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厦门大学

硕士学位论文

同种来源物质及细菌粘膜对肠浒苔孢子附着 的影响

Effects of Conspecific Cues and Bacterial Biofilms on Settlement
of *Ulva intestinalis* Spores

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Abstract

The marine seaweed *Ulva* is the important fouling macroalgae and the main green tide-forming algae, causing serious economic and ecological problems. In the marine environment, *Ulva* usually forms dense aggregation. Settlement of spores plays an important role in *Ulva* aggregation and is the key stage in its life cycle, which changes the life phase from planktonic (spores) into sessile form. Settlement of larvae of marine benthic invertebrates is usually suggested to be guided by the habitat-specific cues, but so far there is only a few of reports on cues for settlement of macroalgae. In present study, we explored cues on settlement of *Ulva intestinalis* spores, which may be released by conspecific fronds (interspecies) or bacteria (interspecific) because both of them existed in high abundance in habitats of *U. intestinalis*. The fronds of *U. intestinalis* were extracted using water and organic solvents and subsequently tested for their activity on settlement of *U. intestinalis*. The compounds active in inducing settlement of spores were analysed, purified and identified. To understand the role of bacteria on settlement of spores, the bacterial community associated with *U. intestinalis* were analyzed and the effect of biofilm of bacteria isolates were also tested for their activity on settlement of spores. The main results were as the followings.

1. The extracts of *U. intestinalis* fronds by organic solvents were found to contain chemical cues that significantly induce settlement of spores. By bioassay-guided isolation, fatty acid C16:4 (hexadeca-4,7,10,13-tetraenoic acid) was isolated from the hexane extract. This fatty acid significantly induced settlement of *U. intestinalis* spores at the concentration as low as 5 $\mu\text{g mL}^{-1}$. Another chemical compound was successfully isolated from the dichloromethane extract, which was identified as dibutyl phthalate. That compound was also active in inducing settlement of spores at 0.5~10 $\mu\text{g mL}^{-1}$.
2. The analysis on composition of fatty acid related compounds in active fractions (HU, FI, and FII) from *U. intestinalis* showed that they all contained saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. Fatty acids from 8 carbon atoms to 22 carbon atoms in chain length were found. Furthermore, seven fatty acids (including six fatty acids founded in *U. intestinalis*) were tested for their activity on

settlement of *U. intestinalis* spores. The effect of fatty acids on settlement of spores was specific to fatty acid. The effect fatty acid on settlement of spores depends on a combination of chain length and the degree of unsaturation. Fatty acids C16:1, C16:4 and C18:2 were found to significantly enhance settlement of *U. intestinalis* spores, indicating they may be the natural inducers from conspecific fronds for controlling settlement and aggregation of spores. This the first report about chemical cues from conspecific fronds to guide the settlement of *Ulva* spores.

3. Analysis of bacterial community structure associated with *U.intestinalis* from Xiamen coastline showed the dominance of phyla Proteobacteria, Bacteriodetes and Cyanobacteria. The result of hightrouput sequencing analysis revealed that bacteria community on algae surface and surrounding rock surface were different in class levels. As much as 35 strains of bacteria were isolated from algae surface and surrounding rock surface. Three isolates (*Hyunsoonleella pacifica*, *Erythrobacter vulgaris* and *Shewanella loihica*) significantly induced settlement and aggregation of *U.intestinalis* spores. Analysis of bacteria community revealed that *Hyunsoonleella* and *Erythrobacter* were in high abundance associated with *U. intestinalis*. It was found here that the bacteria from the *U. intestinalis* habitat could induce settlement and aggregation of *U. intestinalis* spores.

Keywords: *Ulva instestinalis*, spores, settlement, aggregation, fatty acid, bacterial biofilm

摘要

浒苔是重要的海洋污损藻类，同时也是主要的绿潮藻，对海洋产业和海洋生态环境造成严重危害。在天然海区，浒苔常见形成密集群聚。浒苔孢子的附着是其生活史中从浮游态转变为附着态的一个关键阶段，与浒苔形成群聚具密切关系。通常认为海洋底栖无脊椎动物幼体的附着受到许多因子的影响，已有大量相关研究，但目前对大型海藻孢子的附着影响因素研究较少。本文以肠浒苔 (*Ulva intestinalis*) 为研究对象，研究同种藻体来源物质 (种内) 及其栖息地来源菌株 (种间) 对其孢子附着的影响，以检验在肠浒苔栖息地大量存在的同种藻体和微生物是否在其孢子附着中起作用。本文以水和有机溶剂对肠浒苔藻体进行了提取，检测了提取物对肠浒苔孢子附着的影响，从中获得具诱导活性的提取物进一步分离纯化，对活性化合物进行了分析和鉴定，另外对肠浒苔藻体表面及其附着基表面的细菌群落结构进行了分析和比较，并对菌株进行了分离纯化和菌膜制备，检测了细菌粘膜对肠浒苔孢子附着的影响，主要结果如下。

1. 肠浒苔藻体的有机溶剂提取物显著诱导其孢子附着。以活性检测结果为导向，从其正己烷提取物中分离纯化获得了十六碳四烯酸 (C16:4)，该脂肪酸在 $5 \mu\text{g mL}^{-1}$ 浓度下可显著促进肠浒苔孢子的附着。另外，还从其二氯甲烷提取物中分离纯化获得邻苯二甲酸二丁酯，该化合物在 $0.5\sim 10 \mu\text{g mL}^{-1}$ 浓度下对肠浒苔孢子附着具诱导活性。
2. 肠浒苔分离中获得的三个活性组分 (HU, FI 和 FII) 的化学成分分析结果表明，这三个组分中均含有饱和脂肪酸、单不饱和脂肪酸和多不饱和脂肪酸，所含脂肪酸的碳原子数为 8~22。对 7 种脂肪酸 (其中 6 种在肠浒苔中有发现) 的活性检测结果表明，脂肪酸对孢子附着的影响与其碳链长度和不饱和度有关。本文发现 C16:1 和 C18:2 可显著诱导肠浒苔孢子附着，表明这两种脂肪酸与 C16:4 都可能是肠浒苔孢子附着的天然脂肪酸诱导因子。本文首次报道了同种藻体来源的化学物质对浒苔孢子附着具诱导活性。
3. 厦门海岸肠浒苔藻体表面及其附着基表面的细菌群落中变形菌门, 拟杆菌门和蓝菌门均占优势，这三种基底表面的细菌群落结构在纲的水平上存在明显差异。从这两种基底表面共分离纯化获得 35 株菌株，从中筛选出 3 株菌株 (*Hyunsoonleella pacifica*, *Erythrobacter vulgaris* 和 *Shewanella loihica*) 的菌膜可显著诱导肠浒苔孢子附着，其中 *Hyunsoonleella* 和 *Erythrobacter* 在上述基底表面的细菌群落中具较高丰度，表明来

源于肠浒苔栖息地的细菌在肠浒苔群聚附着中可能也发挥重要诱导作用。

关键词：肠浒苔，孢子，附着，群聚，脂肪酸，细菌粘膜

厦门大学博硕士论文摘要库

Chapter 1 General Introduction

1.1 Overview of Marine Biofouling

1.1.1 Marine Biofouling and Impacts

Biofouling can be defined as the undesirable aggregation of organisms on structures submerged in seawater (Hellio & Yebra, 2009). The Aggregation phenomena of microorganisms, plants and invertebrates on man-made structure surfaces give a serious effect on economy, ecological diversity and biosecurity (Davidson et al., 2016; Gule et al., 2016).

Marine industry sector receives the most severe loss due to higher fuel consumption as the increasing of roughness surface due to biofouling, thus subsequently affecting the velocity of the ship (Champ, 2000). Furthermore, dry-docking operations need to be more frequent and longer with fouling organism, that make more expensive and time-consuming for hull maintenance of vessel (Abbot et al., 2000). For the US Navy alone the annual extra cost caused by fouling on ship hulls was estimated to be 1 billion US\$ (Callow & Callow, 2002)

Culture species and aquaculture infrastructures are exposed to a varied range of fouling organisms, which significantly affect the production (Fitridge et al., 2012). In shellfish aquaculture fouling on stock caused physical impairment, biological competition and environmental alteration, while infrastructure is also impacted. Nevertheless, the primary effect of biofouling on finfish farming is directly related to aquaculture facilities (Fitridge et al., 2012). The economic costs associated with biofouling control in aquaculture are considerable, where estimates costly between 5–10% of production costs (equivalent to US\$ 1.5 to 3 billion yr⁻¹ globally). (Lane & Willemsen, 2004).

Biofouling also affect the ecological diversity. Some biofoulers are tolerant to environmental changes and quickly adapt to the new habitat. Green mussels, Asian kelp, *Ulva*, barnacle are examples of biofoulers as well as invasive species. They affect the diversity and stability of ecology due to rapid grow, competitor and become dominant, subsequently outpace dominance of local species (Jackson, 2008; Marris, 2008). Research

conducted by Molnar et al., (2008) found that from 329 of marine invasive species as much as 228 species are translocated to new habitat through fouling and/or ballast tank of ships.

Even though there is not enough evidence to testify that biofouling threaten biosecurity, especially aquatic ecology, several reports have been paying attention to this issue (Fitridge et al., 2012). Spreading of bonamiosis via oysters on barges in the UK (Howard, 1994), spreading salmon anemia (Murray et al., 2002) and spreading gill disease (Tan et al., 2002) disease in salmon culture were examples of diseases that translocated and affected the aquaculture. Biofilms on the surfaces of the vessel also involved spreading of microorganisms and pathogens (Drake et al., 2005, 2007).

Many research have been conducted to prevent fouling formation (Bhattarai et al., 2006; Burgess et al., 2003; Farrapeira et al., 2011; Feng et al., 2013, Yebra et al., 2004,) and studied the formation of biofouling including settlement, aggregation, and adhesion process (Callow et al., 1997; Drake et al., 2005; Hong & Cho, 2013; Petrone, 2013; Prieto et al., 2002).

1.1.2 General Process of Fouling Formation

Because biofouling gives a diverse impact on economy and environment, the study about its formation process is important. The general process formation of biofouling on surfaces begun on a clean surface immersed in seawater as shown in Figure 1.1. Biofouling is a greatly vigorous process. The substratum, geographical location, season, biological factor (competition and predation) influences the specific organisms that develop in a fouling community (Callow & Callow, 2011). On a surface immersed in seawater, it immediately captivates macromolecules consisting of dissolved organic macromolecules (e. g. proteins and tannins) and forming a conditioning film on the surface (Yebra et al., 2004). Furthermore, the conditioning film will attract the primary colonizer e.g. bacterial unicellular algae, protozoa and fungi to adhere within an hour (Cao et al., 2010). The primary colonizer gather together and create a biofilm. This primary colonization is often mentioned as microfouling (Callow & Callow, 2011). The aged biofilm can attract the larvae of invertebrate and/or spores of algae to attach and grow into adult fouling organisms (Dobretsov & Qian, 2004; Goecke et al., 2010; Qian et al., 2007). The settlement, aggregation of the macroalgae and invertebrates in surfaces is usually referred as

macrofouling.

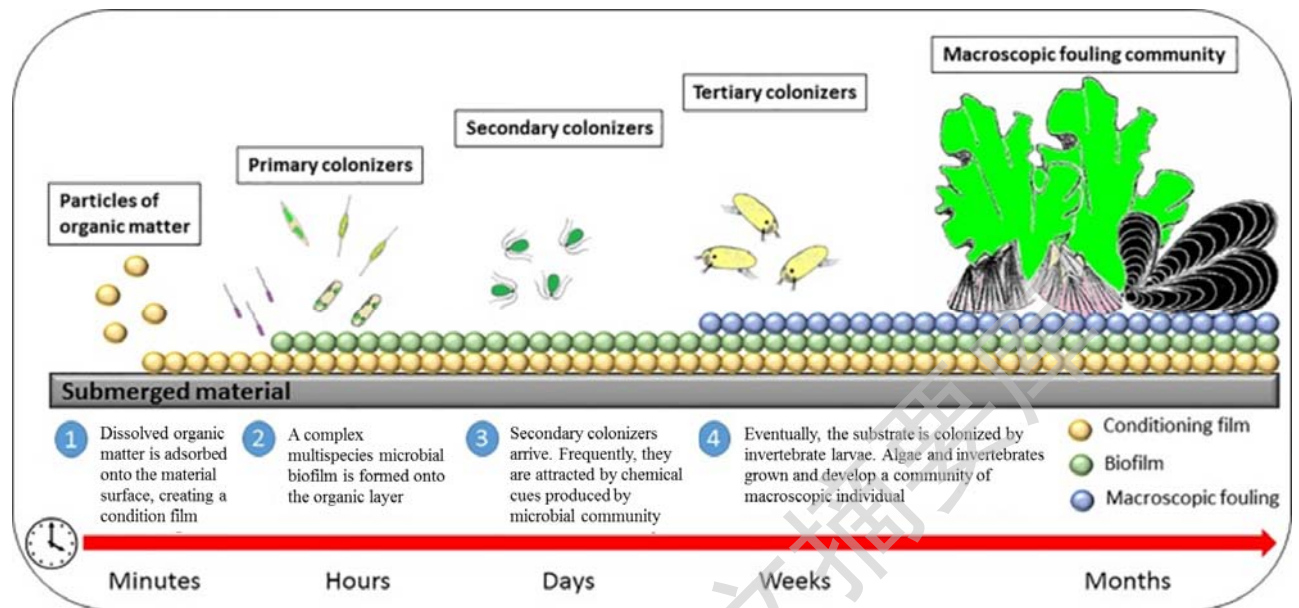


Figure 1.1 Temporal structure of settlement biofouling organism on substrate surface (Martín-Rodríguez et al., 2015; Yebra et al., 2004)

1.2 Macroalgae as Biofouling Organism

1.2.1 Algae as Biofouling

Algae from phylum Chlorophyta, Phaeophyta and Rhodophyta involved in biofouling are listed in Table 1.1. Fouling by marine algae on man-made structure also bring several impacts to economic and ecological diversity. Algae growth on aquaculture facility and buoys can cause such a weight increase that they can subsequently sink (Lebret et al., 2009)

There are plenty of algae fouling on hulls because vessel moves between areas with different environmental condition and always in the photic zone (Chambers et al., 2006). Sticky marine macroalgae on surfaces of ship increase the resistance penalty for ships by 34% (Schultz, 2007).

Moreover, the colonization on ship hulls and in ballast waters has been linked to the introduction of new species which can potentially become invasive (Jackson, 2008; Molnar et al., 2008). The International Maritime Organization has listed a macroalgae species, wakame seaweed as fouling related invasive species (Anonymous, 2017a). Macroalgae *Caulerpa taxifolia* also were identified as strong invasive species and could reduce the overall diversity (Jousson et al., 2000). *Ulva* is strong invasive species because its can

change the overall diversity of new habitat. (Jackson, 2008; Molnar et al., 2008),

Table 1.1 List of macroalgae involved in biofouling

Phylum	Species	
Chlorophyta	<i>Chaetomorpha fibrosa</i>	<i>Stigeoclonium sp.</i>
	<i>Cladophora sp.</i>	<i>Ulothrix zonata</i>
	<i>Codium fragile</i>	<i>Ulva intestinalis</i>
	<i>Fucus vesiculosus</i>	<i>Ulva lactuca</i>
	<i>Rhizoclonium sp.</i>	<i>Urospora</i>
		<i>penicilliformis</i>
	<i>Siphonales</i>	
Phaeophyta	<i>Desmarestia sp.</i>	<i>Sargassum muticum</i>
	<i>Ectocarpus sp.</i>	<i>Scytosiphon</i>
		<i>lomentaria</i>
	<i>Laminaria sp.</i>	<i>Undaria pinnatifida</i>
	<i>Petalonia fascia</i>	<i>Vaucheria sp.</i>
	<i>Pilayella littoralis</i>	
Rhodophyta	<i>Acrochaetium sp.</i>	<i>Furcellaria</i>
		<i>lumbricalis</i>
	<i>Antithamnion sp.</i>	<i>Hildenbrandia sp.</i>
	<i>Bangia fuscopurpurea</i>	<i>Polysiphonia sp.</i>
	<i>Ceramium rubrum</i>	<i>Rhodomela</i>
	<i>confervoide</i>	

Source: (Lebret et al., 2009)

However, the existence of algae as biofoulers has gotten less attention than invertebrate foulers such as barnacle, ascidian, mussels and tubeworm. In other hands, the structure and diversity of marine biofouling are objected to climate changes (Anonymous, 2015; Peck et al., 2015), so the structure of fouling community will change from hard fouling species dominant into soft fouling species dominant. Dobretsov (2009) predicted that the domination of biofouling communities by mussel and barnacle will be taken place by algae and tunicates (soft bodies). This prediction in along with the prediction that all marine creatures with calciferous structure affected by oceanic acidification

Even though there were not enough data talking about the effect of ocean acidification on algae settlement and aggregation. Literatures have reported that algae get the benefit as effects of increased dissolved inorganic carbon which can promote the growth of algae and also allow them to alter more resource into anti-herbivore and photoprotective compounds (Brodie et al., 2014; Cornwall et al., 2012; Harley et al., 2012). This part gives us reasons

why studies of algae as marine biofouling are important.

1.2.2 Green Tide

Recently, *Ulva* got a lot of attention because of its fast growing and covering huge areas. This massive algae blooming usually refers as green tide (Valiela et al., 1997). The green tide mostly happened in the photic zone of eutrophic coastal water (Teichberg et al., 2009). The blooming of this algae can trigger series of harmful impacts to ecosystem, including interference on the growth of seagrasses, alteration in benthic fauna community, disruption the carbon and nitrogen cycles of marine habitat (Valiela et al., 1997), and production of allelopathic toxin (Nelson et al., 2003). The huge amount of algae biomass on beaches and coastal water causes a big social and economic impact to local people (Ye et al., 2011).

Although green tides recurred annually from May to July in China since 2007 and was cited as the biggest case ever reported in the world (Keesing et al., 2011; D. Liu et al., 2013), actually green tides are not unique to China. This phenomenon have previously noted in three decades in marine and estuaries worldwide (Charlier et al., 2007; Schories et al., 2000). The green macroalgae blooms are usually more insistent compared to the temporary blooms of microalgae (Bonsdorff et al., 1997).

There are two assumptions about the origin of the green tide in Qingdao (China)(Smetacek & Zingone, 2013). First, the blooming of *Ulva* was seeded from the dislodging and discarding of the biomass of *Ulva* in seawater during harvest of *Porphyra* sp. in the spring. This *Ulva* thally were thrown into seawater because their attachment to the net of *Porphyra*, and that seeds grew faster in the mid-summer (D. Liu et al., 2013). Second, based on genetic signature it was found that fragment of this bloom-forming algae could overwinter in sediment and then as the stem on summer bloom (F. Liu et al., 2013)

Actually, bloom-forming *U. prolifera* (dominant species in green tides in Qingdao) is not fully floating species because the seeds came from attached fronds (Geng et al., 2015) and the spores of this species have the ability to attach on its mother frond or call as in situ settlement (Gao et al., 2010; Lin et al., 2008). So the green tide actually has a correlation with settlement and aggregation of the spores.

Green tide is not only associated with the vast growing of “floating *Ulva*” (floating

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