

# Somatic cells serve as a potential propagule bank of *Enteromorpha prolifera* forming a green tide in the Yellow Sea, China

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**Abstract** For the last 2 years, vast accumulations of the unattached filamentous green alga, *Enteromorpha prolifera*, have occurred during summer along the coastal region of the Yellow Sea, China. However, algae do not seem to occur after the end of the fertile season. It has been suggested that banks of microscopic forms of the algae, primarily spores, function as a survival mechanism for this opportunistic alga. Therefore, in this study, field surveys and laboratory cultures were conducted to determine if somatic cells were serving as a propagule bank to enable the algae to survive through periods of unfavorable conditions. Laboratory experiments demonstrated that somatic regeneration was one of the most important approaches by which *E. prolifera* colonized and flourished in the study area. Indeed, at least 19.32% of somatic cells from the filamentous segments could survive for 2 months under various temperatures (0, 5, 10, 15, 20, and 30°C at an irradiance of 60 μmol photons m<sup>-2</sup> s<sup>-1</sup>) and irradiances (darkness, 5, 10, 15, 20 and 30 μmol photons m<sup>-2</sup> s<sup>-1</sup> at a temperature of 20°C). Additionally, greater than 35.85% of the somatic cells could survive at 0°C or in darkness for

2 months, and no less than 15.99% of these cells resumed growth when the temperature and irradiance were adjusted to the normal levels (20°C and 60 μmol photons m<sup>-2</sup> s<sup>-1</sup>). Furthermore, the results of field surveys revealed that viable *E. prolifera* was widespread in high quantities in the sediment of the Yellow Sea when the macroalga was absent. Taken together, the results of this study suggest that somatic cells may act as an overwintering stage for the annual spring bloom of *E. prolifera*. These findings should be useful in future studies conducted to behavior of somatic cells in green tide as well as in the management of future spring blooms of *E. prolifera*.

**Keywords** *Enteromorpha prolifera* · Green tide · Microscopic forms · Propagule bank · Regeneration · Somatic cells

## Introduction

Algae belonging to the genus *Enteromorpha* are commonly found in sandy or muddy intertidal flats along the coastline of China (Tseng 1984; Jiang et al. 2008); however, they were never reported in thick mats prior to 2007. *Enteromorpha* spp. bloomed for the first time in the middle area of the Yellow Sea in 2007 (Liang et al. 2008; Sun et al. 2008). In addition, a large-scale *Enteromorpha prolifera* (O.F. Müller) J. Agardh forming a green tide broke out in the middle and south area of the Yellow Sea in late May, 2008 (Sun et al. 2008; Ye et al. 2008; Zhang et al. 2008). By 16 July 2008, 1 million tons of algae had been cleared from one of the severely affected areas, Qingdao City, China (Leliaert et al. 2008). Green tides are not unique to China, but have been recorded for three decades in marine

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and estuarine environments worldwide (e.g., Fletcher 1996, Schories et al. 2000; Charlier et al. 2007). In addition to having negative effects on tourism, large algal mats can also have deleterious ecological effects, including the uncoupling of biogeochemical cycles in sediments from those in water column (Valiela et al. 1997), a negative impact on seagrass beds due to shading, disruption of feeding by wading birds (Raffaelli et al. 1998), development of a lethal environment due to oxygen deficiency (Charlier et al. 2007), and a shift from a high-diversity mixture to low-diversity assemblages of fast-growing annuals (Worm et al. 2001). Because of their ecological and economic impact, green tides have drawn considerable attention from scientists and governments (Fletcher 1996; Valiela et al. 1997; Raffaelli et al. 1998).

Eutrophication is the primary cause of green tides and may explain the extensive growth of opportunistic macroalgal species that are able to take advantage of such conditions (Fletcher 1996; Morand and Briand 1996; Largo et al. 2004). However, little is known about the survival and successful development of green macroalgae, which may play a role in regulating the seasonal metagenesis. The free-living green alga mats can be produced by overwintering adult plants that persist partially embedded in the sediment and begin growing in the following spring (Kamermans et al. 1998). However, such mats can also be produced from settled spores, vegetative fragments, or other microscopic forms of the life cycle that remain dormant or survive with little growth until environmental conditions become favorable (Santelices 1990; Hoffmann and Santelices 1991; Worm et al. 2001).

Banks of microscopic forms (Chapman 1986) that survive long periods of unfavorable environmental conditions and then recover following severe disturbances are crucial to the persistence of algae populations that occur in temporally variable environments (Carney and Edwards 2006). *Enteromorpha* have developed life histories in which the microscopic stages suspend growth during unfavorable conditions and then re-establish activity when the conditions improve (Schories and Reise 1993; Schories 1995; Kolwalkar et al. 2007). Although previous studies have revealed that the reproduction of *Enteromorpha* is primarily due to the presence of asexual zoospores that are derived from the distal end of the thalli (Callow 1996; Worm et al. 2001; Eriksson and Johansson 2005), pathways of reproduction for *E. prolifera* are known to be multifarious, and include sexual, asexual, and vegetative propagation (Dan et al. 1997; Lin et al. 2008; Ye et al. 2008). Additionally, Schories (1995) reported that in calm areas, overwintering of adult plants or plant fragments was a common process to ensure the development of a mass during the next season, whereas the distribution of *Enter-*

*omorpha* on exposed sandy tidal flats depends on recruitment by juvenile stages.

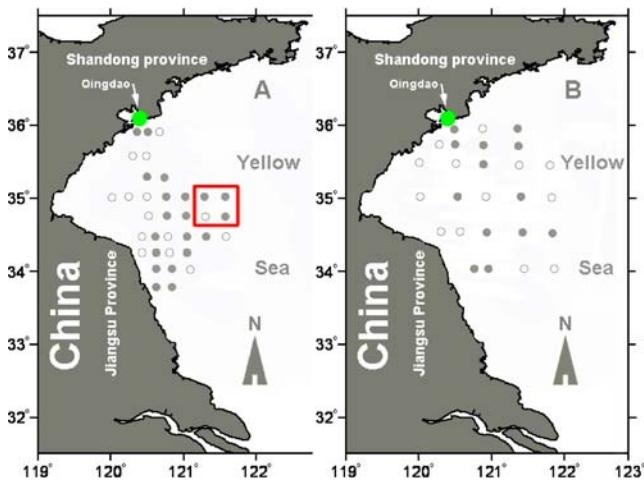
Previously, we demonstrated that somatic cells of *E. prolifera* thalli can regenerate into new individuals under laboratory conditions (Ye et al. 2008). In this study, both laboratory experiments and field surveys covering most of the area in the Yellow Sea that was affected by the green tide were conducted to explore the likelihood of somatic cells as a major element that leading to the development of large algal mats.

## Materials and methods

Floating specimens of *Enteromorpha prolifera* were collected between May and early June of 2008 from the intertidal zone (35°35'N, 119°30'E, 20–50 cm depth) of Zhanqiao Wharf, Qingdao, China, and then cultured in the laboratory. Seawater was collected at the time of sampling using a pump placed 2 m below the surface and filtered with nested plankton nets (200  $\mu\text{m}$  net with a 20  $\mu\text{m}$  net inside). The collected water was autoclaved and made into ES-enriched seawater (McLachlan 1979). In the laboratory, the thalli were examined and those that were intact were isolated, washed several times with the sterile seawater, sterilized with 1% sodium hypochlorite for 2 min, and then rinsed with the autoclaved seawater. The sterilized material was then placed into an aquarium ( $d=40$  cm,  $h=30$  cm) containing enriched seawater, aerated and maintained at 20°C, under a 12:12 h LD photoperiod with 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  being provided by cool-white fluorescent tubes.

## Field survey

A number of rapid-response studies, including shipboard surveys along the coastline, were conducted during the bloom and post-bloom periods in 2008 (Sun et al. 2008). In addition, from June to August of 2008, samples were collected from a large scale of alongshore sediment that was covered with a deposit of viable *E. prolifera* and found by a scuba diver. To avoid repeating tasks, two oceanographic comprehensive surveys were conducted offshore throughout the green tide affected area (120°00'–122°00'E, 33°50'–36°00'N) from June 9–16 and August 9–13 of 2008 using the Marine Scientific Research Vessel, Beidou (Fig. 1). Free-floating samples were collected using a filtration net and the sediment samples were obtained using an Agassiz trawl or a stainless sediment corer. Some of the samples were cultured in Petri dishes immediately under the same conditions described above, after which the somatic cells were



**Fig. 1** Map showing locations in the Yellow Sea investigated by the Beidou Marine Scientific Research Vessel. **a** Locations of the first survey conducted from June 9–16, 2008. Solid circles represent the sites where floating samples were collected. The box indicates the area in which the green tide was first detected on May 15, which is approximately 150 km southeast of Qingdao City. **b** Locations of the second survey conducted from August 9–13, 2008. Solid circles represent the sites where sediment samples were obtained

checked for viability and to ensure they were intact by staining with neutral red and Evans blue. Others samples were stored at  $-18^{\circ}\text{C}$  for future study.

#### Effect of temperatures and photon flux densities on somatic cells

For the survival and regeneration investigations, *E. prolifera* thalli were sterilized again with 1% sodium hypochlorite for 2 min and then rinsed with autoclaved seawater. The surface moisture was then immediately removed with three layers of absorbent paper, after which the clean algae were cut into approximately 1.5-cm-length segments and cultured in Petri dishes containing ES-enriched seawater in a cultivation chamber. At least 20 branchlets were placed in every Petri dish, and every treatment included ten Petri dishes. Temperature treatments included six temperatures (0, 5, 10, 15, 20,  $30^{\circ}\text{C}$ ) under  $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light provided by 40 W cool-white fluorescent tubes. In addition, samples were subjected to one of the following irradiance treatments: darkness, 5, 10, 15, 20, or  $30\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . All photo experiments were conducted at  $20^{\circ}\text{C}$  and all the materials were subjected to a 12:12 LD photoperiod throughout the experiment. Total darkness was obtained by placing the culture dishes into light-tight boxes. The culture medium used in the experiment was replaced every 3 days, at which time observations were made.

To stress the cultures, algae were initially cultured under normal temperature and irradiance ( $20^{\circ}\text{C}$ ,  $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), after which the temperature was changed  $2.5^{\circ}\text{C}$  per day and an additional  $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$  per day were provided. After 2 months of stress culture, the temperatures and irradiances of four combinations ( $0^{\circ}\text{C}$ ,  $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ;  $5^{\circ}\text{C}$ ,  $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ;  $20^{\circ}\text{C}$ , darkness;  $20^{\circ}\text{C}$ ,  $5\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) were gradually adjusted to normal following the same procedure levels described above. When the culture medium for samples that were cultured under darkness was changed, a  $5\mu\text{mol m}^{-2} \text{s}^{-1}$  red light lamp was used. Each individual culture was exposed to this irradiance source for 20 and 40 s when the culture medium was changed (Santelices et al. 2002).

#### Microscopy and photography

Observations were conducted using a light microscope (Nikon, ECLIPSE 50i, Tokyo, Japan). The number of the viable and germinated somatic cells was determined using an inverted microscope (ZEISS, HBO 50, Jena, Germany) at a magnification of  $\times 200$ . Briefly, three Petri dishes were selected at random from each treatment and at least five randomly selected branchlets were enclosed in each dish. Next, three full microscope fields of view were observed randomly on each selected segment. The survival rate (%) was the number of cells that survived divided by all of the observed cells  $\times 100$ ; whereas the germination rate (%) was the number of germinated cells divided by all of the observed cells  $\times 100\%$ .

When more than half of a cell appeared in the field of vision, it was included in the analysis, whereas when less than half of a cell appeared it was excluded. In addition, several of the branchlets that developed into individuals were excluded and dead segments were counted as zero. Some of the branchlets became pale and suffered from cell lyses; therefore, the exact survival rate could not be determined. However, prior to the start of the experiment, the mean value of the number of somatic cells in one field of view at a magnification of  $\times 200$  was determined to be 216.47 ( $n=32$ ) using fresh filaments; therefore, this value was used when dead cells were not observed.

#### Statistical analysis

Analysis of variance (ANOVA) was followed by Duncan's New Multiple Range (DNMR) test when appropriate. All statistical analyses were conducted using SPSS and  $p < 0.01$  was considered to indicate significance. All data are reported as means  $\pm$  S.D.

## Results

### Samples from scientific research vessel surveys

The first survey was conducted during the period when the green tide covered the largest area and the algae were the most prolific. Twenty floating samples were collected, but no sediment algae were found during the voyage (Fig. 1a). Our previous results revealed that barcodes, ITS (ITS1, 5.8 S rDNA and ITS2) and the Rubisco gene of 20 samples collected throughout of the area affected by the green tide were all the same, even though the morphological characteristics and colors were diverse (Leliaert et al. 2008; Zhang et al. 2008). These findings indicated that one species of algae forms the green tide.

The second survey was performed in August when most of the floating algae had disappeared and those that remained were obviously in senescence. Large quantities of algae were recovered from sediment samples in 13 sites during the second study (Fig. 1b). The deposit filaments were much darker in the samples recovered from the sediment than in the floating algae. Staining revealed that 89.79% of the cells collected from the sediments were viable, and nearly 60% of filaments (by weight) resumed growth after 3 days of culture under the normal conditions.

The investigated area was characterized by a wide range of environmental parameters that fluctuated sharply. The surface temperature of the first survey ranged from 13 to 25°C, and the bottom temperatures of the second survey ranged from 12 to 27°C. The depth of the sites in the second survey ranged from 14 to 25 m, with irradiances that were approximately zero. Data from both surveys revealed that the salinities varied from 29 to 35 PSU and the pH ranged from pH 7.8 to 8.3.

### Development of somatic cells under different temperatures

A major release of spores and gametes occurred within the first 3 weeks of the laboratory experiment in treatments that were maintained at 15°C or above, while filaments cultured at 0 and 5°C seldom released reproductive cells throughout the entire experimental period. After 2 months of culture, the status of the somatic cells was recorded (Fig. 2, A–D, F). Most of the cells cultured at 30°C turned white, while others survived and were distributed sporadically among the dead cells (Fig. 2A). The viable cells in the cultures that were maintained at 20°C grew rapidly and developed into seedlings approximately 200 µm in length (Fig. 2B), whereas cells that were cultured at 15°C did not grow beyond germination (Fig. 2C). The cells that survived at 10 and 5°C swelled, were full of chromatophores and did not germinate (Fig. 2D). Plasmolysis was widespread in

somatic cells cultured at 0°C (Fig. 2F); however, staining revealed that most of these cells were viable.

Analysis of variance (ANOVA) indicated that the temperature variations significantly affected the survival rate of somatic cells ( $F=4.050$ ,  $P<0.01$ ; Fig. 3). Specifically, the survival rate at 5°C was approximately 80.67%, which was much higher than at other temperature levels. In addition, the optimal germination temperature was found to be 20°C ( $F=14.885$ ,  $P=0.000$ ), with significantly lower germination rates being observed at lower temperatures, and no germination being observed at 0 and 5°C. Somatic cells that were cultured for 2 months under lower temperatures (0 and 5°C) could germinate into seedlings when shifted to normal conditions (Fig. 2E and G). Specifically, cells that were cultured at 5°C required 4 days of culture under normal conditions to resume growth, while those that were cultured at 0°C required 2 weeks. Finally, the germination rate of the somatic cells was observed and calculated 1 month after onset of the culture. Cells that were cultured at 0 and 5°C had germination rates of 16.24% and 20.35%, separately (Fig. 4).

### Development of somatic cells under different irradiances

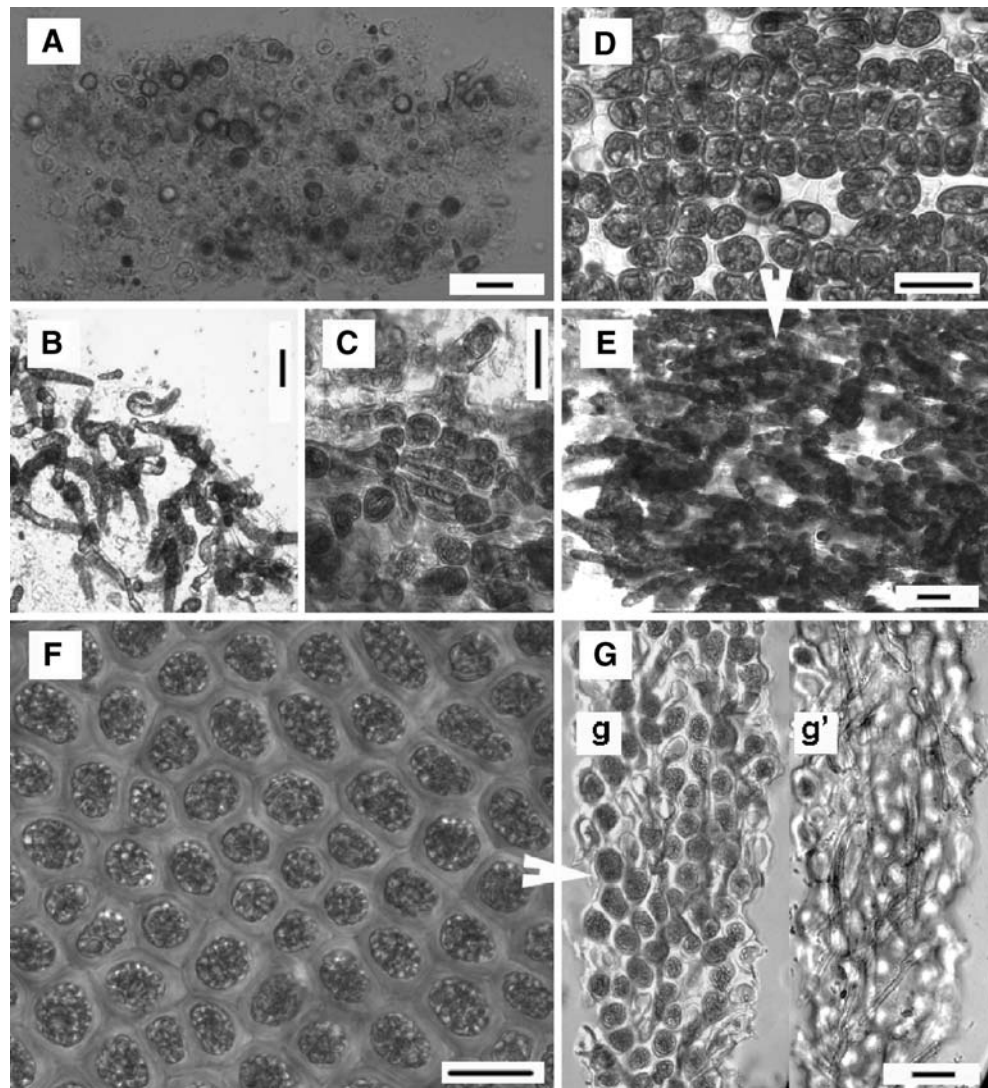
Branchlet cultures under different treatments turned white to different degrees with time, and some did not survive after 2 months of culture. In addition, no spores or gametes were released in treatments that were subjected to irradiances of less than 10 µmol photons m<sup>-2</sup> s<sup>-1</sup>. The survival rate and germination rate of somatic cells cultured under different treatments for 2 months are shown in Fig. 5. Analysis of variance (ANOVA) indicated that the variance in the photo flux densities resulted in different patterns of survival and germination. Specifically, the survival rate did not vary among cultures treated with 0–30 µmol photons m<sup>-2</sup> s<sup>-1</sup> ( $F=1.123$ ,  $P=0.363$ ); however, germination rate varied significantly among cultures subjected to different irradiances ( $F=8.454$ ,  $P=0.000$ ). The maximum germination rates were obtained in response to treatment with 20 µmol photons m<sup>-2</sup> s<sup>-1</sup>, whereas the minimum value, zero, was obtained in response to treatment with 0 and 5 µmol photons m<sup>-2</sup> s<sup>-1</sup>. Furthermore, it took approximately 2 weeks for somatic cells cultured under lower irradiances (0 and 5 µmol photons m<sup>-2</sup> s<sup>-1</sup>) to grow into germlings after being shifted to normal conditions. The germination rate of samples cultured under darkness and at 5 µmol photons m<sup>-2</sup> s<sup>-1</sup> was 15.99% and 17.51%, respectively (Fig. 6).

## Discussion

In the marine environment, macroalgal propagule banks have only recently been described in detail, and the



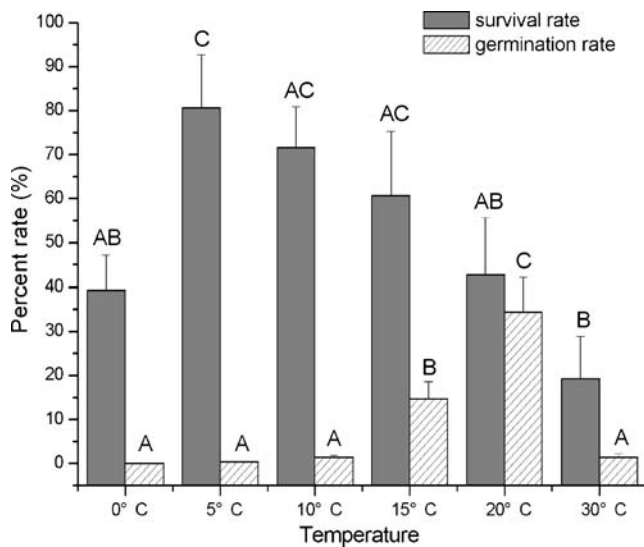
**Fig. 2** The status of the somatic cells cultured under different temperatures. *A–D*, and *F* show the status of somatic cells after 2 months of culture under 0, 5, 15, 20, and 30°C, respectively; *E* and *G* show the status of somatic cells that resumed growth following 1 month of culture under normal conditions after being cultured under 5 and 0°C, respectively, for 2 months. *g* and *g'* show the same cell mass with different foci, with *g* showing the mother cells and *g'* showing newly germinated thalli. Arrows show the shift of the cultured conditions from stress to normal



pertinent information is still far from being understood (Lotze et al. 1999, 2000; Worm et al. 2001; Carney and Edwards 2006). However, it is known that the intensity and composition of destructive macroalgal blooms are strongly affected by the presence of macroalgal propagule banks (Lotze et al. 1999; Worm et al. 2001). Worm et al. (2001) showed that the competitive dominance of the opportunistic *Enteromorpha* spp. was apparently mediated through its extreme dominance in the propagule bank, in which it was 500 to 20,000 times more abundant than other species present in the bank. Fixed macroscopic thalli of *E. prolifera* were not found along the coastline of the Yellow Sea during the past 2 years (Sun et al. 2008), which indicates that it relies on banks of microscopic forms in order to persist through unfavorable seasonal conditions. Thallus segment regeneration of *E. prolifera* has been reported previously (Dan et al. 1997, 2002; Lin et al. 2008; Ye et al. 2008). However, most currently available data regarding the survival capacity of tissues/cells was generated by experi-

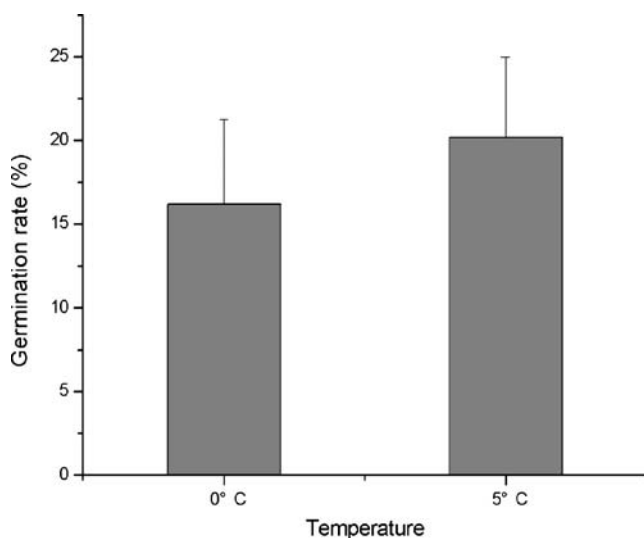
ments conducted to evaluate different problems, and their ecological meaning has not been thoroughly investigated. Dan et al. (1997, 2002) used the chopped tissue of *E. prolifera* for artificial seeding by discharging moveable germ cells. Lin et al. (2008) found that there were seven pathways of reproduction for *E. prolifera*, including cell regeneration. In addition, it has been shown that a large quantity of somatic *E. prolifera* cells regenerated into new individuals when the filaments enter senescent stages (Ye et al. 2008). Finally, Kamermans et al. (1998) found that sexual reproduction was negligible for *Ulva* spp., but that thalli buried in the sediment were able to survive the cold winter when kept in darkness, which was likely responsible for the rapid increase in *Ulva* biomass that was observed in the spring.

*Enteromorpha prolifera* is a benthic species that is widely distributed in the intertidal zones of most global oceans (Sun et al. 2008), especially in subtropical areas (e.g., Tseng 1984). The massive green algae bloom that occurred in the

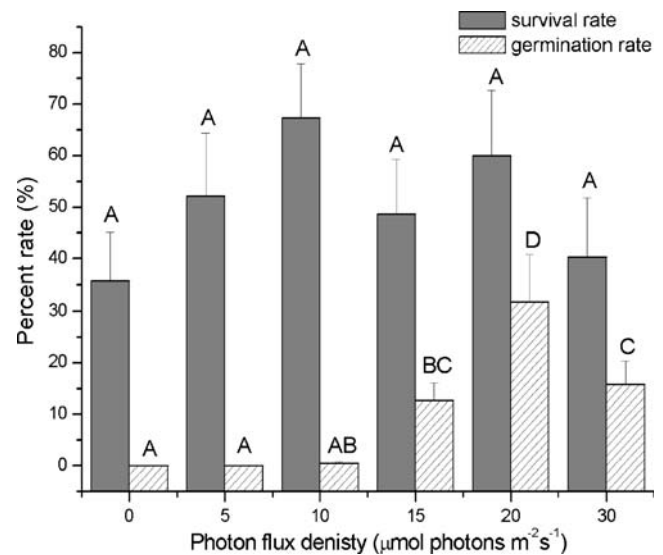


**Fig. 3** The survival rate and germination rate of somatic cells cultured under different temperatures. Values are the means±S.D. In each graph, statistically significant difference (ANOVA, DNMR) are indicated by capital letter superscripts ( $P < 0.01$ )

Yellow Sea in 2008 originated from an offshore area 150 km southeast of Qingdao (Fig. 1A), and then drifted to its final destination on seasonal wind and coastal currents (Sun et al. 2008). The temperature of the sediment environment in the surveyed area is usually above zero; therefore, the minimum temperature we set in the experiment was 0°C. In addition, the light intensity at 3 m below the surface is less than  $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  in the study area, and all of the sites we investigated were sampled to a depth of greater than 14 m. As a result, we set  $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  as the maximum irradiance. Wiencke and Dieck (1990) reported that a cold-water species, *E. bulbosa*, could grow



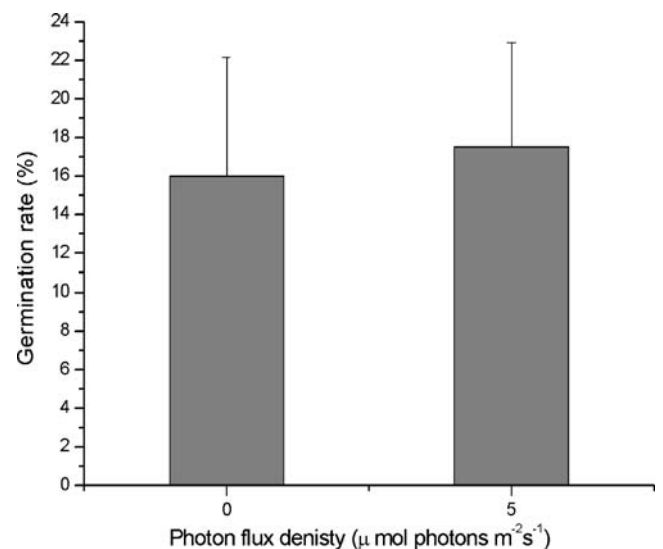
**Fig. 4** The germination rate of somatic cells transferred from 5 and  $5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  to normal conditions. Values are the means±S.D.



**Fig. 5** The survival rate and germination rate of somatic cells cultured under different flux densities. Values are the means±S.D. In each graph, statistically significant differences (ANOVA, DNMR) are indicated by capital letter superscripts ( $P < 0.01$ )

between 0 and 20°C, and optimum growth was demonstrated between 10 and 15°C.

Somatic cells are the central focus of this study, although, a large quantity of spores that were released from the sporangia of a few of the branchlets developed into new plants during the culture process. It has been shown that only coarse sediment is suitable as a substratum for *E. prolifera* zoospores, and that subsequent germination (Schories 1995) and stable substrata that remain at the illuminated sediment surface are essential for small prop-



**Fig. 6** The germination rate of somatic cells transferred from 0 and  $5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  to normal conditions. Values are the means±S.D.

agules to germinate (Schories et al. 2000). It is likely that thalli buried in the sediment are crucial additions to the propagule bank for the impending bloom. The results of our experiment demonstrated that seedlings derived of somatic cells will detach from their dead matrix when they reach a certain length. This finding explains why there were no attached filaments along the coastline of the Yellow Sea during the bloom season (Sun et al. 2008), and why the first bloom was initially detected a great distance from the shore (Fig. 1A). The somatic cells are also more resistant to predation than spores, which further support their importance in formation of the bloom. Because the zoospores of some algae lack a cell wall, it has been suggested that spores are vulnerable to predation by invertebrates until a protective wall is formed (Dayton 1985, Reed et al. 1988). Additionally, Vadas et al. (1982) proved that nearly 100% of the zygotes of *Ascophyllum nodosum* were killed within 2 months in the natural environment. For these reasons, the function of the bank of spores may be masked. However, there has been little evaluation of spore banks due to methodological difficulties (Hoffmann and Santelices 1991). Furthermore, thalli buried in the offshore sediment would suffer less competition pressure from other species because spores that have settled on the rocky substratum are lost easily due to shading by the cover of turf species (Chapman 1984). In our experiment, millions of somatic cells, including cells cultured under stress conditions, were able to regenerate into new plants when the conditions were appropriate. These results suggest that somatic cells function as one of the survival mechanisms for this benthic macroalgae. Burial and winter survival of the somatic cells may explain the rapid increase in *Enteromorpha* biomass that occurs during spring. Taken together, these findings suggest that the initial spring biomass is one of the factors that determine the maximal biomass in summer.

Green tide has been occurring more frequently due to increased discharge of nitrogen-rich chemical pollutants, sewage, and fertilizers from industrial, urban, and agricultural activities (Fletcher 1996; Hermández et al. 1997; Wang et al. 2008). The distances that algal spores may travel away from parent plants are generally short, usually not exceeding a few meters (Sousa 1984; Hoffmann 1987; Santelices 1990). However, floating filaments of green alga carrying prolific cells can travel great distances because they are driven by seasonal winds and currents and can survive in a wide variety of stressful environments. As a result, somatic cells regeneration poses a serious ecology threat. Additionally, *E. prolifera* spread to more areas within the sea over time, which may explain the increase in the scale of green tide that was observed in the Yellow Sea in 2008. Although the lack of knowledge regarding the behavior of somatic cells prevents the establishment of any relationship between the duration of

banks and their survival value, somatic cell regeneration suggests possible directions for further green tide research and management.

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