49″N, 121°36′51″E	14 Nov. 2011	24 Apr. 2012	8260.1 ± 1154.0^A	95% *U. linza*; others: *U. flexuosa*
		14 Mar. 2012		699.3 ± 160.0^B	90% *U. prolifera*; others: *U. linza*, *U. flexuosa*

Values with statistically significant differences ($P < 0.05$) are indicated by different letters.

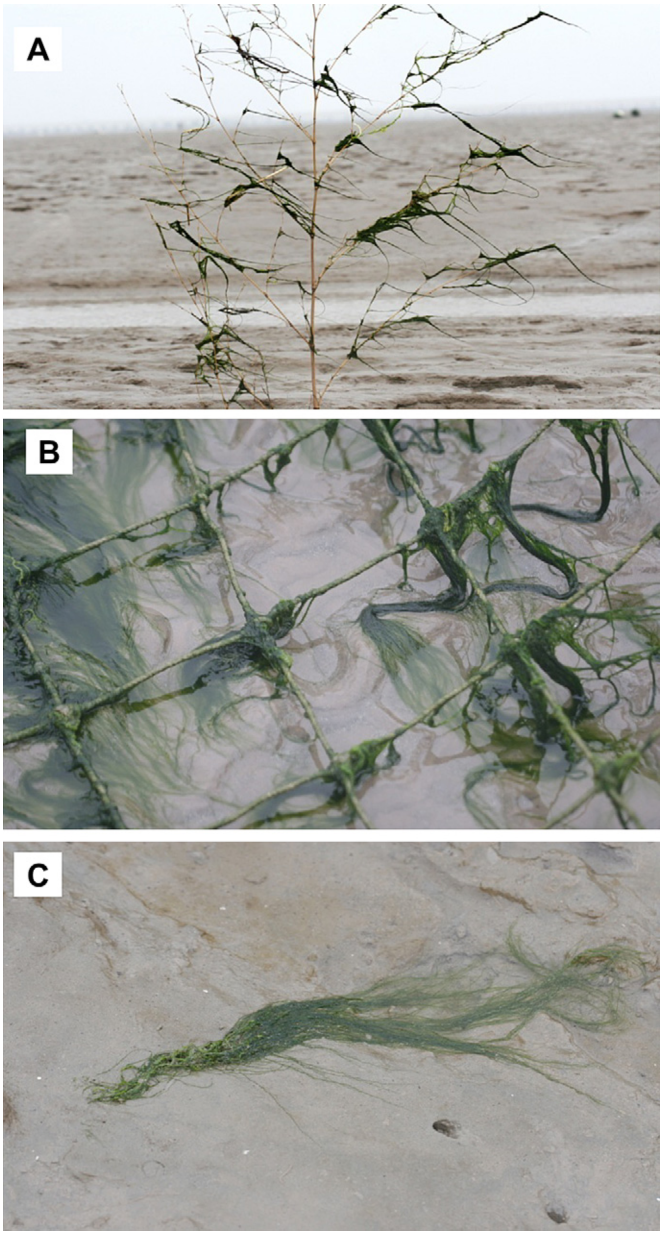

Fig. 5. (A) *Ulva prolifera* thalli on the guiding bamboo during low tides. (B) *Ulva prolifera* thalli on the *Porphyra* nets. (C) *Ulva prolifera* thalli stranded on the bottom during low tides.

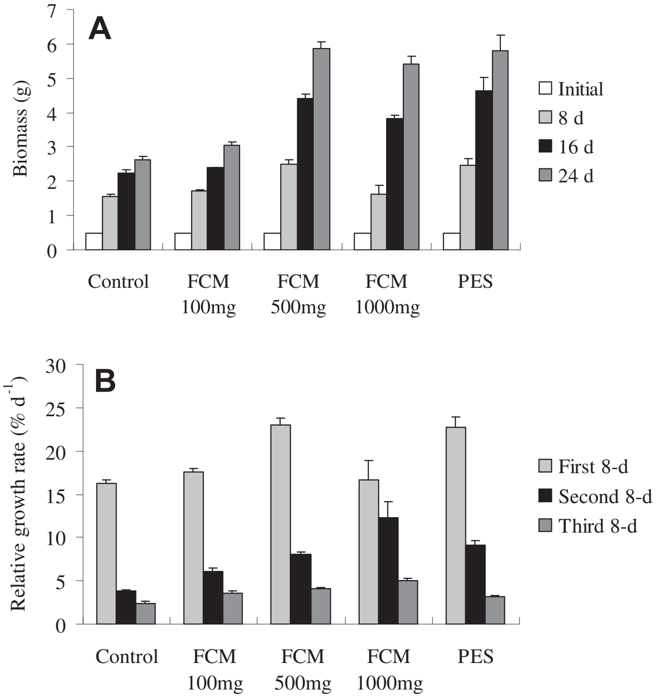

Fig. 6. Effect of fermented chicken manure (FCM) on (A) biomass and (B) relative growth rate (RGR) of *Ulva prolifera* at 18° C and at an irradiance of 100 μmol photons m^{-2} s^{-1} in a 12 h light per day light–dark regime during the 24-day culture period. Means and SE are shown ($n = 4$).

of FCM in large quantity from 2005 to present, dramatically intensified the eutrophication situation in Jiangsu Province coastal waters. In the Sheyang region, local rotifer farmers usually applied 30–45 tons of FCM per hectare of rotifer ponds, while in the Rudong region, they applied 30 tons of FCM and 750 kg of $(NH_4)_2CO_3$ per hectare (Fig. 8). Today, at least 50,000 tons of FCM are estimated to be applied in those ponds annually for production of rotifers to feed the juvenile freshwater crabs. Water from these ponds is periodically discharged to the coastal zone through canals and ditches at the end of the rotifer production from the beginning of March until May–June (Wang et al., 2010). The discharged water contained high amount of nutrients, degrading coastal water quality and leading to eutrophication, as is happening along other coasts in the world (e.g. Anderson et al., 2002; Charlier et al., 2007). Due to the unique geographic structure of the radial mudflat of this coast, this impact is brought as far as 90 km offshore. Culture results of this investigation demonstrated that enrichment of 500 mg FCM was similar to the PES medium in promoting the growth of *U. prolifera*. FCM is a very cheap fertilizer, releasing high levels of nutrients, especially ammonium when dissolved in water. Previous study found that the discharged waste water from the Sheyang rotifer pond contained plenty of ammonium, far beyond 250 mM (Liu et al., 2012a). The opportunistic *Ulva* species showed higher *Vmax/Ks* values in ammonium uptake comparing with nitrate uptake (Luo et al., 2012). In late April, when temperature rose above 10° C, densely-branched *U. prolifera* grew fast in eutrophic water column based on its physiological and morphological features (Liu et al., 2010b; Kennison et al., 2011). In laboratory conditions, the filamentous thalli of *U. prolifera* could be filled up with photosynthetic oxygen quickly under high irradiance and achieved flotation

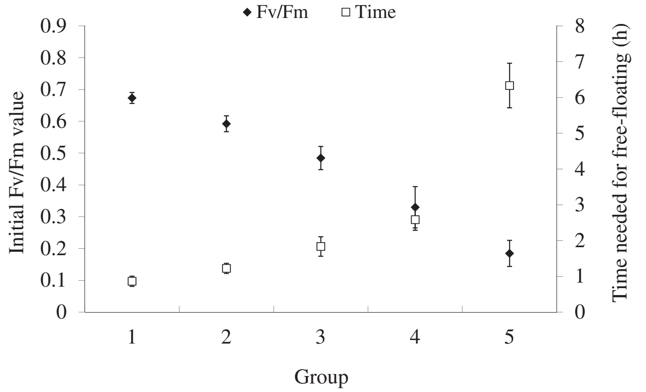

Fig. 7. Floating experiment under a high irradiance of 800 ± 50 μmol photons m^{-2} s^{-1}. Fv/Fm was taken as an indicator to evaluate the health condition of the algal biomass of *Ulva prolifera*. Means and SE are shown ($n = 3$).

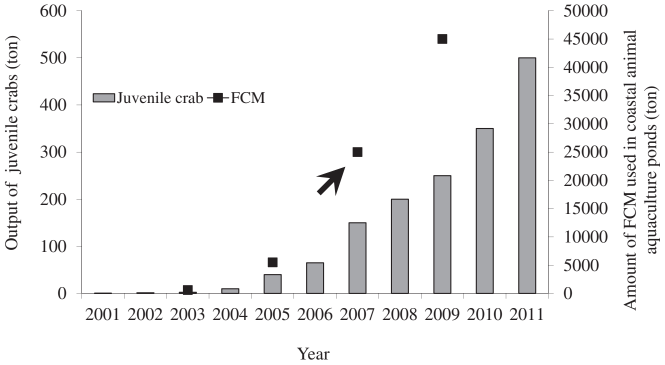

Fig. 8. The output of juvenile crabs and the estimated amount of fermented chicken manure (FCM) used in coastal animal aquaculture ponds (CAAPs) in the Sheyang region from 2001 to 2011. The arrow indicates the year when large-scale green tides started to break out.

within an hour. Although the seawater of Jiangsu Province coast is turbid and the underwater irradiance is low (less than 1 μmol photons $m^{-2}\ s^{-1}$), the *U. prolifera* thalli stranded on the bottom of the mudflat, or twisted on nori rafts or other substrates, could experience strong irradiance during low tides for 3–5 h. Such a strong irradiance was beneficial to the photosynthesis of the bloom-forming alga and the achievement of its drifting condition.

4.3. Infrastructures of the nori nets function as substrate to strand the floating biomass and further enlarge it in the radial mudflat

Results of the field net-setup investigation showed that green algae on the nets were derived from the seawater and sediments with a rapid biomass accumulation from March to April, either growing from the attached microscopic propagules or from the free-floating filaments in the water. Based on differences in species composition and biomass between the two sites, abundance and species composition of *Ulva* propagules in seawater and sediments varied in different locations and seasons in the immense radial mudflat. *Ulva prolifera* biomass on the nets in WS4/5 was six times that on the nets in Neisha, indicating more *U. prolifera* propagules existed at WS4/5 location. This result confirmed our previous finding that *U. prolifera* propagules were detected only at Waisha not Neisha in December 2010 (Liu et al., 2012a). Much longer low tide periods at Neisha (about 1 km offshore) leave *U. prolifera* exposed for longer time to the air and high air temperature in April, limiting the growth of the filamentous algae. This may explain why the *U. prolifera* biomass on the nets installed in March at Neisha was much less than that at WS4/5.

The nori farming nets have been hypothesized as the original source of the floating biomass of the green tide alga *U. prolifera*. Approximately 4,900 tons of green algal biomass was estimated to be stripped off the nets and forming the initial floating biomass (Liu et al., 2009, 2010a). This conclusion was based on the expansion of the farming area, the observation of the attached green algae on the nori nets during farming and the coincidence of nori harvesting time with the floating time of the green alga in that region. The fact that the microscopic propagules of the bloom-forming alga were detected over-wintering in the sediments and seawater of Waisha, where *P. yezoensis* farming is intensively carried out, indicates that the nets of *P. yezoensis* are not the original source of the green-tide forming alga. Rather, to a large extent, they served as the substrate for the propagules or the free-floating filamentous thalli to attach at a later stage, in a similar manner as we obtained in the net setup experiment in this investigation.

One may wonder what the situation would be if there was no nori farming infrastructure on the large radial intertidal mudflat from Sheyang to Rudong? Will *U. prolifera* still propagate in large quantity under intensified release of nutrients from coastal activities? In our field investigation at about 50 km offshore of Waisha during low water, stranded and free-living filamentous *U. prolifera* were consistently present. One of the natural examples to show how the release of nutrients from the land facilitates the growth of *U. prolifera* is at Xiangshan Bay (29°26′–29°34′N, 121°27′–121°50′E) of Zhejiang Province. This bay has been famous for its eutrophication, red tide events and production of natural *U. prolifera* (Liu et al., 2012b). In field investigation, green-carpet-like *Ulva* biomass has been present on the muddy intertidal zone during low tide in this bay in early spring (China Network Television, 2011; Liu et al., unpublished data). Local fishermen have been harvesting, cleaning and processing this biomass into foods for many years. However, along the Jiangsu Province coast, nori nets certainly help in enlarging the biomass in that area due to the elevation by the nets of the biomass to the surface of the water to experience stronger irradiance.

Consequently, we re-emphasize the need for early management actions in the sequence of events leading to the recurrent and massive green tides in the Yellow Sea. For a truly integrated management of the coastal zone, reduction in nutrient inputs and control of the effluents of the coastal animal aquaculture pond systems, are needed in the land-based operations. If the green tides are to be managed, and, hopefully, reduced or eliminated, their development needs to be stopped at the sources on land, not at intermediate steps on the radial intertidal mudflat when it is already too late for preventing their massing blooming.

Acknowledgments

The authors thank Lu Qinqin, Zhu Miaoxian, Zhu Jianyi, Hu Chuanming, Zhu Zhu, Mu Xinwu, Zhang Tao, Xia Bujun, Sun Zhengping and local fishermen for their assistance in the algal collection and culture. This investigation is financially supported by Qingdao Municipal Science and Technology Commission (10-4-1-14-hy, 11-3-1-3-hy), a project from Science and Technology Commission of Qingdao Shinan District (2011-5-030-QT), a project from Qingdao National Oceanographic Center (QNOC) and the Leading Academic Discipline Project of Shanghai Municipal Education Commission (J50701).

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