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

3

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13

#### 14 Abstract

15 In 2008, Qingdao (36°06' N, 120°25' E, P. R. China) experienced the world largest  
16 drifting macroalgal bloom composed of the filamentous macroalga *Ulva prolifera*. No  
17 convincing biological evidence regarding the algal source is available so far. A series  
18 of field collections of both *Ulva* sp. and waters in various sites along Jiangsu coasts  
19 were conducted in March to May of 2009. Density of microscopic *Ulva* germlings in  
20 the waters sampled from different sites ranged from 7 to 3140 individuals L<sup>-1</sup>,  
21 indicating the wide-spreading and long-term existence of the algae in the investigated  
22 region. Morphological and the nuclear ribosomal internal transcribed spacer ITS

23 nrDNA and the chloroplast-encoded *rbcL* gene comparisons of 26 algal samples  
24 revealed that the algae collected from land-based animal aquaculture ponds mostly  
25 resembled the dominating blooming alga in 2008. Mismatch of *Porphyra* farming  
26 period with the occurrence of the green tide bloom, as well as the negative  
27 identification results of the sampled green algae from the *Porphyra* rafts eliminated  
28 *Porphyra* rafts as the principal and original source of the dominating blooming alga.

29

30 **Key words:** *Ulva prolifera*; *Porphyra*; green tide; algal bloom; eutrophication; ITS  
31 nrDNA, *rbcL*

32

### 33 **1. Introduction**

34 Green tides are massive accumulations of unattached green macroalgae, principally  
35 belonging to the genus *Ulva*, and are intimately associated with eutrophicated marine  
36 environments (Nelson et al., 2008). One of the green tide algae, the filamentous alga  
37 *Ulva prolifera*, formerly known as *Enteromorpha prolifera* (Hayden et al., 2003), is  
38 broadly distributed along the nearshore coasts of the north-eastern Asia (Tseng, 1983,  
39 Shimada et al., 2008). Conspicuous growth of this alga was usually found in  
40 environments with sufficient input of nutrients, such as estuaries, from where  
41 land-derived nutrient rich effluents are combined and discharged into coastal waters  
42 (Leskinen et al., 2004; Conley et al., 2009). This alga can tolerate a wide range of  
43 temperatures, salinities and irradiances (Tan et al., 1999; Dan et al., 2002; Cohen and  
44 Fong, 2006). From May to July 2008 before the Olympic sailing competition,

45 Qingdao coasts experienced an attack of the world's largest drifting green tide,  
46 evaluated at a level of one million tons of harvestable biomass (FW). The bloom once  
47 covered approximately 13,000-30,000 km<sup>2</sup> of the Yellow Sea (Sun et al., 2008). The  
48 dominating species was identified as being the filamentous, intensively ramificated *U.*  
49 *prolifera* (Müller) J. Agardh (Chlorophyta, Ulvophyceae) (Leliaert et al., 2008, 2009,  
50 Sun et al., 2008; Ye et al., 2008). Recent phylogenetic analyses showed that this  
51 unique strain forms a clade with representatives of the *Ulva linza-procera-prolifera*  
52 (LPP) complex and seems to be ubiquitous in several countries (Leliaert et al., 2009).  
53 Field-collected algal samples, as well as those maintained in culture, were both  
54 characterized as intensively ramified and demonstrated outstanding capacity of  
55 vegetative growth under favorable conditions.

56 Accurate localization of the origin and persistence of this green algal bloom is the  
57 first step in understanding this large-scale green tide and finding solutions to the  
58 problems it could potentially bring. According to satellite images the drifted biomass  
59 initiated offshore of the coasts of Jiangsu province and was transported across the  
60 Yellow Sea to Qingdao coasts by seasonal winds and surface currents (Liu et al.,  
61 2009). The original "seed" source of the bloom remained unidentified, although  
62 hypothesis was recently proposed. Liu et al. (2009) thought that the rapid expansion  
63 of *Porphyra* farming along the Jiangsu coasts was the principal cause. It is therefore  
64 necessary to analyze the time series of this red alga farming in relation with the green  
65 tide event in terms of reproduction and growth of both algae. Simple morphological  
66 identification has proven to be insufficient to distinguish species in the genus *Ulva*

67 because often unattached thalli demonstrate considerable morphological plasticity  
68 (Malta et al., 1999). Use of appropriate molecular markers can both identify the algae  
69 and provide important information concerning the origins and dynamics of the blooms  
70 (Malta et al., 1999; Largo et al., 2004). Nuclear ribosomal internal transcribed spacer  
71 ITS nrDNA and the chloroplast-encoded *rbcL* gene sequences were used to combine  
72 the previous *Ulva* and *Enteromorpha* into one genus (Hayden et al., 2003) and were  
73 popularly used to discern the taxonomic positions of the strains in *Ulva* (Leliaert et al.,  
74 2009, Shimada et al., 2008).

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

83

## 84 **2. Materials and methods**

### 85 ***2.1. Choices and description of sample collection sites***

86 The entire coast of Jiangsu province (30°44'-35°4'N) is characterized by an extended  
87 shallow and muddy intertidal zone, constituting an ideal environment for performing  
88 *Porphyra* cultivation by use of floating cultivation methods (Shang et al., 2008). The

89 world's largest cultivation of *Porphyra yezoensis* has been carried out in this province  
90 since 1970s. Until today, *Porphyra* farming occupies 21,000 hectares of intertidal area,  
91 producing 126,000 tons (FW) annually (P. Xu, personal communication). Parallel to  
92 the *Porphyra* farming area along the coasts are animal aquaculture pond systems on  
93 land (AAPs) in which *Eriocheir sinensis* (a fresh water crab with larval stage in the  
94 marine environment), and *Penaeus vannamei* (a white prawn species introduced from  
95 America) are farmed (Fig. 1). *Porphyra yezoensis* is farmed by use of semi and  
96 full-floating rafts composed of bamboo and nets on which the conchospores attach  
97 and grow into blades in 2-3 months during the cold season from December to March.  
98 Young *E. sinensis* (ca. 1 cm) are produced from February to May in seawater in  
99 coastal AAPs, locally called "Natural Ecological Ponds" (NEPs), developed from  
100 2001 onwards. The NEP method, because of its low cost and easiness to manage,  
101 became rapidly the dominating one to produce young crabs in Jiangsu province and is  
102 characterized with intensive application of organic fertilizers. Jiangsu province is thus  
103 becoming the largest young crab production centre in China. *P. vannamei* is  
104 principally farmed along the northern coast of Jiangsu province from March to July in  
105 shallow coastal ponds with water depth ranging from 1 to 1.5 m. Typical coastal  
106 aquaculture areas in this province are characterized by *P. yezoensis* cultivation in the  
107 intertidal zone and large numbers of land-based AAPs separated by a dam. Waters  
108 from AAPs are collectively discharged from a main sluice channel (Fig. 1). Large  
109 volumes of water are exchanged frequently between AAPs and the intertidal water  
110 during rainfall seasons. Salinity, temperature and nutrient levels in the water of these

111 ponds fluctuated, thus making the pond a special niche for species that could tolerate,  
112 survive and reproduce, such as species in the genus *Ulva*.

113 Along the coasts of Jiangsu province, open-sea *Porphyra* cultivation starts with the  
114 transfer of the seeded nets to the sea each year in November and ends up with the  
115 withdrawal of the nets and bamboos from the sea at the end of April (P. Xu, personal  
116 communications). Surface water temperature during this period drops from 15 °C to 3  
117 °C and thereafter increases to 14 °C over the winter with slight variations in different  
118 areas (Fig. 2). Blades of *Porphyra* grow most significantly during low temperature  
119 periods and cover most of the nets. Filamentous green algae are often found to grow  
120 vigorously on the nets where *Porphyra* conchospores sparsely attached. In such cases,  
121 the nets are often sun-dried for more than 12 h to kill the epiphytic green algae, while  
122 *Porphyra* can tolerate such extreme exposure (Y.D. Yu, personal communications).

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

## 128 **2.2. Treatments of water and algal samples**

129 In each of the six locations investigated in Jiangsu province (full-floating rafts of  
130 *Porphyra* cultivation area, nearshore water and sluice gates of AAPs at Liuwei,  
131 coastal pond as well as full-floating rafts of *Porphyra* cultivation area at Xiangshui), 4  
132 L of seawater were collected and mixed thoroughly. Seawater was subsequently



133 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  
134 of Provasoli Enriched Seawater (PES) medium in 1 L glass beakers (2 per sampling  
135 location) placed in temperature-controlled rooms at 15-18 °C under 80-100  $\mu\text{mol}$   
136 photons  $\text{m}^{-2} \text{s}^{-1}$  in a 12 h light per day light-dark regime. The medium was renewed  
137 every 7 d in the following culture period. After 3 weeks, the green algal germlings,  
138 attaching to the wall and bottom of the glass beakers, grew up to 1-5 cm and were  
139 counted and identified by both morphological and molecular analyses as explained  
140 below. These germlings were derived from microscopic spores or gametes or those  
141 that were attached to particles invisible to the naked eye at the time of sampling. After  
142 counting, the algae were removed from the beakers and further grown in suspension  
143 culture under the same conditions.

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

### 150 **2.3. Algal DNA extraction**

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

155 mM Tris-HCl (pH 8.0), 1% polyvinyl pyrrolidone (w/v), 1%  $\beta$ -mercaptoethanol (v/v)  
156 and 10  $\mu\text{g mL}^{-1}$  RNase A was added to the tube. The mixture was incubated at 65 °C  
157 for 60 min with gentle inversion occasionally (Wang et al. 2006). 250  $\mu\text{L}$  of 5 M KAc  
158 (pH 8.0) was added to the mixture and kept on ice for 15 min. Centrifugation was  
159 followed at 12,000 rpm for 15 min. The upper phase was transferred to a new tube.  
160 One volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at  
161 12,000 rpm for 10 min. This step was repeated once more. The aqueous phase was  
162 precipitated with one volume of cold isopropanol at -20 °C for 1 h and collected by  
163 centrifugation at 12,000 rpm for 10 min. Finally, the precipitate was washed with 75%  
164 ethanol, dried and dissolved in double distilled water. The concentration and the  
165 quality of isolated DNA were assessed by electrophoresis on 1.0% agarose gel. DNA  
166 concentration of each sample was adjusted to 50  $\text{ng } \mu\text{L}^{-1}$ .

#### 167 **2.4. ITS nrDNA and *rbcL* gene amplification and sequencing**

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

172 ITS1 (5'-TCTTTGAAACCGTATCGTGA-3'),

173 ITS2 (5'-GCTTATTGATATGCTTAAGTTCAGCGGGT-3'),

174 *rbcL*1 (5'-ATGTCACCACAAACAGAACTAAAGC-3'),

175 *rbcL*2 (5'-AATTCAAATTTAATTTCTTTCC-3').

176 Total genomic DNA (30-40 ng) was added to 50  $\mu\text{L}$  PCR reactions containing final

177 concentrations of 1×PCR buffer (Takara, Japan), 2 mM MgCl<sub>2</sub> (Takara), 0.8 mM  
178 dNTPs (Takara), 25 μM of each primer, and 1.6 U Taq Polymerase(Takara).  
179 Amplification products were separated by 1.0% agarose gel electrophoresis and  
180 fragments of an expected length were cut from the gel and purified by use of a DNA  
181 Gel Extraction Kit (Bio Basic Inc., Canada) according to the manufacturer's  
182 instructions. ITS nrDNA and *rbcL* gene were sequenced on both strands using ABI  
183 3730 XL automated sequencers (Shanghai Biosune Biotechnology Co., Ltd). Each  
184 sequencing reaction was repeated twice.

### 185 **2.5. Phylogenetic analysis**

186 ITS nrDNA and *rbcL* gene were sequenced from 26 algal samples including 20 from  
187 2009's field sampling and 6 from 2008's bloom in Qingdao. Additional 15 ITS  
188 nrDNA and 10 *rbcL* sequences were downloaded from the GenBank. The ITS nrDNA  
189 and *rbcL* sequences were aligned for phylogenetic analyses using Clustal W  
190 (Thompson et al., 1994). For the *rbcL* sequence data set, the alignment was  
191 unambiguous. But the ITS nrDNA alignment contained many insertions and deletions.  
192 The combined data set contained the ITS nrDNA and *rbcL* sequences (Hayden et al.  
193 2003). Prior to analysis of the combined data, the incongruence length difference test  
194 was conducted. The phylogenetic trees were constructed by neighbor-joining (NJ)  
195 method using the program Mega 4.0 (Jiang et al., 2008). *Blidingia* sp., collected from  
196 rafts and sluice gate from Liuwei, served as an outgroup taxon. The bootstrap  
197 consensus tree inferred from 1000 replicates is taken to represent the evolutionary  
198 history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions

199 reproduced in less than 50% bootstrap replicates are collapsed. The percentage of  
200 replicate trees in which the associated taxa clustered together in the bootstrap test  
201 (1000 replicates) is shown next to the branches. The tree is drawn to scale, with  
202 branch lengths in the same units as those of the evolutionary distances used to infer  
203 the phylogenetic tree. The evolutionary distances were computed using the Kimura  
204 2-parameter method (Kimura, 1980) and are in the units of the number of base  
205 substitutions per site.

## 206 **2.6. Sporulation tests**

207 Unialgal culture (individual plant) isolated from Qingdao's bloom was used  
208 throughout the test. In addition, a filamentous strain isolated from Jiangsu coast was  
209 used in the temperature tests for comparison. The filamentous algal material was cut  
210 into short fragments (2-3cm). Ten fragments were selected and put into each well (5-7  
211 ml) of a multi-well culture plates filled with PES. A series of temperature (5, 10, 12,  
212 15, 18 °C) and salinities (30, 24 and 18) were tested. For the salinity test, all cultures  
213 were maintained at 18 °C. All cultures were exposed to  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a  
214 12 h light per day light-dark regime. For each testing condition, sporulation rate was  
215 calculated as the averaged percentage of total sporulated fragments as identified by  
216 microscopic checking on day 6 (n=6, totally 60 fragments were checked and counted  
217 in each condition). The cultures were performed in six GXZ-260C light and  
218 temperature-controlled photo-incubators (Ningbo Jiangnan Instrument, China) with  
219 temperature deviation less than 0.5 °C.

220

### 221 3. Results

#### 222 3.1. Phylogenetic analysis of the algal samples

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

237 The ITS nrDNA sequence analyses showed that 12 algal samples - one from  
238 intertidal rocks (filamentous), two from *Porphyra* rafts (one filamentous, one  
239 ribbon-like), seven from AAPs, one from coastal water (obtained from microscopic  
240 germlings after 3 week culture in the lab) at Liuwei and one from AAPs at Xiangshui  
241 were closely related to the Qingdao algal bloom samples (filamentous) (Fig. 5; 2008  
242 a-e). Samples isolated from *Porphyra* rafts at Neisha, Waisha and some of the AAPs

243 at Liuwei were in the third clade. Two samples from AAPs at Xiangshui were in the  
244 fourth clade together with *U. compressa* (AF035350). Two dominating filamentous  
245 algal samples, isolated from *Porphyra* rafts and from the sluice gate, respectively,  
246 were identified as belonging to the genus *Blidingia* and were treated as an outgroup,  
247 together with *B. minima* (AJ000206).

248 Combined ITS nrDNA and *rbcL* sequence analyses revealed identical results, in  
249 which six filamentous algal samples from AAPs, and one coastal water-derived  
250 filamentous sample, were closely related to the dominating Qingdao algal bloom in  
251 2008 (Fig. 6). Other filamentous algal samples joined together in other clades.

### 252 **3.2. Morphological observations**

253 Green algae including *Cladophora* sp., *Blidingia* sp. and *Ulva* sp. (ribbon and  
254 filamentous in structures) were found in the dominating algal samples collected  
255 from the full and semi-floating *Porphyra* rafts at six cultivation locations including  
256 Xishu (full-floating), Liuwei (semi-floating), Xiangshui (full-floating), Waisha  
257 (full-floating) and Neisha (semi-floating). *Ulva* sp. could be easily distinguished  
258 under the microscope, while *Blidingia* sp. needs molecular data to be identified.

259 While in most of the investigated AAPs, usually only one dominating *Ulva*  
260 species existed as can be judged by the morphology (Fig. 7), probably  
261 determined by the unique water environment in those ponds in comparison with  
262 the apparent versatile environment on the rafts in the surface water in intertidal  
263 zone.

### 264 **3.3. Microscopic germlings in the water**

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

### 273 ***3.4. Sporulation tests in the isolated fragments of the dominating *Ulva* sp. of the*** 274 ***bloom***

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

280

## 281 **4. Discussion**

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

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

300 In tracking the original source of the 2008 Qingdao bloom, Liu et al. (2009)  
301 hypothesized that thousand hectares of *Porphyra* rafts in the intertidal zone along  
302 Jiangsu province were the original source of the algae. This was supported by the  
303 recent rapid expansion of *Porphyra* farming along the coast of blooming area.  
304 However, the authors did not provide biological identification data. Bamboos with  
305 attached green algae were found several times in the algal biomass accumulated in  
306 Qingdao in 2008, bringing people's attention to the *Porphyra* raft systems. Jiang et al.  
307 (2008) pointed out that the dominating alga found in the drifting biomass in Qingdao  
308 was not locally derived based on the ITS phylogenetic analysis. In the present



309 investigation it was shown that *Porphyra* rafts did support the growth of diverse green  
310 algae, but with few identical to the dominating alga of the Qingdao's bloom.  
311 Free-living microscopic stages of *Ulva* sp. isolated from the costal waters of Liuwei,  
312 which were later identified to be very close to the dominating alga in the 2008  
313 Qingdao bloom, were found to be in the waters when the temperature was only 5 °C  
314 and there were small amounts of green algae to be spotted at that time on the  
315 *Porphyra* rafts (personal observations). High density of culture-derived seedlings of  
316 *Ulva* sp. in Jiangsu coastal waters strongly supported the year-round existence of the  
317 algae in the water, even when the *Porphyra* cultivation raft systems are not in place.  
318 The key issue is where the large amount of the algae in their microscopic stages  
319 originated. *Porphyra* cultivation has been carried out for more than 30 years, while  
320 large-scale blooms of *Ulva* in the Yellow Sea are only a recent phenomenon in the  
321 past three years.

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

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

346 It is worth noting is that filamentous *Ulva* were ubiquitous in nearly all observed  
347 AAPs we visited with the only exceptions being those treated with so called  
348 “*Ulva*-killer” (Prometryn,; 2,4-Bio(isopropylamino)-6-methylthio-s-triazine). This  
349 chemical was used in combination with sand and applied in ponds to block the sessile  
350 *Ulva* on the pond bottom. In pond farming, *Ulva* has to be removed periodically to  
351 prevent the competition with microalgae for nutrients. An appropriate amount of the  
352 latter in the water (locally called “colored water”) is thought to be beneficial for the

353 healthy growth of shrimps or crabs. How the wide application of this chemical affects  
354 the balance between the floating *Ulva* biomass and the microalgae populations in the  
355 coastal waters (both are nutrient scrubbers) in Jiangsu province remains to be  
356 resolved.

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

367

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372 for sharing the information on aquaculture activities.

373

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448

449 **Legends of the figures**

450

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

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

461 **Fig.3.** Sampling locations (Xishu, Liuwei, Xiangshui, Waisha and Neisha) along the  
462 coasts of Jiangsu province. The accumulation of large amount of drifted *Ulva* biomass

463 in Qingdao in 2008 is indicated by the area circled with a dashed line in which we had  
464 another sampling location (Shilaoren).

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

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

484 **Fig.6.** Neighbor-joining (NJ) tree constructed from the analysis of combined ITS



485 nrDNA and *rbcL* sequences. The tree was rooted with *Blidingia* sp. 1 collected from  
486 *Porphyra* rafts and *Blidingia* sp. 2 collected from a sluice gate at Liuwei, respectively.  
487 The numbers under the branches represent full heuristic bootstrap values (1000  
488 replicates) greater than 50%. Branch lengths are proportional to the amount of  
489 sequence change, which are indicated by the scale bar below the tree (in the  
490 parentheses, \* refer to the samples that were thin filamentous with ramifications; R  
491 refers to ribbon in morphology).

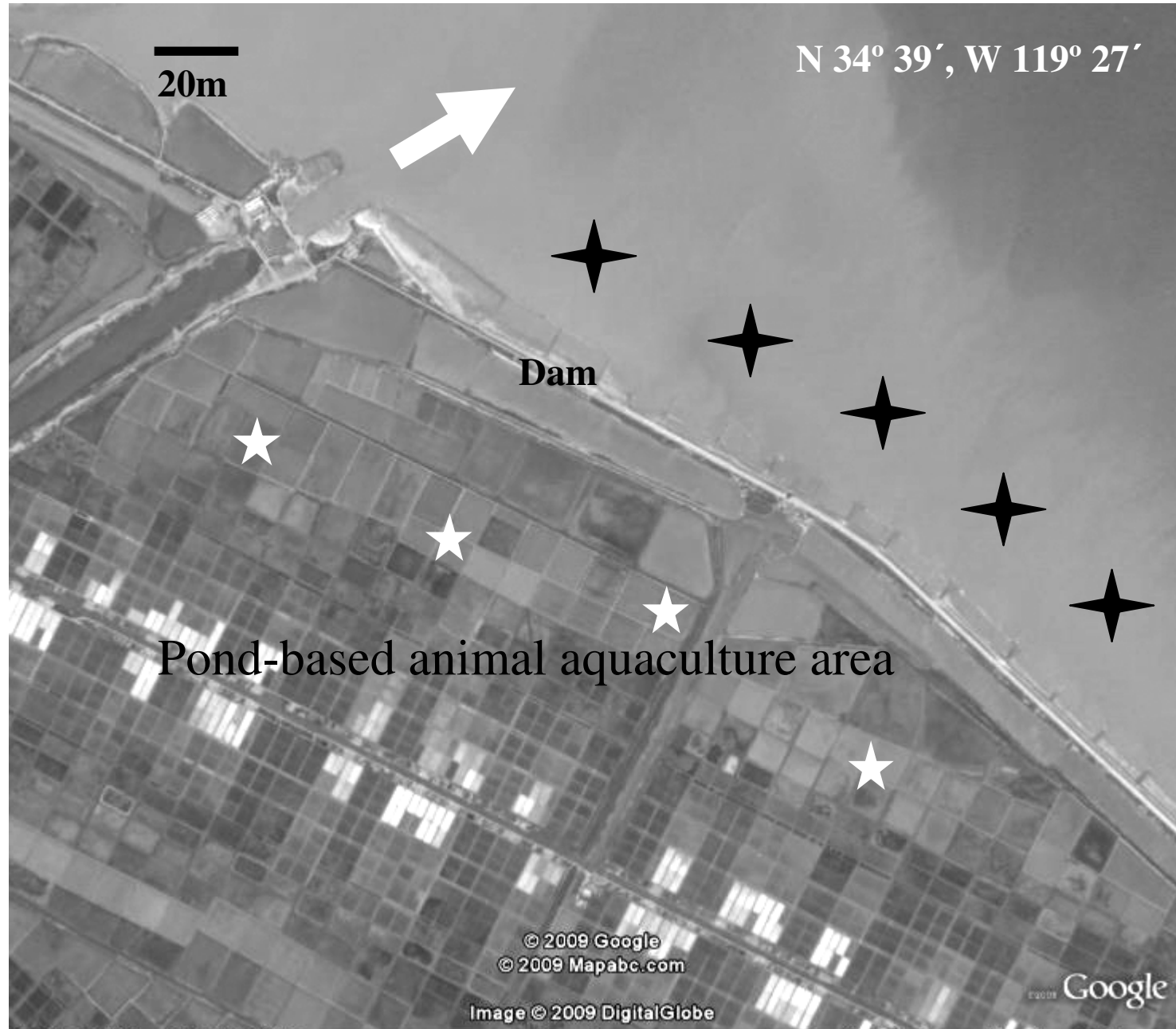
492 **Fig. 7.** Photographs of the filamentous algal samples collected from the Qingdao  
493 2008's bloom (A, B), Liuwei (C, D), Waisha (E, F) and Xiangshui (G, H), Jiangsu  
494 province. They show the typical tubular and filamentous structure with lateral  
495 ramifications.

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

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

504

Fig.1



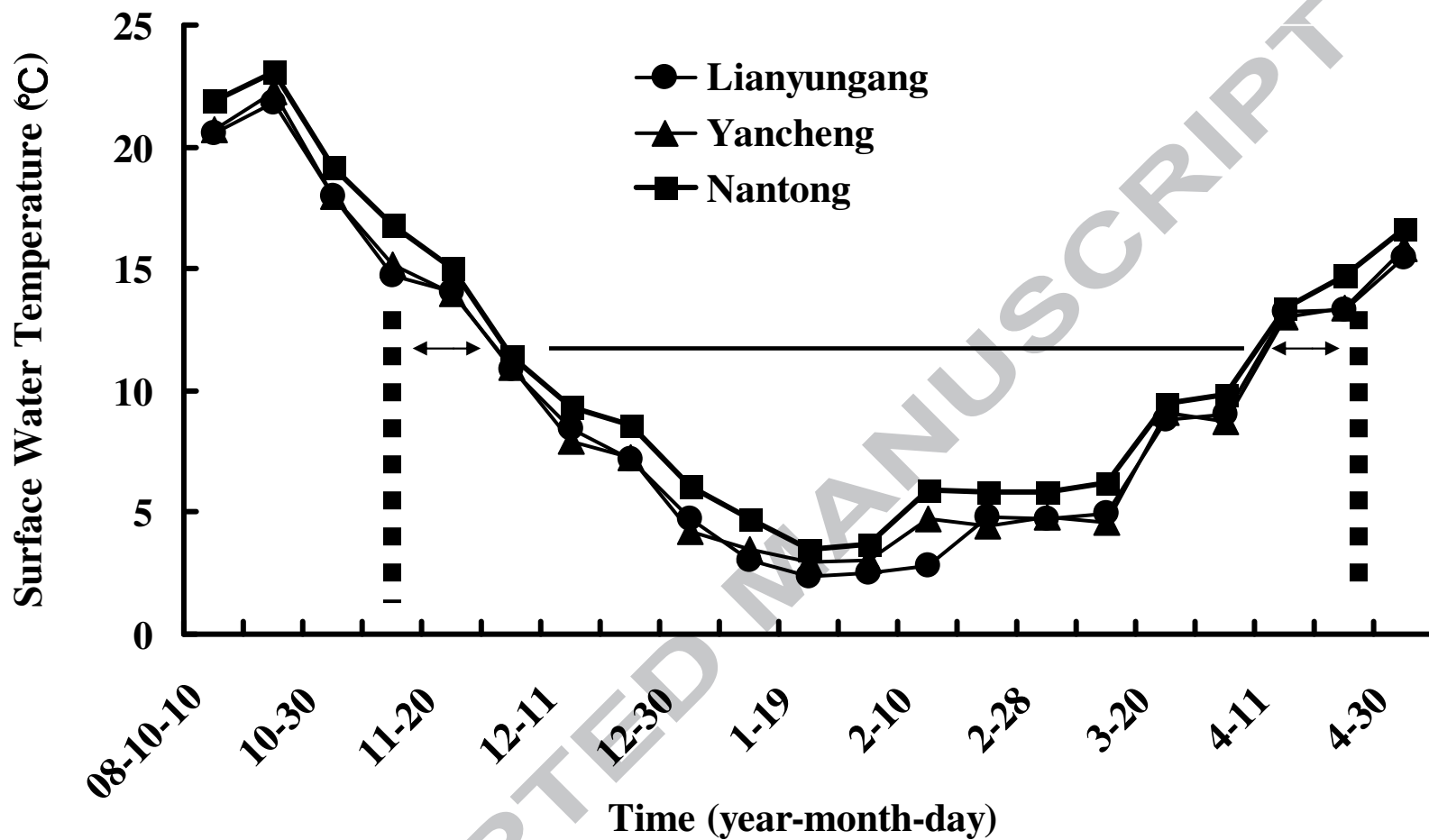


Fig. 3

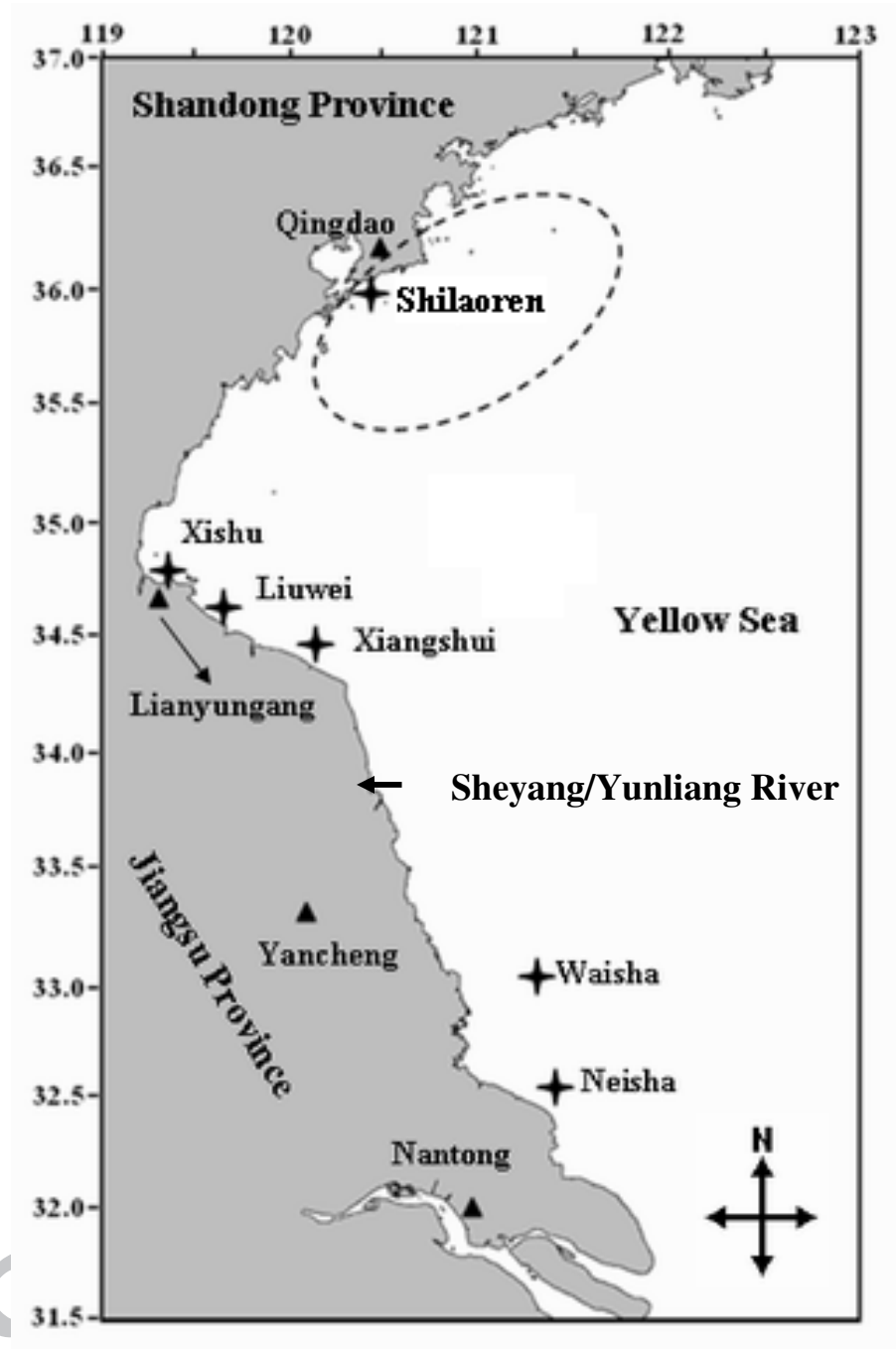


Fig. 4

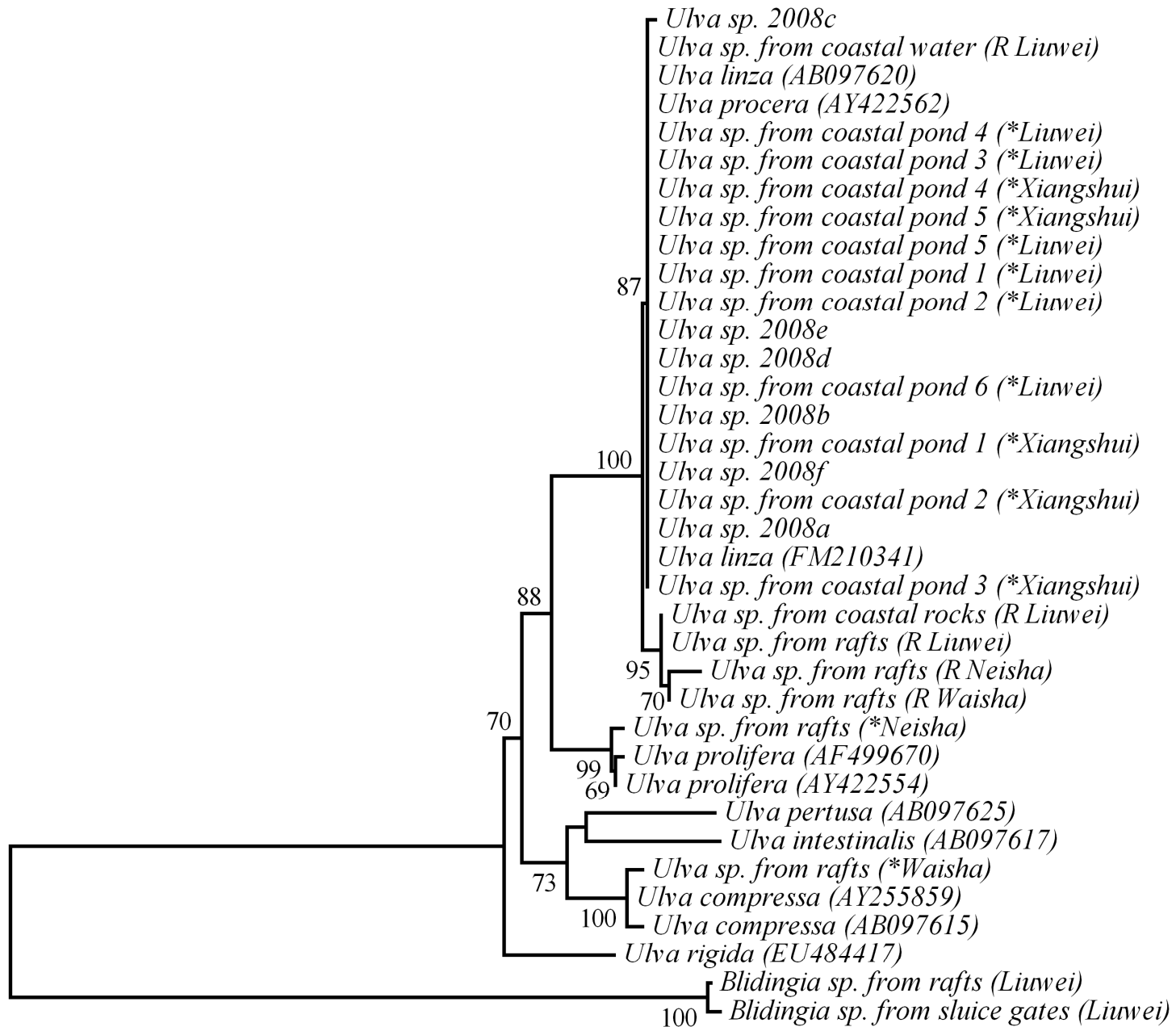


Fig. 5

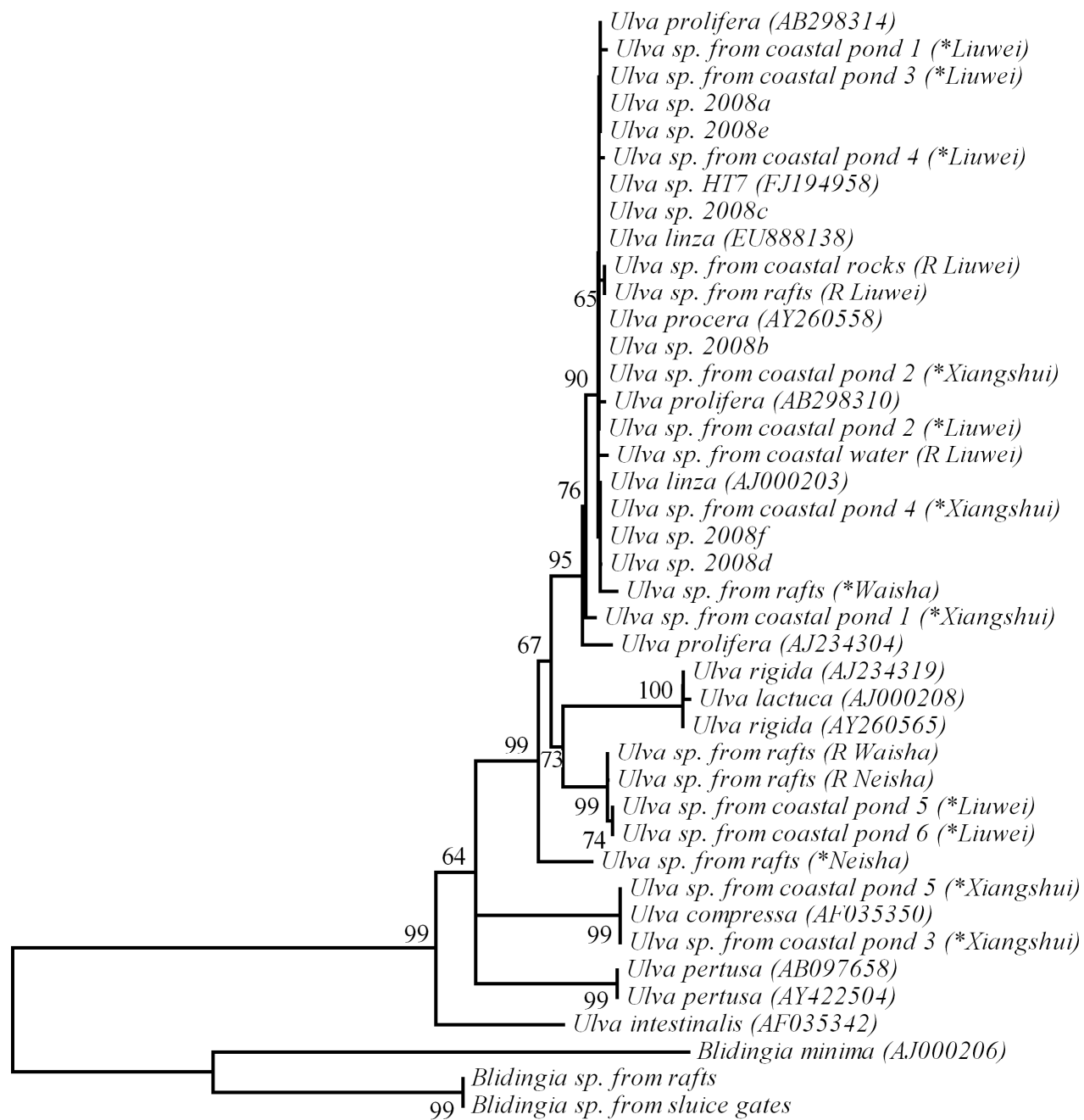


Fig. 6

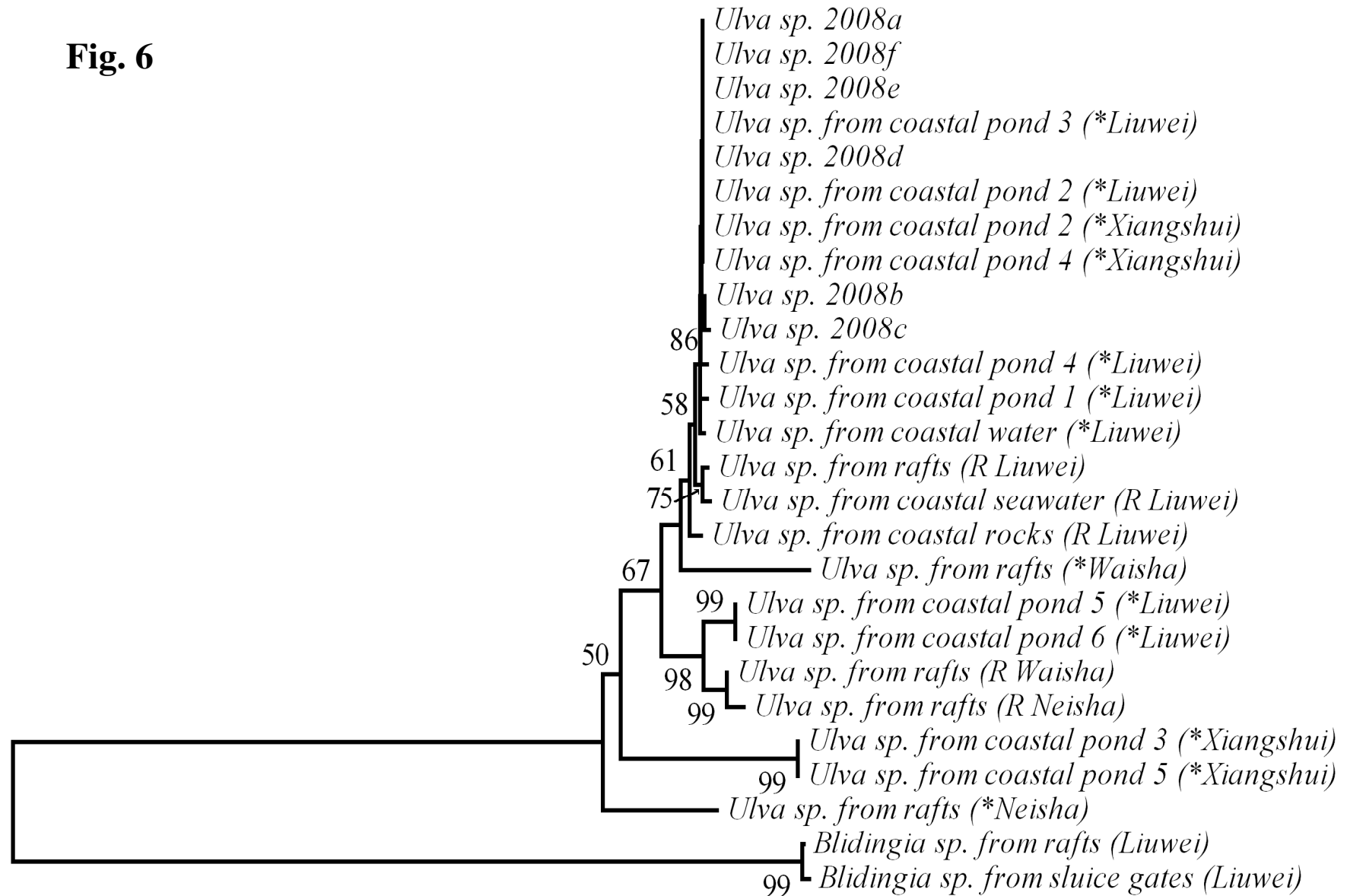


Fig. 7

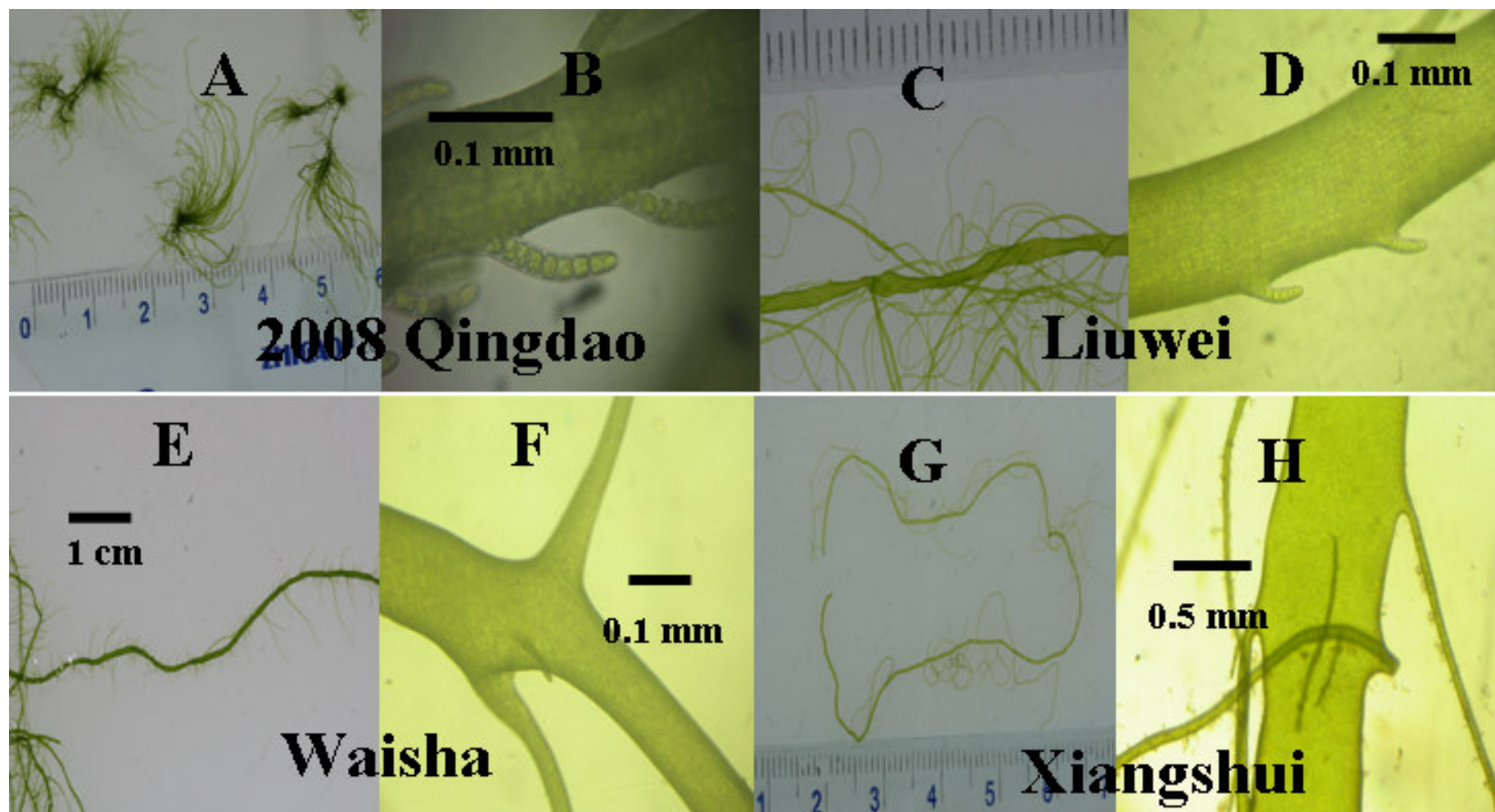




Fig. 8

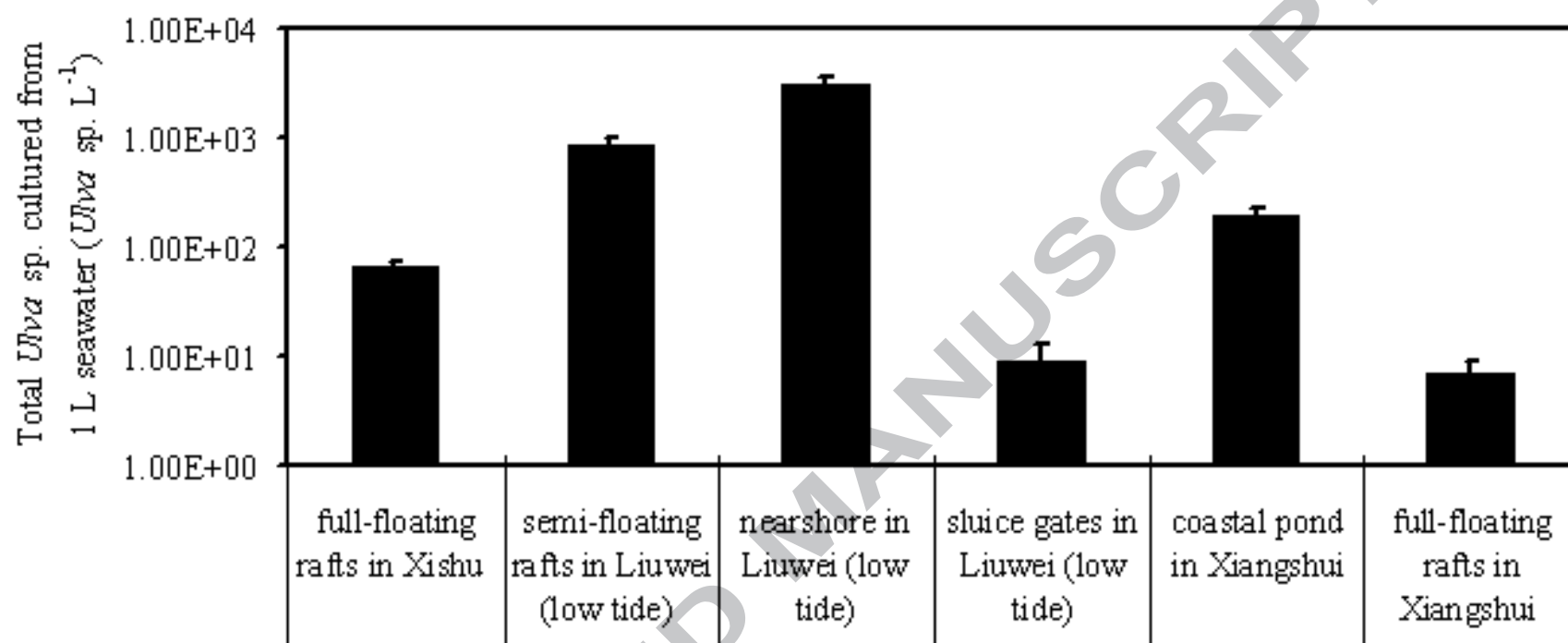


Fig.9

