



## Tracking the algal origin of the *Ulva* bloom in the Yellow Sea by a combination of molecular, morphological and physiological analyses

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### ABSTRACT

In 2008, Qingdao (36°06'N, 120°25'E, PR China) experienced the world largest drifting macroalgal bloom composed of the filamentous macroalga *Ulva prolifera*. No convincing biologic evidence regarding the algal source is available so far. A series of field collections of both *Ulva* sp. and waters in various sites along Jiangsu coasts were conducted in March to May of 2009. Density of microscopic *Ulva* germlings in the waters sampled from different sites ranged from 7 to 3140 individuals L<sup>-1</sup>, indicating the wide-spreading and long-term existence of the algae in the investigated region. Morphological and the nuclear ribosomal internal transcribed spacer ITS nrDNA and the chloroplast-encoded *rbcl* gene comparisons of 26 algal samples revealed that the algae collected from land-based animal aquaculture ponds mostly resembled the dominating blooming algae in 2008. Mismatch of *Porphyra* farming period with the occurrence of the green tide bloom, as well as the negative identification results of the sampled green algae from the *Porphyra* rafts eliminated *Porphyra* rafts as the principal and original source of the dominating blooming algae.

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### 1. Introduction

Green tides are massive accumulations of unattached green macroalgae, principally belonging to the genus *Ulva*, and are intimately associated with eutrophicated marine environments (Nelson et al., 2008). One of the green tide algae, the filamentous alga *Ulva prolifera*, formerly known as *Enteromorpha prolifera* (Hayden et al., 2003), is broadly distributed along the nearshore coasts of the north-eastern Asia (Tseng, 1983; Shimada et al., 2008). Conspicuous growth of this alga was usually found in environments with sufficient input of nutrients, such as estuaries, from where land-derived nutrient rich effluents are combined and discharged into coastal waters (Leskinen et al., 2004; Conley et al., 2009). This alga can tolerate a wide range of temperatures, salinities and irradiances (Tan et al., 1999; Dan et al., 2002; Cohen and Fong, 2006). From May to July 2008 before the Olympic sailing competition, Qingdao coasts experienced an attack of the world's largest drifting green tide, evaluated at a level of one million tons of harvestable biomass (FW). The bloom once covered approximately 13,000–30,000 km<sup>2</sup> of the Yellow Sea (Sun et al., 2008). The dominating

species was identified as being the filamentous, intensively ramified *U. prolifera* (Müller) J. Agardh (Chlorophyta, Ulvophyceae) (Leliaert et al., 2008, 2009; Sun et al., 2008; Ye et al., 2008). Recent phylogenetic analyses showed that this unique strain forms a clade with representatives of the *Ulva linza-procera-prolifera* (LPP) complex and seems to be ubiquitous in several countries (Leliaert et al., 2009). Field-collected algal samples, as well as those maintained in culture, were both characterized with intensive ramification and demonstrated outstanding capacity of vegetative growth under favorable conditions.

Accurate localization of the origin and persistence of this green algal bloom is the first step in understanding this large-scale green tide and finding solutions to the problems it could potentially bring. According to satellite images the drifted biomass initiated offshore of the coasts of Jiangsu province and was transported across the Yellow Sea to Qingdao coasts by seasonal winds and surface currents (Liu et al., 2009). The original “seed” source of the bloom remained unidentified, although hypothesis was recently proposed. Liu et al. (2009) thought that the rapid expansion of *Porphyra* farming along the Jiangsu coasts was the principal cause. It is therefore necessary to analyze the time series of this red alga farming in relation with the green tide event in terms of reproduction and growth of both algae. Simple morphological identification has proven to be insufficient to distinguish species in the genus

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*Ulva* because often unattached thalli demonstrate considerable morphological plasticity (Malta et al., 1999). Use of appropriate molecular markers can both identify the algae and provide important information concerning the origins and dynamics of the blooms (Malta et al., 1999; Largo et al., 2004). Nuclear ribosomal internal transcribed spacer ITS nrDNA and the chloroplast-encoded *rbcL* gene sequences were used to combine the previous *Ulva* and *Enteromorpha* into one genus (Hayden et al., 2003) and were popularly used to discern the taxonomic positions of the strains in *Ulva* (Leliaert et al., 2009; Shimada et al., 2008).

The principal objective of this investigation is to use multiple means to source-track the dominating bloom alga. These means include, (1) standard analyses of ITS nrDNA and the chloroplast-encoded *rbcL* gene sequences of the algal samples collected at different coastal sites of Jiangsu province before the bloom and the samples of 2008 Qingdao's bloom; (2) algal morphological comparisons and sporulation (reproduction) tests under different temperature regimes; (3) quantitative determination of culturable *Ulva* microscopic stages in free seawaters and (4) analyses of time cycle of *Porphyra* farming in relation to the occurrence of the green tide.

## 2. Materials and methods

### 2.1. Choices and description of sample collection sites

The entire coast of Jiangsu province (30°44'–35°4'N) is characterized by an extended shallow and muddy intertidal zone, constituting an ideal environment for performing *Porphyra* cultivation by use of floating cultivation methods (Shang et al., 2008). The world's largest cultivation of *Porphyra yezoensis* has been carried out in this province since 1970s. Until today, *Porphyra* farming occupies 21,000 hectares of intertidal area, producing 126,000 tons (FW) annually (P. Xu, personal communication). Parallel to the *Porphyra* farming area along the coasts are animal aquaculture pond systems on land (AAPs) in which *Eriocheir sinensis* (a fresh water crab with larval stage in the marine environment), and *Penaeus vannamei* (a white prawn species introduced from America) are farmed (Fig. 1). *Porphyra yezoensis* is farmed by use of semi and full-float-

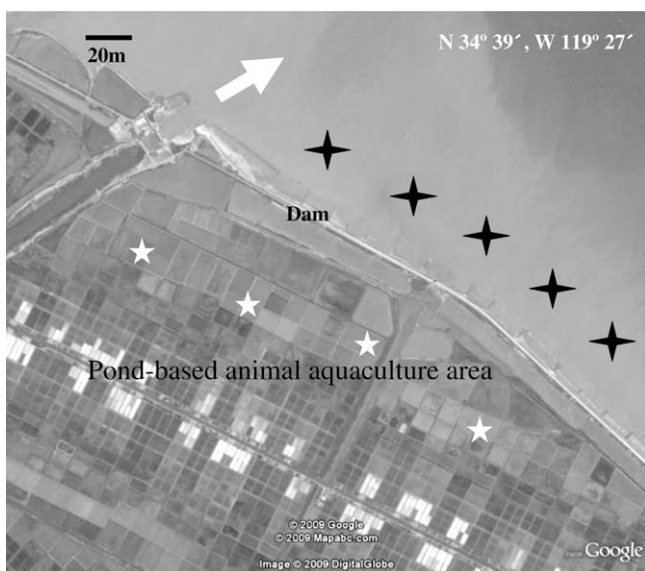
ing rafts composed of bamboo and nets on which the conchospores attach and grow into blades in 2–3 months during the cold season from December to March. Young *E. sinensis* (ca. 1 cm) are produced from February to May in seawater in coastal AAPs, locally called “natural ecological ponds” (NEPs), developed from 2001 onwards. The NEP method, because of its low cost and easiness to manage, became rapidly the dominating one to produce young crabs in Jiangsu province and is characterized with intensive application of organic fertilizers. Jiangsu province is thus becoming the largest young crab production center in China. *P. vannamei* is principally farmed along the northern coast of Jiangsu province from March to July in shallow coastal ponds with water depth ranging from 1 to 1.5 m. Typical coastal aquaculture areas in this province are characterized by *P. yezoensis* cultivation in the intertidal zone and large numbers of land-based AAPs separated by a dam. Waters from AAPs are collectively discharged from a main sluice channel (Fig. 1). Large volumes of water are exchanged frequently between AAPs and the intertidal water during rainfall seasons. Salinity, temperature and nutrient levels in the water of these ponds fluctuated, thus making the pond a special niche for species that could tolerate, survive and reproduce, such as species in the genus *Ulva*.

Along the coasts of Jiangsu province, open-sea *Porphyra* cultivation starts with the transfer of the seeded nets to the sea each year in November and ends up with the withdrawal of the nets and bamboos from the sea at the end of April (P. Xu, personal communications). Surface water temperature during this period drops from 15 °C to 3 °C and thereafter increases to 14 °C over the winter with slight variations in different areas (Fig. 2). Blades of *Porphyra* grow most significantly during low temperature periods and cover most of the nets. Filamentous green algae are often found to grow vigorously on the nets where *Porphyra* conchospores sparsely attached. In such cases, the nets are often sun-dried for more than 12 h to kill the epiphytic green algae, while *Porphyra* can tolerate such extreme exposure (Y.D. Yu, personal communications).

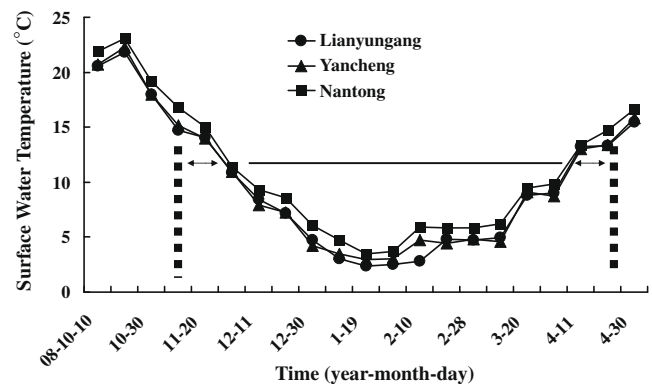
Considering the above observations, we collected green algal and corresponding water samples from the rafts of *Porphyra* cultivation system, the intertidal zone, the coastal AAPs adjacent to the algal farming area and from the sluice gates of the AAPs along both southern and northern coasts of Jiangsu province in April–May 2009 (Fig. 3).

### 2.2. Treatments of water and algal samples

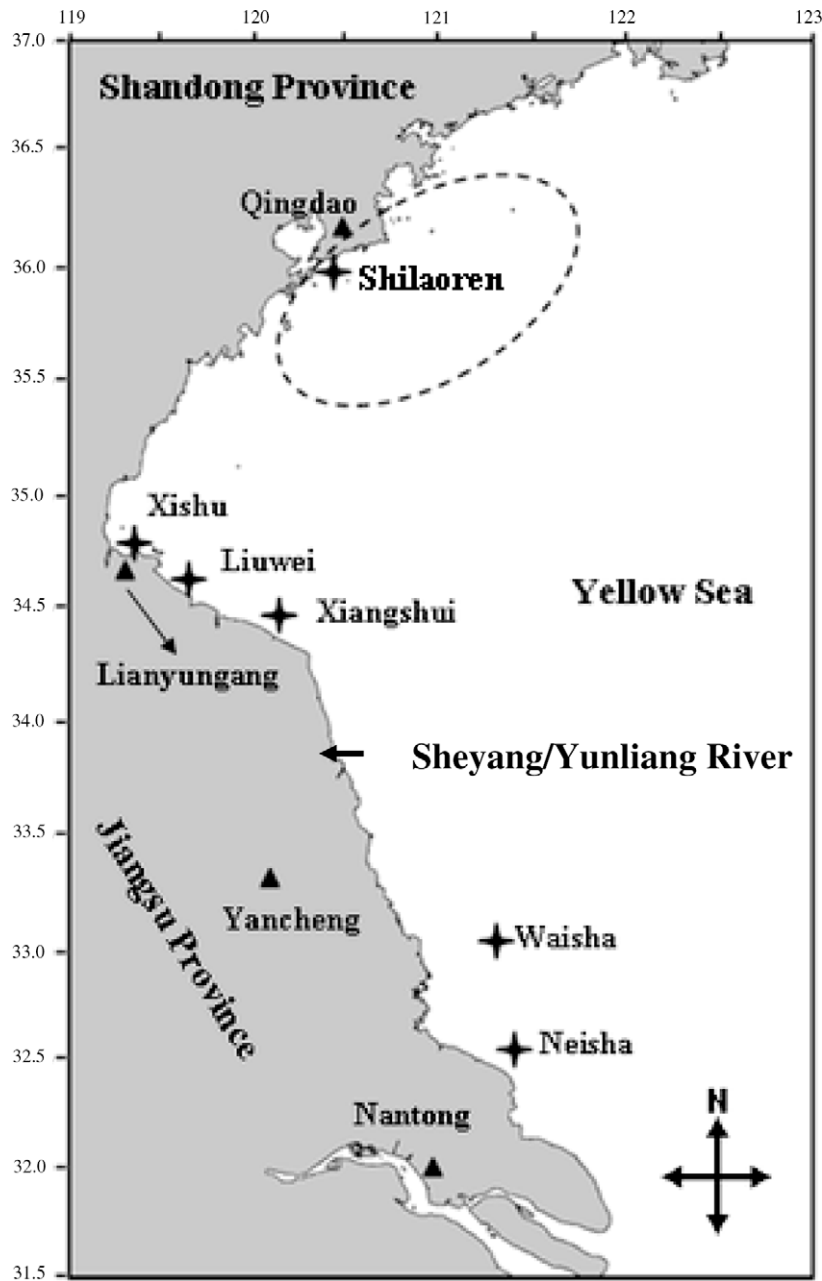
In each of the six locations investigated in Jiangsu province (full-floating rafts of *Porphyra* cultivation area, nearshore water and sluice gates of AAPs at Liuwei, coastal ponds as well as full-



**Fig. 1.** Satellite image of a typical coastal area in Jiangsu province. The seasonal *Porphyra* farming area (crosses) is located in the intertidal zone. A long dam divides the seaweed farming area from the year-round pond-based animal aquaculture system (stars). Waters from the ponds collectively discharge into the coastal water through a principal sluice gate (arrow).



**Fig. 2.** Changes in surface water temperatures during the *Porphyra* farming period at Lianyungang, Yancheng and Nantong in Jiangsu province (culture period in between vertical dashed lines). The horizontal line represents the period with temperatures below 12 °C during which both growth and sporulation of *Ulva* spp. slows down dramatically.



**Fig. 3.** Sampling locations (Xishu, Liuwei, Xiangshui, Waisha and Neisha) along the coasts of Jiangsu province. The accumulation of large amount of drifted *Ulva* biomass in Qingdao in 2008 is indicated by the area circled with a dashed line in which we had another sampling location (Shilaoren).

floating rafts of *Porphyra* cultivation area at Xiangshui), 4 L of seawater were collected and mixed thoroughly. Seawater was subsequently aerated after adding  $\text{NO}_3^-$  ( $823 \mu\text{mol L}^{-1}$ ) and  $\text{PO}_4^{3-}$  ( $73 \mu\text{mol L}^{-1}$ ) to reach the levels of Provasoli enriched seawater (PES) medium in 1 L glass beakers (two per sampling location) placed in temperature-controlled rooms at 15–18 °C under 80–100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a 12 h light per day light–dark regime. The medium was renewed every 7 d in the following culture period. After 3 weeks, the green algal germlings, attaching to the wall and bottom of the glass beakers, grew up to 1–5 cm and were counted and identified by both morphological and molecular analyses as explained below. These germlings were derived from microscopic spores or gametes or those that were attached to particles invisible to the naked eye at the time of sampling. After counting, the algae were removed from the beakers and further grown in suspension culture under the same conditions.

For the field-collected algal samples, the algae were cleaned *in situ* and brought back to the laboratory in cooled box within 24 h. Dominating *Ulva* species in each sample, judged by morphological observations, were individually sorted and cleaned with sterilized seawater and further grown in  $\text{GeO}_2$ -added PES medium for a week to remove epiphytic diatoms for further DNA analyses. In all the analyses we performed, no DNA contamination of epiphytic algae was ever observed.

### 2.3. Algal DNA extraction

The algal samples were washed three times with sterilized seawater, dried with filter paper. Then 100 mg of unialgal material for each sample was ground to fine powder in liquid nitrogen and transferred to a 2 mL tube. 750  $\mu\text{L}$  cetyl trimethyl ammonium bromide (CTAB) buffer including 3% CTAB (w/v), 1.4 M NaCl, 20 mM

EDTA, 100 mM Tris–HCl (pH 8.0), 1% polyvinyl pyrrolidone (w/v), 1%  $\beta$ -mercaptoethanol (v/v) and 10  $\mu\text{g mL}^{-1}$  RNase A was added to the tube. The mixture was incubated at 65 °C for 60 min with gentle inversion occasionally (Wang et al., 2006). 250  $\mu\text{L}$  of 5 M KAc (pH 8.0) was added to the mixture and kept on ice for 15 min. Centrifugation was followed at 12,000 rpm for 15 min. The upper phase was transferred to a new tube. One volume of chloroform–isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 10 min. This step was repeated once more. The aqueous phase was precipitated with one volume of cold isopropanol at –20 °C for 1 h and collected by centrifugation at 12,000 rpm for 10 min. Finally, the precipitate was washed with 75% ethanol, dried and dissolved in double distilled water. The concentration and the quality of isolated DNA were assessed by electrophoresis on 1.0% agarose gel. DNA concentration of each sample was adjusted to 50  $\text{ng } \mu\text{L}^{-1}$ .

#### 2.4. ITS nrDNA and *rbcl* gene amplification and sequencing

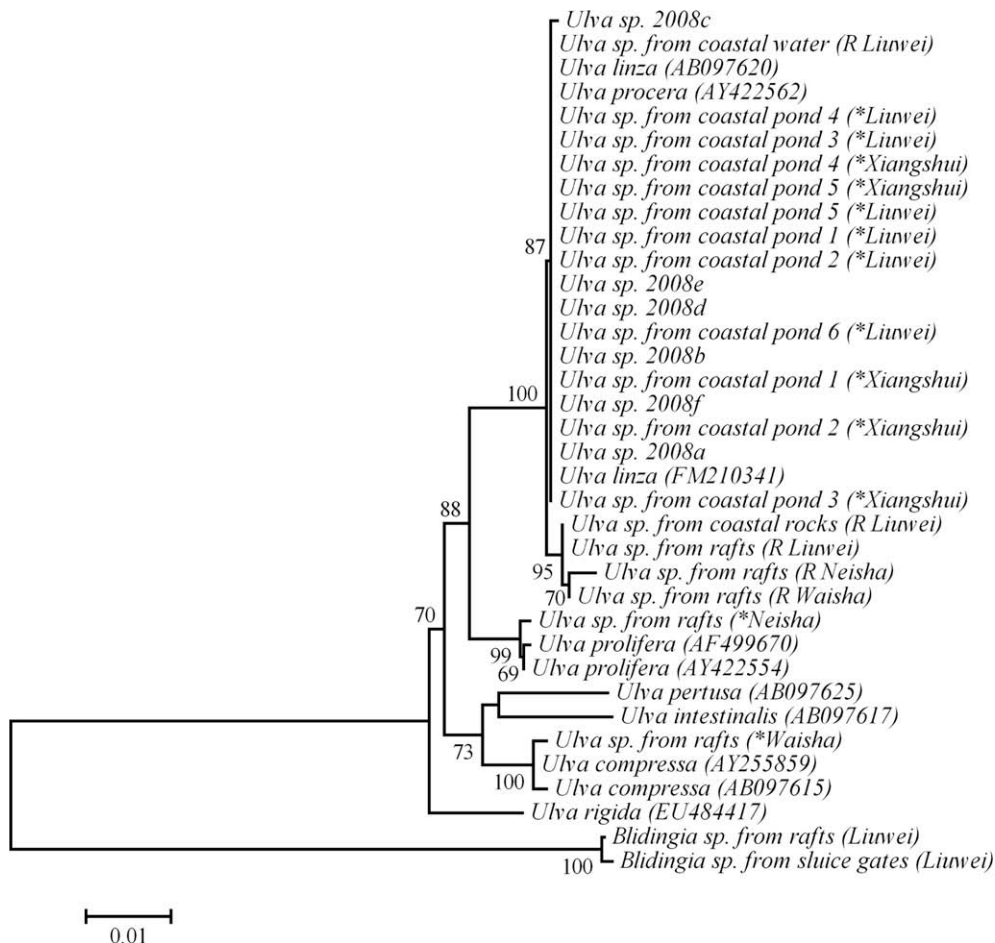
Polymerase chain reaction (PCR) amplification of ITS nrDNA and *rbcl* gene was as described by Hayden et al. (2003). Primers used to amplify and sequence ITS nrDNA and *rbcl* gene were synthesized by Shanghai Sangon Biologic Engineering Technology & Service Co., Ltd., with the following sequence:

- ITS1 (5'-TCTTTGAAACCGTATCGTGA-3'),
- ITS2 (5'-GCTTATTGATATGCTTAAGTTCAGCGGT-3'),
- *rbcl*L1 (5'-ATGTCACCACAAACAGAACTAAAGC-3'),
- *rbcl*L2 (5'-AATTCAAATTTAATTTCTTCC-3').

Total genomic DNA (30–40 ng) was added to 50  $\mu\text{L}$  PCR reactions containing final concentrations of 1  $\times$  PCR buffer (Takara, Japan), 2 mM  $\text{MgCl}_2$  (Takara), 0.8 mM dNTPs (Takara), 25  $\mu\text{M}$  of each primer, and 1.6 U Taq Polymerase (Takara). Amplification products were separated by 1.0% agarose gel electrophoresis and fragments of an expected length were cut from the gel and purified by use of a DNA Gel Extraction Kit (Bio Basic Inc., Canada) according to the manufacturer's instructions. ITS nrDNA and *rbcl* gene were sequenced on both strands using ABI 3730 XL automated sequencers (Shanghai Biosune Biotechnology Co., Ltd.). Each sequencing reaction was repeated twice.

#### 2.5. Phylogenetic analysis

ITS nrDNA and *rbcl* gene were sequenced from 26 algal samples including 20 from 2009s field sampling and 6 from 2008s bloom in Qingdao. Additional 15 ITS nrDNA and 10 *rbcl* sequences were downloaded from the GenBank. The ITS nrDNA and *rbcl* sequences were aligned for phylogenetic analyses using Clustal W (Thompson et al., 1994). For the *rbcl* sequence data set, the alignment was



**Fig. 4.** Neighbor-joining (NJ) tree constructed from the analysis of *rbcl* gene sequences of collected algal samples along Jiangsu coasts and those downloaded from Genebank for comparison. The tree was rooted with *Blidingia* sp. 1 collected from *Porphyra* rafts and *Blidingia* sp. 2 collected from a sluice gate at Liuwei, respectively. The numbers under the branches represent full heuristic bootstrap values (1000 replicates) greater than 50%. Branch lengths are proportional to the amount of sequence change, which are indicated by the scale bar below the tree (in the parentheses, \* Refer to the samples that were thin filamentous with ramifications; R refers to ribbon in morphology).

unambiguous. But the ITS nrDNA alignment contained many insertions and deletions. The combined data set contained the ITS nrDNA and *rbcL* sequences (Hayden et al., 2003). Prior to analysis of the combined data, the incongruence length difference test was conducted. The phylogenetic trees were constructed by neighbor-joining (NJ) method using the program Mega 4.0 (Jiang et al., 2008). *Blidingia* sp., collected from rafts and sluice gate from Liuwei, served as an outgroup taxon. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. 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.

## 2.6. Sporulation tests

Unialgal culture (individual plant) isolated from Qingdao's bloom was used throughout the test. In addition, a filamentous strain isolated from Jiangsu coast was used in the temperature tests for comparison. The filamentous algal material was cut into short fragments (2–3 cm). Ten fragments were selected and put into each well (5–7 mL) of a multi-well culture plates filled with PES. A series of temperature (5, 10, 12, 15 and 18 °C) and salinities (30, 24 and 18) were tested. For the salinity test, all cultures were maintained at 18 °C. All cultures were exposed to 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a 12 h light per day light–dark regime. For each testing condition, sporulation rate was calculated as the averaged percentage of total sporulated fragments as identified by microscopic checking on day 6 ( $n = 6$ , totally 60 fragments were checked and counted in each condition). The cultures were performed in six GXZ-260C light and temperature-controlled photo-incubators (Ningbo Jiangnan Instrument, China) with temperature deviation less than 0.5 °C.



**Fig. 5.** Neighbor-joining (NJ) tree constructed from the analysis of the nuclear encoded internal transcribed spacer DNA (ITS nrDNA) region, including the 5.8S gene, of collected algal samples along Jiangsu coasts and those downloaded from the Genbank for comparison. The tree was rooted with *Blidingia* sp. 1 collected from *Porphyra* rafts and *Blidingia* sp. 2 collected from a sluice gate at Liuwei, respectively. The numbers under the branches represent full heuristic bootstrap values (1000 replicates) greater than 50%. Branch lengths are proportional to the amount of sequence change, which are indicated by the scale bar below the tree (in the parentheses, \* Refer to the samples that were thin filamentous with ramifications; R refers to ribbon in morphology).

### 3. Results

#### 3.1. Phylogenetic analysis of the algal samples

Analyzed together with control sequences downloaded from the Genbank, the more conserved *rbcL* gene sequences (1288 bp in length) revealed that the dominating algal samples collected from 11 AAPs and one sample derived from coastal water, all tubular and filamentous with ramifications, were in the same clade as all the six unialgal cultures isolated from the Qingdao 2008 bloom (Fig. 4; 2008a–f). Three dominating algal samples from *Porphyra* rafts and one from intertidal rocks, all ribbon in structure, were closely forming two adjacent clades. One algal sample from *Porphyra* rafts collected from Neisha (filamentous with ramifications) was in the fourth clade together with *U. prolifera* (AF499670) and *U. prolifera* (AY422554). One filamentous algal sample from Waisha was classified in the same clade as *U. compressa* (AY255859) and *U. compressa* (AB097615). Two fine filamentous algal samples, one from *Porphyra* rafts and one from the pond sluice gate, respectively, were identified as *Blidingia minima* var. *minima* (according to the ITS nrDNA sequences below) and were treated therefore as an outgroup.

The ITS nrDNA sequence analyses showed that 12 algal samples – one from intertidal rocks (filamentous), two from *Porphyra* rafts (one filamentous, one ribbon-like), seven from AAPs, one from coastal water (obtained from microscopic germlings after 3 week culture in the lab) at Liuwei and one from AAPs at Xiangshui were closely related to the Qingdao algal bloom samples (filamentous) (Fig. 5; 2008a–e). Samples isolated from *Porphyra* rafts at Neisha, Waisha and some of the AAPs at Liuwei were in the third clade. Two samples from AAPs at Xiangshui were in the fourth clade together with *U. compressa* (AF035350). Two dominating filamentous algal samples, isolated from *Porphyra* rafts and from the sluice gate, respectively, were identified as belonging to the genus *Blidingia*

and were treated as an outgroup, together with *B. minima* (AJ000206).

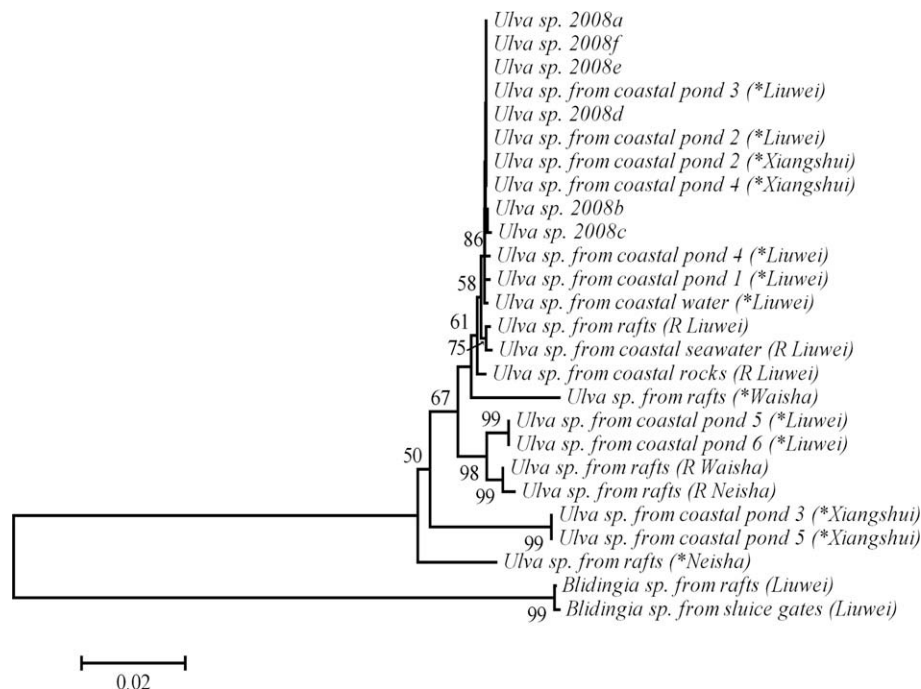
Combined ITS nrDNA and *rbcL* sequence analyses revealed identical results, in which six filamentous algal samples from AAPs, and one coastal water-derived filamentous sample, were closely related to the dominating Qingdao algal bloom in 2008 (Fig. 6). Other filamentous algal samples joined together in other clades.

#### 3.2. Morphological observations

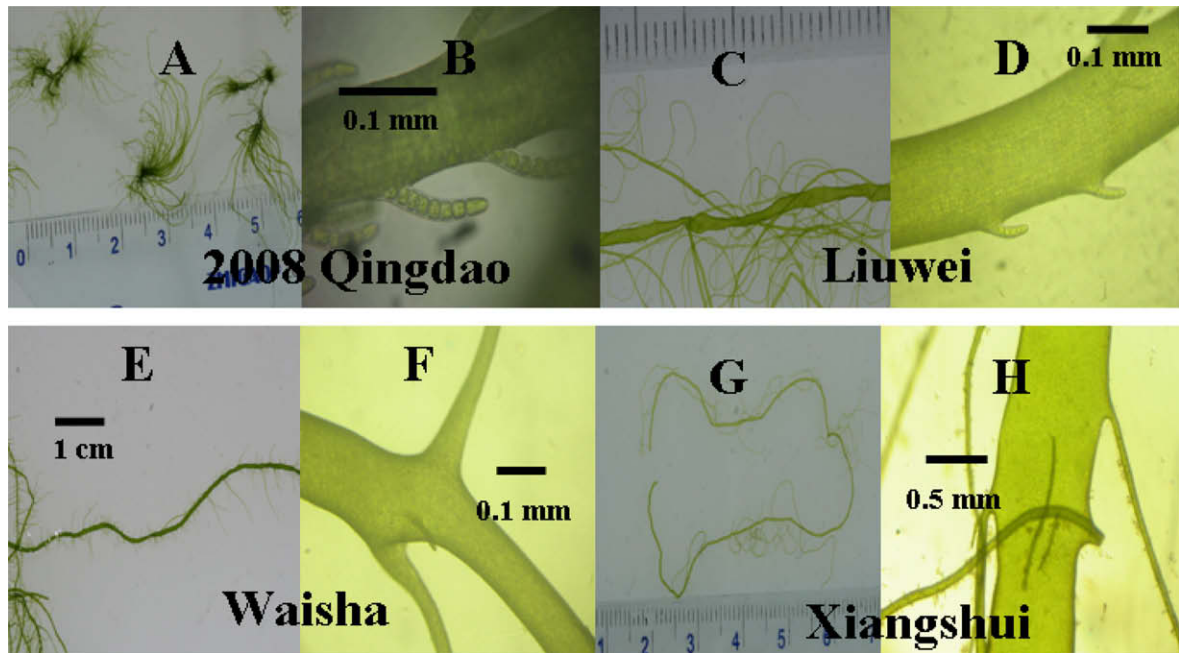
Green algae including *Cladophora* sp., *Blidingia* sp. and *Ulva* sp. (ribbon and filamentous in structures) were found in the dominating algal samples collected from the full and semi-floating *Porphyra* rafts at six cultivation locations including Xishu (full-floating), Liuwei (semi-floating), Xiangshui (full-floating), Waisha (full-floating) and Neisha (semi-floating). *Ulva* sp. could be easily distinguished under the microscope, while *Blidingia* sp. needs molecular data to be identified. While in most of the investigated AAPs, usually only one dominating *Ulva* species existed as can be judged by the morphology (Fig. 7), probably determined by the unique water environment in those ponds in comparison with the apparent versatile environment on the rafts in the surface water in intertidal zone.

#### 3.3. Microscopic germlings in the water

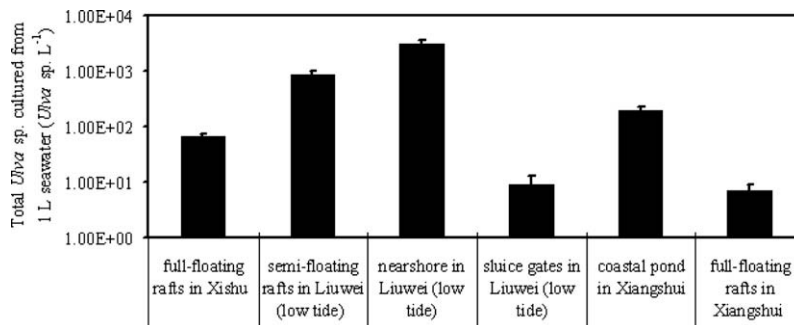
Filamentous *Ulva* were obtained from microscopic germlings in the waters from all six investigated locations. Density varied from less than 7 to 3140 individuals L<sup>-1</sup> (Fig. 8). Higher densities were detected at low tide in Liuwei in nearshore waters. In the relatively clear water sampled from a representative AAPs, the density of the germlings was 500 individuals L<sup>-1</sup>. A surprisingly low number was recorded at the sluice gates where the effluents from the AAPs were collectively discharged into coastal waters. These results



**Fig. 6.** Neighbor-joining (NJ) tree constructed from the analysis of combined ITS nrDNA and *rbcL* sequences. The tree was rooted with *Blidingia* sp. 1 collected from *Porphyra* rafts and *Blidingia* sp. 2 collected from a sluice gate at Liuwei, respectively. The numbers under the branches represent full heuristic bootstrap values (1000 replicates) greater than 50%. Branch lengths are proportional to the amount of sequence change, which are indicated by the scale bar below the tree (in the parentheses, \* Refer to the samples that were thin filamentous with ramifications; R refers to ribbon in morphology).



**Fig. 7.** Photographs of the filamentous algal samples collected from the Qingdao 2008s bloom (A and B), Liuwei (C and D), Waisha (E and F) and Xiangshui (G and H), Jiangsu province. They show the typical tubular and filamentous structure with lateral ramifications.



**Fig. 8.** Density of microscopic stages of *Ulva* spp. in seawater collected from six locations, including *Porphyra* full-floating rafts in Xishu, *Porphyra* semi-floating rafts in Liuwei, nearshore waters at Liuwei, a sluice gate at Liuwei, a coastal pond in Xiangshui and *Porphyra* full-floating rafts in Xiangshui.

showed the universal existence of the microscopic stages of *Ulva* sp. in all the sampling sites investigated.

### 3.4. Sporulation tests in the isolated fragments of the dominating *Ulva* sp. of the bloom

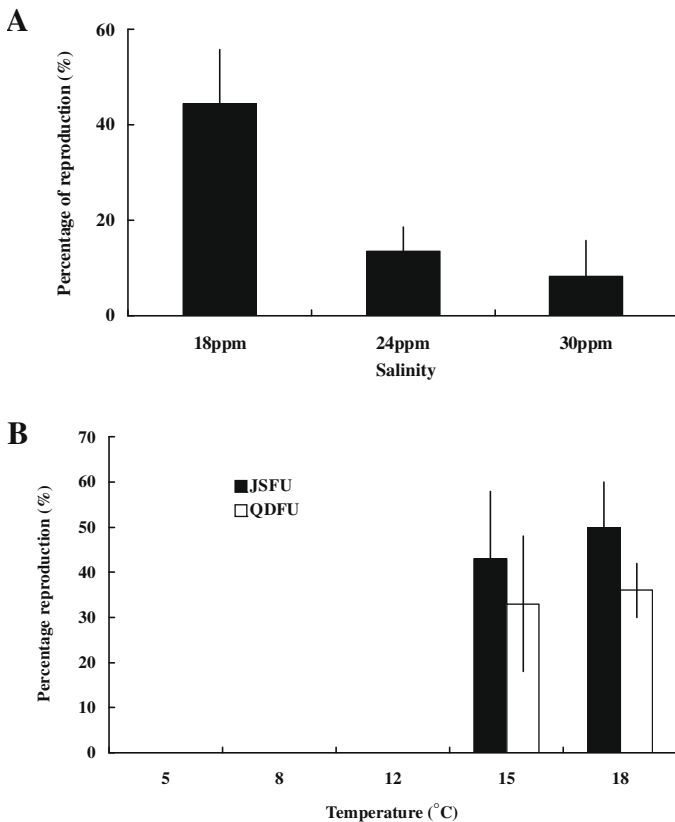
In culture conditions, low salinities and higher temperature facilitated sporulation in isolated filamentous *Ulva* fragments (Fig. 9A and B). At temperatures of 12 °C, sporulations were sparsely spotted in the tested fragments, indicating that low temperature greatly hampered the production of the gametes. No significant difference was found in the two dominating filamentous *Ulva* species in the temperature test.

## 4. Discussion

In elucidating the “seed” source of the largest drifting macroalgal bloom so far observed, data obtained in this investigation points to three important aspects: (1) both morphological and molecular evidences showed that the filamentous *Ulva* sp. ubiquitously living year-round in the AAPs along the Jiangsu coasts mostly resemble the dominating algal species isolated in the 2008 Qingdao green tide event, in comparison to those isolated

from *Porphyra* rafts. AAPs, at the scale of thousands of hectares along the coasts of Jiangsu province, are characterized by higher light penetration, nutrient levels after being artificially fertilized to enrich the pond water and higher temperature in early summer. Thus they constitute the most efficient year-round *Ulva* nurturing niche to sustain this tolerant green algae; (2) the green algal species attached on *Porphyra* rafts were diverse and came originally from the microscopic stages of the algae that were free-living in the water along the entire coast of Jiangsu province. Therefore *Porphyra* raft itself could not become the principal and original source of the blooming algae; and (3) high levels of nutrients derived from AAPs and land-based effluents discharged in the coastal waters of Jiangsu province, in combination with the year-round existence of the green tide forming algae in thousands of hectares of AAPs are potentially constituting two most important triggering factors of the recurrent spring green tides in the Yellow Sea.

In tracking the original source of the 2008 Qingdao bloom, Liu et al. (2009) hypothesized that thousand hectares of *Porphyra* rafts in the intertidal zone along Jiangsu province were the original source of the algae. This was supported by the recent rapid expansion of *Porphyra* farming along the coast of blooming area. However, the authors did not provide biologic identification data. Bamboos with attached green algae were found several times in



**Fig. 9.** Sporulation (percentages of reproduction) of fragments of the dominating alga in the 2008 Qingdao's bloom under different salinities (A) and temperatures (B). In B, JSFU refers to Jiangsu filamentous *Ulva* for comparison; QDFU refers to Qingdao filamentous *Ulva*.

the algal biomass accumulated in Qingdao in 2008, bringing people's attention to the *Porphyra* raft systems. Jiang et al. (2008) pointed out that the dominating alga found in the drifting biomass in Qingdao was not locally derived based on the ITS phylogenetic analysis. In the present investigation it was shown that *Porphyra* rafts did support the growth of diverse green algae, but with few identical to the dominating alga of the Qingdao's bloom. Free-living microscopic stages of *Ulva* sp. isolated from the coastal waters of Liuwei, which were later identified to be very close to the dominating alga in the 2008 Qingdao bloom, were found to be in the waters when the temperature was only 5 °C and there were small amounts of green algae to be spotted at that time on the *Porphyra* rafts (personal observations). High density of culture-derived seedlings of *Ulva* sp. in Jiangsu coastal waters strongly supported the year-round existence of the algae in the water, even when the *Porphyra* cultivation raft systems are not in place. The key issue is where the large amount of the algae in their microscopic stages originated. *Porphyra* cultivation has been carried out for more than 30 years, while large-scale blooms of *Ulva* in the Yellow Sea are only a recent phenomenon in the past 3 years.

The data of this investigation support the view that AAPs along the Jiangsu province coast are the principal source of the green tide algae. Both morphological identifications and gene sequence data confirmed this point. Accurate identification of *Ulva* species has been puzzling scientists for a long time. Independent analyses of either morphology or gene sequence comparison alone had been shown to be insufficient. A combination of both approaches can lead to reliable identification of the morphotypes in nearly all the cases (Hayden et al., 2003). The dominating alga in the 2008 Qingdao's bloom is filamentous, tubular in structure and characterized

with intensive ramifications (Sun et al., 2008). The ITS nrDNA and *rbcl* sequences were highly uniform in all the samples collected from the Yellow Sea (Ye et al., 2008; Jiang et al., 2008). In this investigation, similar filamentous *Ulva*, with few or intensive ramifications were found in nearly all observed AAPs and were closely related with the 2008 Qingdao bloom in ITS nrDNA and *rbcl* gene sequences. Furthermore, AAPs are operating year-round and, thus constantly contributing to coastal waters the microscopic stages of *Ulva* as well as the thallus fragments. While fragmented thalli tend to undergo sporulation rapidly, releasing even more spores in coastal waters, especially under elevated light and temperature regimes (Lüning et al., 2008; this observation). Spore contribution could be accelerated during early spring when temperature and light levels start to increase. Frequent and efficient sporulation secures a rapid and wide spreading of gametes or spores in the water. The longevity of the gametes or spores that were attached to the muddy particles remains to be resolved in future work. The propagule-attached particles might accumulate in the coastal waters of Jiangsu province over time, thus forming the potential base for future blooming biomass when light, temperature, nutrient and current conditions coincide.

It is worth noting that filamentous *Ulva* were ubiquitous in nearly all observed AAPs we visited with the only exceptions being those treated with so called "*Ulva*-killer" (Prometryn 2,4-bio(iso-propylamino)-6-methylthio-s-triazine). This chemical was used in combination with sand and applied in ponds to block the sessile *Ulva* on the pond bottom. In pond farming, *Ulva* has to be removed periodically to prevent the competition with microalgae for nutrients. An appropriate amount of the latter in the water (locally called "colored water") is thought to be beneficial for the healthy growth of shrimps or crabs. How the wide application of this chemical affects the balance between the floating *Ulva* biomass and the microalgae populations in the coastal waters (both are nutrient scrubbers) in Jiangsu province remains to be resolved.

In summary, this investigation has shown that the *Ulva* biomass in year-round operated AAPs along the coasts of Jiangsu province constitutes a potential, principal source of the bloom-forming alga in the adjacent Yellow Sea. How the seasonal climatic parameters, surface water currents, intensive nutrient applications are all converging to prepare the conditions triggering the development of large floating green tide biomass along the coast needs to be comprehensively investigated both in the field and in the laboratory. Results of such investigation will be applicable within the wider perspective of integrated coastal zone management to hopefully find biomitigative solutions to what appears to be a significant and recurrent ecological problem, not only in the Qingdao region but also in other places throughout the world.

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#### References

- Cohen, R.A., Fong, P., 2006. Using opportunistic green macroalgae as indicators of nitrogen supply and sources to estuaries. *Ecological Applications* 16, 1405–1420.
- Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., Lancelot, C., Likens, G.E., 2009. Controlling eutrophication: nitrogen and phosphorus. *Science* 323, 1014–1015.
- Dan, A., Hiraoka, M., Ohno, M., Critchley, A.T., 2002. Observations on the effect of salinity and photon fluence rate on the induction of sporulation and rhizoid formation in the green alga *Enteromorpha prolifera* (Müller) J. Agardh (Chlorophyta, Ulvales). *Fishery Sciences* 68, 1182–1188.



- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Hayden, H.S., Blomster, J., Maggs, C.A., Silva, P.C., Stanhope, M.J., Waaland, J.R., 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology* 38, 277–294.
- Jiang, P., Wang, J.F., Cui, Y.L., Li, Y.X., Lin, H.Z., Qin, S., 2008. Molecular phylogenetic analysis of attached Ulvaceae species and free-floating *Enteromorpha* from Qingdao coasts in 2007. *Chinese Journal of Oceanology and Limnology* 26, 276–279.
- Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.
- Largo, D.B., Sembrano, J., Hiraoka, M., Ohno, M., 2004. Taxonomic and ecological profile of “green tides” species of *Ulva* (Ulvales, Chlorophyta) in central Philippines. *Hydrobiologia* 512, 247–253.
- Leliaert, F., Malta, E.J., Engelen, A.H., Mineur, F., De Clerck, O., 2008. Qingdao algal bloom culprit identified. *Marine Pollution Bulletin* 56, 1516.
- Leliaert, F., Zhang, X., Ye, N., Malta, E.J., Engelen, A.H., Mineur, F., Verbruggen, H., De Clerck, O., 2009. Identity of the Qingdao algal bloom. *Phycological Research* 57, 147–151.
- Leskinen, E., Alström-Rapaport, C., Pamilo, P., 2004. Phylogeographical structure, distribution and genetic variation of the green algae *Ulva intestinalis* and *U. Compressa* (Chlorophyta) in the Baltic Sea area. *Molecular Ecology* 13, 2257–2265.
- Liu, D.Y., Keesing, J.K., Xing, Q.G., Shi, P., 2009. World's largest macroalgal bloom caused by expansion of seaweed aquaculture in China. *Marine Pollution Bulletin*. doi:10.1016/j.marpolbul.2009.01.013.
- Lüning, K., Kadel, P., Pang, S.J., 2008. Control of reproduction rhythmicity by environmental and endogenous signals in *Ulva pseudocurvata* (Chlorophyta). *Journal of Phycology* 44, 866–873.
- Malta, E., Draisma, S.G.A., Kamermans, P., 1999. Free-floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. *European Journal of Phycology* 34, 443–454.
- Nelson, T.A., Haberlin, K., Nelson, A.V., Ribarich, H., Hotchkiss, R., Van Alstyne, K.L., Buckingham, L., Simunds, D.J., Fredrickson, K., 2008. Ecological and physiological controls of species composition in green macroalgal blooms. *Ecology* 89, 1287–1298.
- Shang, Z.T., Jiang, M.S., Pu, M.J., 2008. Analysis of the general situations of Laver culture in Jiangsu province climatic suitability. *Journal of Anhui Agricultural Sciences* 36 (13), 5315–5319 (in Chinese with English abstract).
- Shimada, S., Yokoyama, N., Arai, S., Hiraoka, M., 2008. Phylogeography of the genus *Ulva* (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. *Journal of Applied Phycology* 20, 979–989.
- Sun, S., Wang, F., Li, C.L., Qin, S., Zhou, M.J., Ding, L.P., Pang, S.J., Duan, D.L., Wang, G.C., Yin, B.S., Yu, R.C., Jiang, P., Liu, Z.L., Zhang, G.T., Fei, X.G., Zhou, M., 2008. Emerging challenges: massive green algal blooms in the Yellow Sea. *Nature Proceedings*. hdl:10101/npre.2008.2266.1.
- Tan, I.H., Blomster, J., Hansen, G., Leskinen, E., Maggs, C.A., Mann, D.G., Sluiman, H.J., Stanhope, M.J., 1999. Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds, *Ulva* and *Enteromorpha*. *Molecular Biology and Evolution* 16, 1011–1018.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Tseng, C.K., 1983. *Common Seaweeds in China*. Science Press, Beijing.
- Wang, D., Wang, X.L., Li, D.P., Wang, F.J., Duan, D.L., 2006. The genetic analysis and germplasm identification of the gametophytes of *Undaria pinnatifida* (Phaeophyceae) with RAPD method. *Journal of Applied Phycology* 18, 801–809.
- Ye, N.H., Zhuang, Z.M., Jin, X.S., Wang, Q.Y., Zhang, X.W., Li, D.M., Wang, H.X., Mao, Y.Z., Jiang, Z.J., Li, B., Xue, Z.X., 2008. China is on the tracking *Enteromorpha* spp. forming green tide. *Nature Proceedings*. hdl:10101/npre.2008.2352.1.