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The expansion of *Ulva prolifera* O.F. Müller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction

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ABSTRACT

Since 2007, *Ulva* macroalgal blooms have occurred along the coastal areas of the Yellow Sea, China. These blooms are dominated by fragments of *Ulva prolifera* in the early stages of development. The objectives of this study were to identify the primary mode of asexual reproduction for *U. prolifera* and to evaluate the contribution of these thalli fragments to the formation of blooms. Four different growth and reproductive strategies of *U. prolifera* segments were found including: 1) tubular diameter becoming larger; 2) formation of new branches; 3) release of zoids; and 4) polarized growth. This is the first report showing the development of numerous blade-lets from a single segment, which is remarkably different from previous studies on other *Ulva* species. The results in the present study provide critical information to understand how this species is able to support its explosive growth during a bloom.

1. Introduction

Over the last few decades, macroalgal blooms have increased worldwide in frequency and size. The impacts have caused significant ecological and economic losses (Fletcher, 1996a; Blomster et al., 2002; Largo et al., 2004; Lyons et al., 2009). *Ulva* has been identified as the major genus in most of these bloom events (Fletcher, 1996b). It is a cosmopolitan species in marine and estuarine habitats. *Ulva* grows rapidly especially in nutrient-rich waters and is highly tolerant to stresses, such as salinity, temperature, and light (Tan et al., 1999).

Ulva prolifera has been identified as the dominant species of the green tide in the Yellow Sea of China (Wang et al., 2008; Liu et al., 2010; Zhao et al., 2011), which is considered as the site of the world's largest macroalgal blooms (Ye et al., 2011). Although three other *Ulva* species also appeared on the sea surface at the early stages of the blooms, only *U. prolifera* could drift into the northern Yellow Sea (Cui et al., 2015). Recent studies suggested that *Ulva* was attached to *Pyropia* aquaculture rafts from the Subei Shoal (Liu et al., 2009, 2010, 2013; Keesing et al., 2011; Zhang et al., 2014). Through field investigations, Zhang et al. (2014) and Wang et al. (2015) found that the attached *Ulva* blooms were dragged down from the *Pyropia* rafts by fishermen, resulting in numerous *Ulva* fragments being released in the water column. Although *Ulva* has the capability of high, sustained levels of biomass production in blooms, many *Ulva* species are difficult to maintain in a sustained vegetative state (Subbaramaiah, 1970; Oza and Rao, 1977). Fragmentation has been shown to promote zoid formation in vegetative *Ulva* thalli (Dan et al., 2002). The objectives of this study were to identify the different modes of asexual reproduction of *U. prolifera*, and to further elaborate upon the contribution of fragmentation to the formation of the macroalgal blooms in the Yellow Sea, China.

2. Material and methods

2.1. Sample collection and preparation of the Ulva material

Ulva prolifera was originally collected from the *Pyropia* aquaculture rafts in the intertidal zone of Jiangsu coast, China on April 8th, 2012. Single thalli were cleaned of debris and epiphytes, rinsed gently using sterile seawater and transported to the laboratory in seawater at 10 °C. The healthy thalli were checked carefully under a microscope to make sure that there was no germ cell formation. The thalli were then chopped into small pieces and cultured in von Stosch's enriched (VSE) seawater at 12:12 L:D photoperiod, 100 μmol m^{-2} s^{-1} photon fluence rate and at 20 °C. The zoids that were released from mature thalli were individually collected and cultivated into new germlings. We were not able to differentiate if the zoids were zoospores or parthenogametes.

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2.2. Developmental pathways of the Ulva segments

The fresh grown unbranched tubular thalli were selected and cut into 3 mm segments in all experiments. These segments were transferred into Pyrex Petri dishes (6 cm in diameter) containing 50 mL von Stosch's enriched seawater medium (Ott, 1965) (15–20 segments per dish). The dishes were placed in incubators at photon fluence rates of 50, 75 and 100 μmol m^{-2} s^{-1}, temperatures of 10, 15, 20 and 25 °C for 10 days, respectively. A microscope equipped with "PixeLINK" digital camera (PixeLINK, Ottawa, Ontario, Canada) was used daily to observe and record the morphological variations of *Ulva prolifera* fragments at The University of Connecticut.

2.3. Growth experiment for the polarized Ulva segments

The polarized segments were transferred to new Petri dishes and flasks containing sterile VSE medium as described above and cultivated at photon fluence rates of 10, 50, 100 and 200 μmol m^{-2} s^{-1}, and temperatures of 10, 15, 20, 25 and 30 °C for 8 days. During the culture period, the algae were harvested every four days and weighed after blotting the thalli dry with paper towels. The specific growth rate (SGR) was calculated based on the equation: SGR ($\%d^{-1}$) $= [\ln (W_t/W_o)] / t \times 100\%$, where W_o and W_t were the initial and final weight of cultured algae, t was the culture period in days.

The thalli under the 20 °C and 100 μmol m^{-2} s^{-1} condition were kept in the incubator for 2 months. The media were refreshed every 3 days. Meanwhile, the morphological changes and developmental processes of thalli were recorded. Growth of *Ulva* segments was analyzed with two-way ANOVA and Tukey's test for multiple mean comparison using SAS statistical software. Positive significance was based on $p < 0.05$.

3. Results

3.1. Different developmental ways of Ulva prolifera expansion

Four different growth and reproductive strategies of *Ulva prolifera* segments were observed (Fig. 1). The frequency of occurrence in the developmental pathways of *Ulva* segments was different in each of the environmental conditions (Table 1). At low temperatures (10–15 °C), most *Ulva* fragments produced inflated tubular thalli. This was most significant at low photon fluence rate (50 μmol m^{-2} s^{-1}). However, when the segments were cultivated at high temperature conditions (20–25 °C) and at a high photon fluence rate (100 μmol m^{-2} s^{-1}), 87% fragments released zoids within 4 days. The frequency of branch formation reached up to 68.7% under 20 °C and at 75 μmol m^{-2} s^{-1}. Polarized growth was observed at high temperatures (20–25 °C) and at a low photon fluence rate (50 μmol m^{-2} s^{-1}).

3.2. Developmental process of Ulva prolifera polarized fragments

Once the *Ulva* segments were place in petri dishes (Fig. 2-a), the upper cut surfaces of *Ulva* thalli gave rise to a great number of new blade-lets (Fig. 2-b, c). At the beginning, some papillate structures appeared on the apical cut edges. Afterwards, the protoplast filled the voids of the papillae, which formed new cells (Fig. 2-d). Through continuous mitotic cell divisions, the new cells gradually grew into long intact blade-lets. On average, 25 new blade-lets were generated from the upper side of one segment. At the basal cut surfaces, cells were constantly elongated forming rhizoids, with the protoplasts unequally distributed in these elongated cells (Fig. 2-e, f).

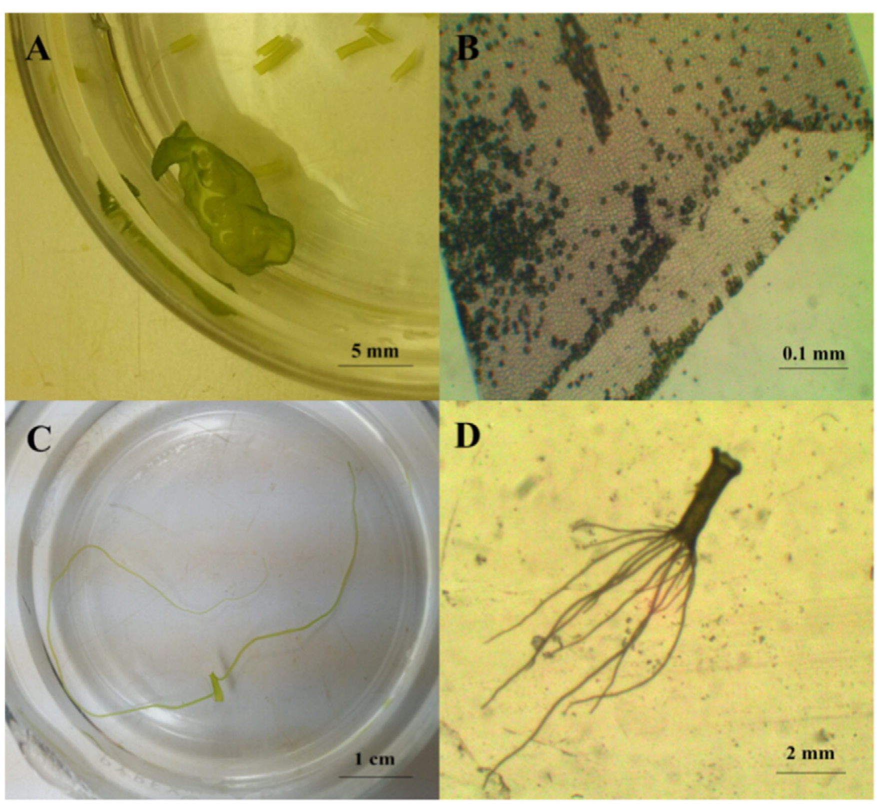

Fig. 1. Four developmental ways of *Ulva prolifera* fragments or segments (A: an inflated *U. prolifera* thallus, the fragments developed into an inflated tubular shape thallus, with the diameter approximately six times larger than that of the original segment; B: the *Ulva prolifera* segments released germ cells, which quickly attached on the sidewalls of flask and rapidly germinated into new germlings in less than 24 h; C: the thalli produced adventitious branches; D: polarized growth of an *U. prolifera* fragment, the upper cut surface formed a number of new blade-lets, and the lower cut surface gave rise to a few elongated rhizoids).

Table 1
Effect of combinations of temperature and photon fluence levels on the frequency of occurrence in the developmental strategies of *Ulva prolifera* fragments.

Probability (%)	50 μmol m^{-2} s^{-1}				75 μmol m^{-2} s^{-1}				100 μmol m^{-2} s^{-1}			
	A	B	C	D	A	B	C	D	A	B	C	D
10 °C	93.4	0.0	6.6	0.0	77.4	2.8	19.8	0.0	71.3	6.3	22.4	0.0
15 °C	92.1	4.5	3.4	0.0	76.8	4.2	14.2	4.8	71.7	18.8	6.7	2.8
20 °C	21.4	7.1	35.7	35.8	10.5	14.6	68.7	6.2	0.0	72.4	27.6	0.0
25 °C	7.6	68.3	12.2	11.9	0.0	68.6	31.4	0.0	0.0	86.6	13.4	0.0

3.3. Effect of temperature and irradiance on the growth of the polarized Ulva segments

The results indicated that growth rate of the polarized *Ulva* segments was significantly affected by both irradiance and temperature ($p < 0.05$). The growth rates vary at different temperature–irradiance combinations (2.5%–28%d^{-1}). The growth rate at the lowest temperature (10 °C) and the lowest irradiance (10 μmol m^{-2} s^{-1}) was only 2.5% d^{-1}, while the maximum growth rate was observed 20 °C and 100 μmol m^{-2} s^{-1} (29.9% d^{-1}) (Fig. 3).

When the polarized segments were cultivated at optimum conditions (20 °C and 100 μmol m^{-2} s^{-1}), the new blade-lets grew rapidly. The original segments were almost negligible in size as compared to the length of the blade-lets (Fig. 4-a). In 15 days, the blade-lets grew into fresh green healthy thalli (Fig. 4-b). On the 27th day, thalli became very inflated on some parts of the blade-lets (Fig. 4-c). Then a great number of sporelings were observed on the bottom of the flask (Fig. 4-d). Six days after that, the *Ulva* sporelings grew into hundreds of young thalli (Fig. 4-e). Another 5 days later, the flask was full of the slender tubular thalli (Fig. 4-f).

4. Discussion

4.1. Polarized growth of Ulva prolifera segments

In this study, we found that one 3 mm fragment or segment of *Ulva prolifera* could form 25 new thalli on average, and each thallus grew up to 35 cm long within 15 days. This is the first report of polarized growth

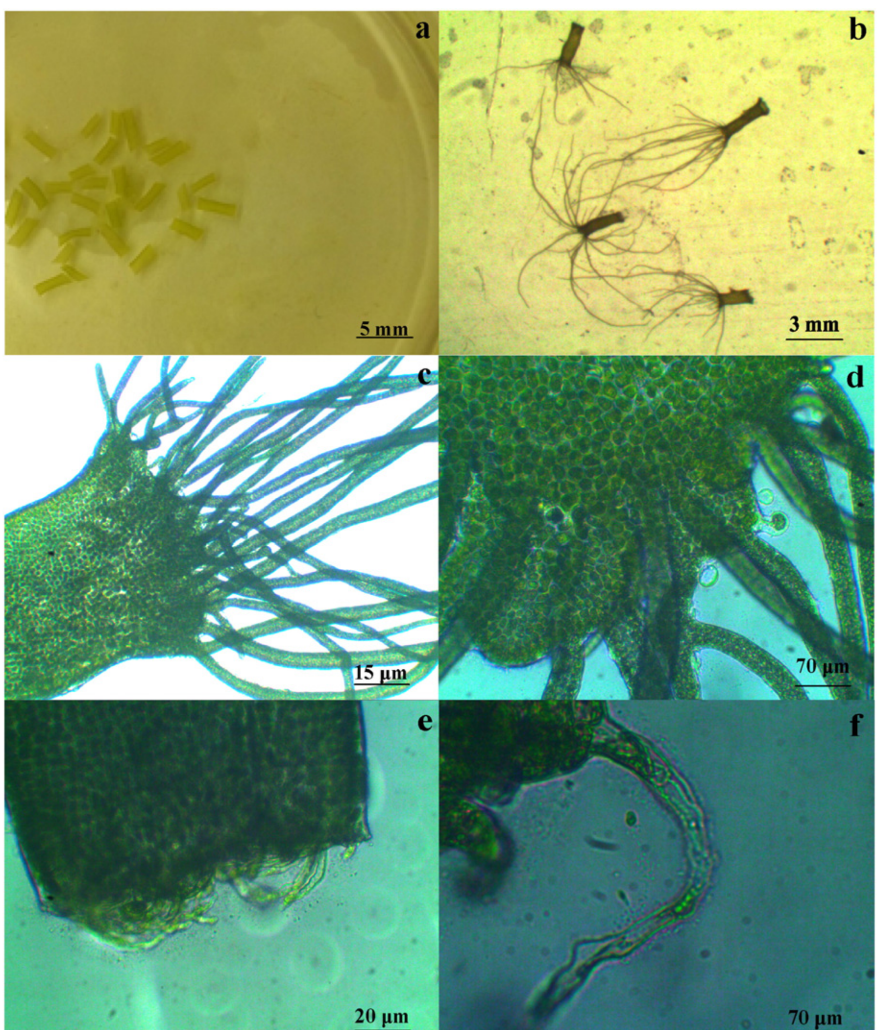

Fig. 2. Formation of *Ulva prolifera* polarized segments. a, initial *U. prolifera* segments; b, polarized growth of *U. prolifera* segments; c–d, new blade–lets from the upper cut surface of the *Ulva* segment; and e–f, rhizoids generated from the basal cut surface of the *U. prolifera* segment.

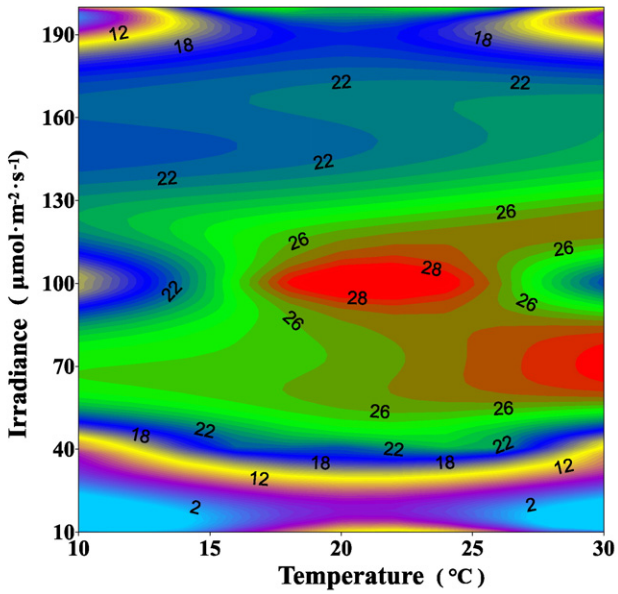

Fig. 3. Contour plot of averaged SGR of *U. prolifera* polarized segment during the culture period as a function of temperature and irradiance.

with formation of numerous blade-lets from a small fragment of *U. prolifera*. This pattern of growth may explain, in part, why *U. prolifera* becomes the dominant species in the world's largest macroalgal blooms in the Yellow Sea, China. In Ulvalean algae, this is a unique growth strategy reported only in *U. prolifera.* Previous studies also showed polarized growth patterns in other *Ulva* species. However, other *Ulva* species including, *Ulva intestinalis* and *Ulva compressa*, formed only one or two new blade-lets, which Müller–Stoll named 'papillae' from the upper cut surfaces (Müller-Stoll, 1952; Eaton et al., 1966; Lee and Wichroski, 1996). Although the regeneration of broken fragments have been known and studied over many decades, the phenomenon of vigorous blades finally developing into new fronds from the upper cut surface, was not described in previous studies (Burrows, 1959; Bliding, 1963; Eaton et al., 1966; Moss and Marsland, 1976; Lin et al., 2008). During experimentation, we also found that the lower side of the fragments could release zooids (Fig. 1). In some cases the released zoospores could attach on the original thallus fragment or germinate within the vegetative cell forming new blades, making the thalli look like a polarized fragments. The polarized growth was mostly observed at high temperature (20–25 °C) and low irradiances (50 μmol m^{-2} s^{-1}). However, the frequency of occurrence was only 35.7%, suggesting that the developmental patterns might also be determined by other factors. Previous research has shown that endogenous hormones may play an important role in the cell differentiation, morphological constructing process and developmental pathways of macroalgae (Bradley and Cheney, 1990; Liu and Kloareg, 1991). Therefore, further studies are needed to understand this unique growth strategy in *U. prolifera.*

4.2. The contribution of Ulva segments to the macroalgae blooms in the Yellow Sea of China

Morphological changes in *Ulva prolifera* induced by temperature and light were observed in the present study. This finding provides critical information to understand the dynamics of *Ulva* blooms in the Yellow Sea, China. *Ulva prolifera* was mechanically removed from *Pyropia* aquaculture rafts along the coast of Jiangsu Province during the harvest period, from April to May. This mechanical removal might have created a greater number of *U. prolifera* segments in the water column than if left undisturbed (Zhang et al., 2014).

In April in the Southern Yellow Sea, the sea surface temperature (SST) is generally ranges from 9 to 12 °C. It then increases to 16 °C in May and to 24 °C by June (Keesing et al., 2011). Although the photon fluence rate in this region maintains a high value even on rainy days at the sea surface, the light is considered to be very low due to the abundant sediment supply associated with large rivers, silt-dominated sediments and an offshore radial sand ridge (He et al., 2012). Therefore, *Ulva prolifera* segments may vegetatively grow and become widespread in April. As the SST increases in May, the floating *U. prolifera* segments

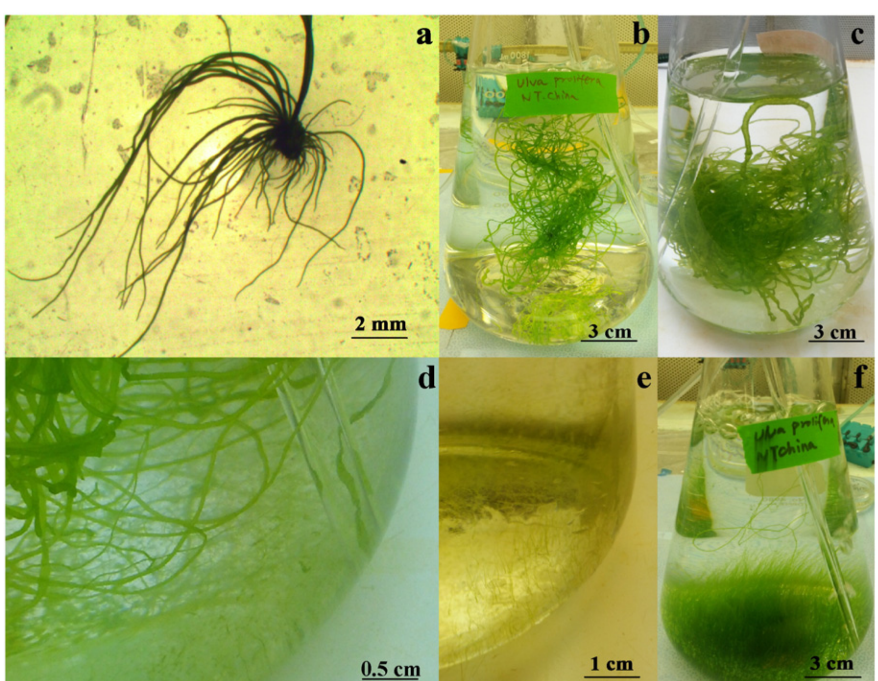

Fig. 4. Developmental process of the polarized *Ulva prolifera* segments. a–c, continuous growth of blade-lets from one polarized *U. prolifera* segment; and d–f, new *U. prolifera* juvenile thalli generated from the vegetative cells of *U. prolifera* grown segments.

may reproduce and form a numerous new thalli from the segments, and the settled segments gradually develop polarized growth, which will enable the rapid expansion of the green tide at this early stage. In June, although there are only a small number of *Pyropia* rafts remaining in the field, *Ulva prolifera* is known to have a great reproductive capacity. Zhang et al. (2013) reported that one cm^2 of thallus can release up to 2.84–6.62 $\times$ 10^6 zoospores or 1.14–2.65 $\times$ 10^7 gametes. More importantly, 91.6–96.4% of the zoospores or parthenogametes could germinate and rapidly become *Ulva* sporelings. Besides, Huo et al. (2013) found that the density of zoids distributed in June in the Southern Yellow Sea was much higher than those in April. At the same time, the area of floating blooms increased from 10 km^2 to 169 km^2. These results indicated that the zoids released by *Ulva* segments developed into new sporelings and supported the increase of biomass of the floating blooms of macroalgae during this period. At the early stage of the green tide, the polarized growth pattern of the *Ulva* segments showed high growth rates and promoted the rapid formation of these blooms. Afterwards, the zoids released by *Ulva* segments will also contribute to the amazing development of these expansive green tides. In this study, although we did not do the experiments of nutrients on the growth of *Ulva* segments, all the experimented segments were cultivated in an enriched seawater medium, the von Stosch's enriched medium has a similar nutrient concentration to the coastal waters of the Southern Yellow Sea. We suggest that the laboratory conditions of this study simulated the *U. prolifera* blooming forming conditions *in situ*.

In conclusion, this is the first report showing the development of numerous blade-lets from a single *U. prolifera* segment, which is remarkably different from previous studies on other *Ulva* species. The findings in the present study provide critical information to understand how this species is able to support its explosive growth during a bloom. Although *U. prolifera* is a fouling seaweed in the *Pyropia* aquaculture raft systems, this species is also considered as an important sea crop in some countries. *Ulva* has been cultivated in Japan since 1985 and consumed in various ways (Kawashima et al., 2013; Dan et al., 2002). *Ulva* has also been used as a very efficient biofilter to reduce the nutrient load from fish farm effluents (Neori et al., 1991, 1998, 2003; Hernández et al., 2002). Besides, *Ulva* seaweed forming green tides can be produced into biofuels (Ceylan and Goldfarb, 2015) and animal feeds (Bolton et al., 2009). Therefore, resource utilization of the attached *Ulva* seaweeds on the *Pyropia* rafts should be a better way to control green tides in the Yellow Sea.

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