Estuarine, Coastal and Shelf Science 163 (2015) 103–111

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

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

Development of a fluorescence *in situ* hybridization (FISH) method for rapid detection of *Ulva prolifera*

Qing-Chun Zhang [a], Qing Liu [a, b, 1], Zhen-Jun Kang [a, b], Ren-Cheng Yu [a, *], Tian Yan [a], Ming-Jiang Zhou [a]

[a] Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Accepted 4 September 2014
Available online 16 September 2014

Keywords:
fluorescence *in situ* hybridization (FISH)
Ulva prolifera
green tide
Yellow Sea

ABSTRACT

Large-scale green tides have occurred consecutively since 2007 in the Yellow Sea (YS), China. The dominant causative species of the green tides has been identified as *Ulva prolifera*. The origin of green tides in the YS has been traced back to the Subei Shoal based on the results of remote-sensing, numerical simulations and field investigations. However, it is difficult to study the early development of green tides in the Subei Shoal because of the mixture of multiple green algae and the morphological diversity of *U. prolifera* when under variable environmental conditions. In this study, a rapid and accurate fluorescence *in situ* hybridization (FISH) method was developed to detect *U. prolifera* from the community of green algae targeting the 5S rDNA spacer region of *U. prolifera*. Two specific probes, 5S-1 and 5S-2, were designed based on the sequences of the 5S rDNA spacer regions of *U. prolifera*, *Ulva linza* and *Ulva flexuosa*. Specificity of the FISH method was tested using the six species of green algae commonly occurring in the Subei Shoal, including *U. prolifera*, *U. linza*, *U. flexuosa*, *Ulva compressa*, *Ulva pertusa* and *Blidingia* sp. The results showed that only *U. prolifera* could be labeled with both probes. Probe 5S-1, which showed a much higher labeling efficiency on *U. prolifera*, was ultimately selected as the probe for the FISH detection. The sample preparation method was optimized, particularly for the mature green algae, by the addition of cellulase and proteinase K in the pre-hybridization solution. Labeling efficiency with the probe 5S-1 reached 96% on average under the optimized conditions. The successful development of the FISH method has been applied to qualitative and quantitative analysis of field samples collected from the YS, and the results indicate a potential use in future green algae studies.

1. Introduction

Over the last two decades, new-type harmful algal blooms (HAB) have continued to emerge in the coastal waters of China. Massive, free-floating green tides, for example, first emerged in the Yellow Sea (YS) in the summer of 2007 and became a recurrent phenomenon after that. Green tides significantly collapsed tourism, aquaculture industry and coastal ecosystems (Wang et al., 2011, 2012). In 2008, the large-scale green tide posed a significant threat to the Olympic sailing event in Qingdao and resulted in a huge economic loss. Based on previous studies, several green algae species have been identified during green tides (Duan et al., 2012), and the dominant causative species has been confirmed as *Ulva* (*Enteromorpha*) *prolifera*, based on morphological features as well as molecular evidence (Ding et al., 2009; Hiraoka et al., 2011; Duan et al., 2012; Zhao et al., 2013).

Data from both satellite remote-sensing and numerical simulations have suggested that floating green algae in the YS originate from the Subei Shoal on the coast of Jiangsu province (Hu et al., 2010; Qiao et al., 2011). Field investigations have also found large quantities of green algae in aquaculture ponds and aquaculture rafts of *Porphyra yezoensis* along the coastline of the Subei Shoal (Liu et al., 2009, 2010a; Pang et al., 2010). Among them, the culture zone for *P. yezoensis*, with lots of rafts made of bamboo poles and ropes, were believed to be an important nursery region for the green algae before the formation of large-scale green tides in the YS (Liu et al., 2009, 2010a). Large amounts of green algae have been found attached to the bamboo poles and ropes in spring (Fan et al., 2015), but it has been hard to identify *Ulva prolifera* from other co-

* Corresponding author.
E-mail address: rcyu@qdio.ac.cn (R.-C. Yu).
[1] Equal contribution to this manuscript as the first author.

http://dx.doi.org/10.1016/j.ecss.2014.09.004
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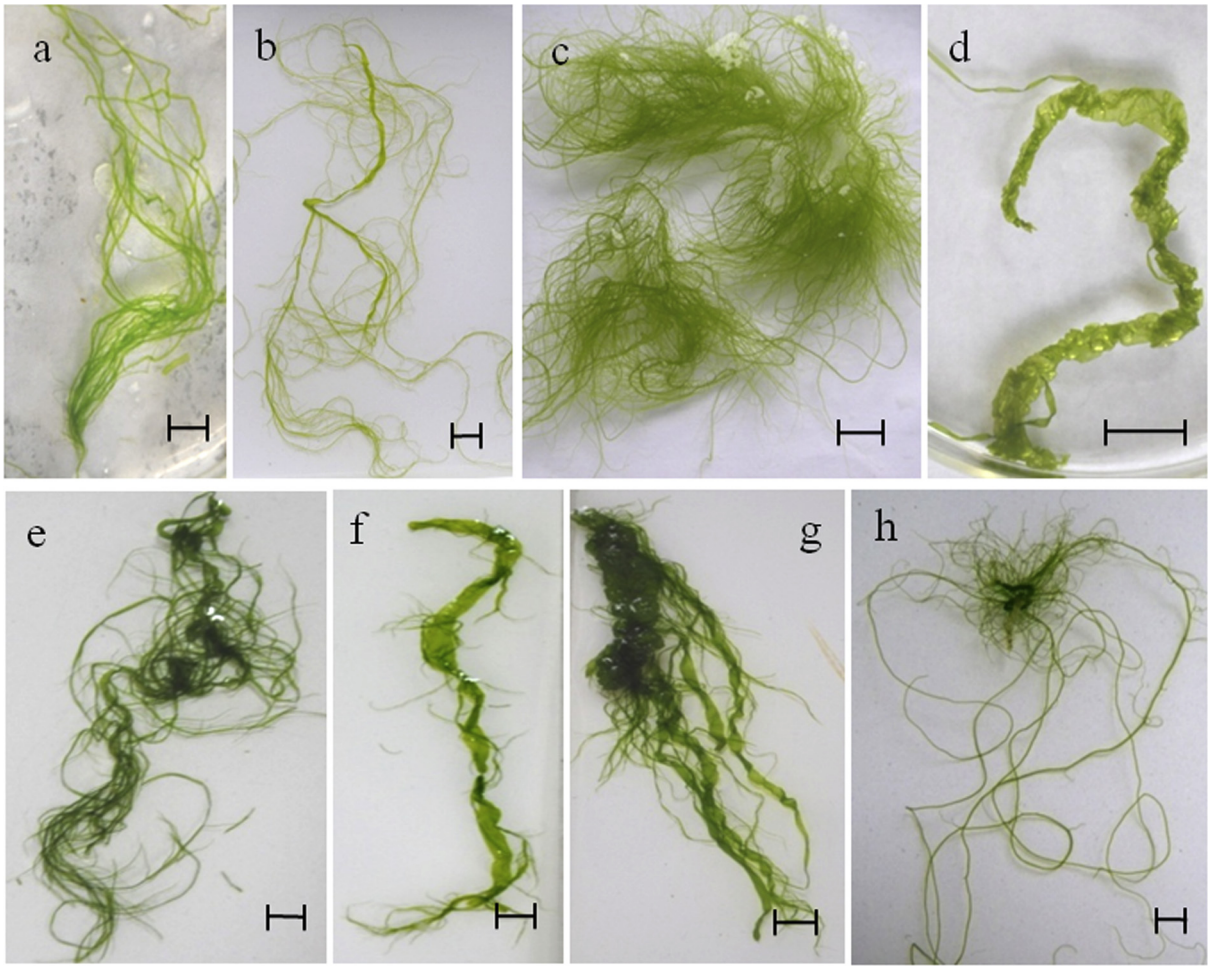

Fig. 1. Samples of *Ulva prolifera* collected from the Yellow Sea with different morphological features. (a–b: selected samples of *U. prolifera* attached to the *Porphyra* culture rafts in the Subei Shoal in 2012; c–h: selected samples of *U. prolifera* floating in the Yellow Sea in 2012. Length of the bars in the photos represent 1 cm. All these samples were identified as *U. prolifera* based on the sequence information of the ITS and 5S rDNA spacer regions.)

existing green algal species and make a quantitative assessment of relative abundance of *U. prolifera* in the community of green algae. The reason for this is that morphological features of *U. prolifera* are quite diverse when under variable environmental conditions. For example, floating *U. prolifera* in the YS showed significantly different morphological features from those attached to the rafts (Fig. 1). Therefore, whether *U. prolifera* present in the attached green algae community or not is a key issue in studies of green tides in the YS, and it is necessary to develop a rapid and accurate method to identify *U. prolifera* from the assembly of green algae attached to the *Porphyra* aquaculture rafts.

Previous studies suggest that *Ulva prolifera* are grouped in the *Ulva linza–prolifera–procera* species complex (*Ulva* LPP species complex), based on the phylogenetic analysis of the 18S rDNA, the ITS regions of rDNA, cytochrome *c* oxidase 1 (*cox1*) and the RuBisCO large subunit (*rbcL*) genes (Morand and Merceron, 2005; Leliaert et al., 2009; Pang et al., 2010; Wang et al., 2010a, 2010b; Liu et al., 2010a, 2010b, 2010c; Duan et al., 2012). However, it is impossible to discriminate *U. prolifera* from the other two species based on the sequence information of these genes. Fortunately, they can be distinguished from each other based on the sequence of the 5S rDNA spacer (Hiraoka et al., 2011). This provides a good opportunity for the development of molecular methods to detect *U. prolifera* in the field samples rapidly and accurately, and the technique selected was a fluorescence *in situ* hybridization (FISH) assay.

This assay is a cytogenetic technique developed by biomedical researchers in the early 1980s to detect and localize specific DNA sequences on chromosomes (Langer-Safer et al., 1982). FISH methods have been applied successfully to qualitatively and quantitatively detect harmful algae in HAB studies (Tanabe et al., 2002; Sako et al., 2004; Groben and Medlin, 2005; Kim and Sako, 2005; Hosoi-Tanabe and Sako, 2005, 2006; Chen et al., 2008; Touzet et al., 2009; Zhang et al., 2010). However, there have not been any reports on the application of the FISH method in studies of macroalgae identification so far. In this study, we developed a FISH method to rapidly identify *Ulva prolifera* from other green algae species, which can assist in ecological studies of green tides in the YS.

2. Materials and methods

2.1. Algal strains and culture conditions

Six species of green algae isolated along the coast of the YS were used in this study, including *Ulva prolifera* (4 strains), *Ulva linza* (1 strain), *Ulva flexuosa* (2 strains), *Ulva compressa* (2 strains), *Ulva pertusa* (1 strain), and *Blidingia* sp. (1 strain) (Table 1, the firs 11 lines). The algae were cultured in f/2-Si medium with additional SeO_2 to control the growth of diatoms. Seawater collected from the Huiquan Bay, Qingdao (salinity 30 ± 1) was filtered through a 0.45-