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Conspecific cues that induce spore settlement in the biofouling and green tide-forming alga Ulva tepida provide a potential aggregation mechanism



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ABSTRACT

Ulva, an important biofouling and green tide-forming alga, forms aggregations that have adverse economic and ecological impacts. However, little is known regarding the aggregation mechanism of Ulva. Knowledge of Ulva aggregation provides clues for controlling this alga in marine biofouling and green tides. We hypothesized Ulva contains conspecific settlement cue(s). Hexane and dichloromethane extracts of U. tepida fronds were found to induce the settlement of conspecific spores. From these extracts, two bioactive compounds that induce spore settlement in U. tepida were isolated: C16:4 at 5 μ g mL⁻¹ and di-n-butyl phthalate at 0.1–10 μ g mL⁻¹. Fatty acids with different carbon chains (C16-C18) and degrees of saturation, C16:1 and C18:2, previously reported to be present in U. tepida fronds, showed significant inducing activity for spore settlement. C16:1, C16:4, C18:2, and di-n-butyl phthalate all triggered dense settlements of U. tepida spores. The results support our hypothesis and provide insight into possible aggregation mechanisms for Ulva.

1. Introduction

Ulva (= Enteromorpha) spp. are cosmopolitan intertidal green macroalgae (Kirkendale et al., 2013; Wichard et al., 2015) and are the most common macroalgal biofoulers in the world (Callow, 1986; Hoipkemeier-Wilson et al., 2004). The production of motile spores and settlement of the spores on substrata are key stages in the Ulva life cycle. Habitat selection occurs during the progression from planktonic spores to benthic sporelings. Spores actively swim, explore, and sense potential surfaces (Reed et al., 1992; Pickett-Heaps et al., 2010; Heydt et al., 2012), following which they attach to a surface and develop into new plants.

In the field, Ulva settles in aggregates (Fig. 1) often on manmade surfaces, causing adverse economic and ecological impacts. Biofouling results in increased fuel consumption and more costly dry-docking for ships (Abbot et al., 2000; Callow and Callow, 2002) as well as other substantial damage to marine artificial structures (Messano et al., 2009; Venkatesan et al., 2017). Green tides are another example of Ulva aggregation (Teichberg et al., 2010; Bast et al., 2014; Hu et al., 2014; Gao

et al., 2017, 2018). Green tides are large volumes of algal biomass on beaches and in coastal waters. Green tides harm tourism-based economies, interrupt traditional fisheries and impact aquaculture (Ye et al., 2011; Smetacek and Zingone, 2013). However, in the current literature, little is known regarding the aggregation mechanism of Ulva.

Transition from a planktonic propagule to a benthic mode of life is common in sessile fouling organisms. Gregarious settlement, aggregation, is also very common. The biological advantages of living in dense groups include enhanced cross-fertilization success of propagules, decreased probability of predation (Highsmith, 1982), and reduced turbulence from wave action (Railkin, 2004). Many studies demonstrate chemical cues associated with conspecific juveniles and adults of invertebrates induce the settlement of planktonic larvae on or near adults, resulting in aggregation. The original work on gregarious settlement resulting in aggregation dates to the 1950s (Barnes, 1953; Crisp and Knight-Jones, 1953; Hidu, 1969). The settlement of Pacific oyster (Crassostrea gigas) larvae is guided by glycoproteins contained in the shell of conspecifics (Vasquez et al., 2013). The larvae of the barnacle Balanus (= Amphibalanus) amphitrite are induced to settle by water-

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Fig. 1. *Ulva* as a biofouling and green tide-forming species. **(A)** Scanning electron microscope (SEM) image of an *U. tepida* spore taken with a SNE-3200M SEM at 20k magnification. **(B)** Green tide of *Ulva* in Qingdao coastal waters in China. **(C)** Gregarious settlement of *Ulva* on the beach during late winter until late spring on the Xiamen coast in China. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

borne odors of conspecifics (Rittschof et al., 1984), by molecules from adults that attract predatory snails (Rittschof, 1985; Rittschof and Bonaventura, 1986), as well as by a settlement-inducing protein complex (SIPC) extracted from conspecific adults (Yoshinaga et al., 2000; Dreanno et al., 2006). Larvae of the sand dollar *Echinarachnius parma* are induced to settle and metamorphose by an adult-associated factor with a molecular weight < 1000 Da (Pearce and Scheibling, 1990). Although the larval settlement of several invertebrate species has been found to be induced by chemical cues from conspecifics, few conspecific cues have been isolated and structurally identified because they have historically been difficult to isolate in sufficient quantity. Instead, some mimics of peptide pheromones for aggregation have been identified (Rittschof and Cohen, 2004).

Chemical cues play important roles in the life of algae. For example, *U. reticulata* releases chemical compounds with a molecular weight > 100 kD as deterrent signals for settlement by larvae of the polychaete *Hydroides elegans* (Harder and Qian, 2000). Similarly, *U. mutabilis* releases fatty acid-derived metabolites into the culture medium, which are believed to act as signaling molecules between the algae and bacteria (Alsufyani, 2014). Female gametes of brown algae release unsaturated, nonfunctionalized acyclic, and/or alicyclic C₁₁ hydrocarbons to attract conspecific male gametes (Boland, 1995). Callow and Callow (1998, 2000) report the effects of fatty acids on spore settlement in *Ulva*. Inspired by their observation that silicone elastomers cured with dibutyltin dilaurate attracted the attachment of more *U. compressa* spores, they found that the saturated fatty acids C₁₂–C₁₆ induced the settlement of *U. compressa*. The saturated fatty acids C₁₇–C₂₁ possessed no inductive activity, and monounsaturated fatty acids (C14:1, C16:1,

and C18:1) induced greater spore settlement than the equivalent saturated fatty acids (C14:0, C16:0, and C18:0). Since the frond of *Ulva* contains a relatively high proportion of lipids and bacterial films that might release fatty acids, Callow and Callow (1998, 2000) discussed the possibility of fatty acids as chemical cues to guide spore settlement in the natural environment. However, there are no reports of conspecific cues inducing the settlement of macroalgal spores.

In the present study, we tested the hypothesis that *Ulva* contains inducing compound(s) for settlement of its spores.

2. Materials and methods

2.1. Extraction and isolation of active compounds from U. tepida

Ulva fronds were collected from the intertidal zone (24°35'20.584" N, 118°06′58.114″ E) in Xiamen, Fujian Province, China during January 2016. Species identification was performed based on the internal transcribed spacer (ITS) nucleotide sequence analysis (Zhang et al., 2018). The ITS sequence of the algal sample was compared with sequences in GenBank using BLAST. The ITS sequence of our sample showed the highest similarity with Ulva tepida (MK426953.1) (100%). The obtained sequence data was submitted to GenBank with the accession number MK850208. The reason for choosing this species was that we found this alga as a common biofouler on artificial submerged structures in Xiamen. U. tepida has also been reported as a bloomforming alga (Chávez-Sánchez et al., 2019). The fronds of U. tepida were thoroughly rinsed with freshwater to remove epiphytes and debris. Fronds were air dried for 48 h, obtaining a dry weight of 11.52 kg. Dried fronds were extracted with 80 L hexane (HE), then the residue was extracted with 80 L dichloromethane (DI). Extraction was performed three times with each organic solvent. The HE and DI extracts were dried under reduced pressure. For an aqueous extract (AQ), fresh fronds were lyophilized, resulting in a dry weight of 17.24 g, and extracted with 1 L distilled water. The aqueous extract was then lyophilized. All extracts were stored at -20 °C prior to the bioassays.

Bioassays with the extracts indicated that the AQ extract did not significantly induce spore settlement, whereas the HE and DI extracts significantly induced spore settlement (Fig. 2). Therefore, the HE and DI extracts were chosen for isolation of the bioactive compounds. The HE extract was fractionated using a Solid-Phase Extraction (SPE) column packed with NH₂ (aminopropyl) resin and successively eluted with HE: ethyl acetate (EA) 10:1, 4:1, 2:1, 1:1, 100% EA and then 100% methanol to yield seven fractions (FH1-FH7), among which the active fraction FH6 was a pure compound (compound 1). The DI extract was fractioned on a silica column eluted with a gradient of petroleum ether (PE)/EA (starting with PE: EA 5:1, followed by PE: EA 4:1, 3:1, 2; 1, and then 1:1) to give eight subfractions (FDI-FDVIII). Fraction FDI showed settlement-inducing activity and was therefore selected for further purification. This fraction was subjected to preparative thin-layer chromatography (TLC) on silica plates, using HE: diethyl ether: acetic acid 70:30:1 (v/v/v) as the developing solvent, to obtain compound 2.

2.2. Compound elucidation

The structures of the compounds were identified based on the NMR and MS spectral data. The NMR spectra were obtained in deuterochloroform (CDCl₃) on a Bruker Avance III 600 instrument (¹H NMR, 600 MHz; ¹³C NMR, 150 MHz) with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in parts per million (ppm, δ), and coupling constants (*J*) are expressed in Hz. The MS data were measured on a Bruker electrospray ionization quantitative timeof-flight (ESI-Q-TOF) mass spectrometer.



Fig. 2. Extraction and isolation of settlement-inducing active compounds for *U. tepida* spore settlement from conspecific fronds. Significant differences compared with the control ($0 \ \mu g \ mL^{-1}$) are indicated by * (Tukey's post-hoc test, *P* < 0.05). Data are expressed as the mean + standard deviation (n = 3). HE: hexane; DI: dichloromethane; AQ: aqueous; RFU: relative fluorescent unit; SPE: solid phase extraction; TLC: thin-layer chromatography.

2.3. Effects of fatty acids with different chain lengths and saturation degrees on spore settlement

Chlorophyta contain large amounts of fatty acids with 16 and 18 carbon chains (Jamieson and Reid, 1972; Aknin et al., 1992; Khotimchenko, 1993). Compound 1, which induces spore settlement, was identified as the fatty acid hexadeca-4,7,10,13-tetraenoic acid (C16:4). To assess the possible involvement of other fatty acids as spore settlement inducers we tested other fatty acids palmitic acid (C16:0, \geq 99%), margaric acid (C17:0, \geq 98%), stearic acid (C18:0, \geq 98.5%), palmitoleic acid (C16:1, \geq 98.5%), oleic acid (C18:1, \geq 99%), linoleic acid (C18:2, \geq 99%), and α -linolenic acid (C18:3, \geq 99%) (Sigma-Aldrich, St Louis, MO, US). Additionally, we evaluated the effects of different chain lengths and saturations of the fatty acids on spore settlement.

2.4. Spore settlement assay

Spores of *U. tepida* were released from fertile *U. tepida* plants collected a few days prior to spring tide by placing plants into a glass tank containing filtered (0.22 µm) seawater (FSW, pH 8.14, salinity 31 psµ). Spore settlement assays were carried out following Callow et al. (1997) and Mieszkin et al. (2012) with modifications. Briefly, assays were conducted in six-well plates (Greiner Bio-One, Frickenhausen, Germany). The concentration of spore suspension was adjusted to 1×10^6 spores mL⁻¹ (OD_{600 nm} = 0.16) using FSW. Organic extracts and fractions were dissolved in dimethyl sulfoxide (DMSO), and the aqueous extract was dissolved in FSW. A volume of 50 µL of each solution was added into each triplicate well containing 4.95 mL spore suspension and a glass coverslip (24 × 24 mm). For the assays with the organic

extracts and fractions, a 1% (v/v) solution of DMSO in the spore suspension was used as a control. For the assay with the aqueous extract, spore suspension without any chemicals added was used as a control. After incubation of the plates for 1 h in the dark at room temperature, unsettled spores were removed from the coverslips by passing the coverslips back and forth 10 times in FSW (Callow et al., 1997). The coverslips were fixed with 10 mL of 2.5% (v/v) glutaraldehyde in FSW. The biomass of the attached spores was estimated by quantifying the chlorophyll fluorescence in a plate reader (TECAN GENios Plus) (Mieszkin et al., 2012). Fluorescence was measured as relative fluorescence units (RFUs). The RFU value for each coverslip was the average fluorescence reading of 36 points taken randomly within the coverslip.

In the assays with pure compounds, toxicity to spores was evaluated in addition to the effect on spore settlement. Compounds were dissolved in DMSO. The spore settlement assays with pure compounds **1**, **2** and the aforementioned fatty acids were performed as described above, and there were six replicates for each compound and control (1% DMSO). After removing unattached spores from the coverslips, three replicates of coverslips were subjected to fluorescence measurement as described above. The other three replicates were placed into new six-well plates, to which 5 mL FSW was added to each well and cultured for a further 48 h. The plates were placed in an incubator at 24 °C with a 12:12 light: dark cycle using cool white fluorescent light (3000–4000 lux). Toxicity was determined by lysed/dead settled spores after 48 h.

2.5. Statistical analysis

Differences in algal spore settlement between the treatments and control were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. The significance level was defined as P < 0.05. Statistical analyses were performed using SPSS v.21 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Isolation and identification of conspecific chemical cues inducing U. tepida spore settlement

Fronds of U. tepida were extracted with hexane (HE), dichloromethane (DI), and water (AQ), and the effects of these extracts on the settlement of *U. tepida* spores are shown in Fig. 2. Exposure to the HE and DI extracts at low concentrations (5 μ g mL⁻¹ for the HE extract and 0.5 ug mL^{-1} for the DI extract) significantly increased spore settlement compared to the control (P < 0.001). In contrast, the AQ extract did not increase spore settlement from 0.5 to $100\,\mu g\,m L^{-1}$ (P = 0.255). This result suggested that the U. tepida fronds contain inducing compounds for the settlement of conspecific spores and that these compounds have medium or low polarity. Subsequently, two active compounds were isolated from the HE and DI extracts of the U. tepida fronds (Fig. 2). Compound 1 was obtained as a colorless oil with ESI-MS m/z 247.1703 [M-H]⁻. Compound 2 was obtained as a colorless liquid with ESI-MS m/z 301.1407 $[M + Na]^+$. Their ¹H NMR and ¹³C NMR data are listed in Supplementary Tables 1 and 2 Based on their spectral data and comparisons with the literature [Alamsjah et al. (2005) and Lin et al. (2014)], compounds 1 and 2 were identified as hexadeca-4,7,10,13-tetraenoic acid (fatty acid C16:4, molecular formula of C16H24O2) and di-n-butyl phthalate (molecular formula of $C_{16}H_{22}O_4$), respectively. Their chemical structures are shown in Fig. 3.

The effects of the fatty acid C16:4 and di-n-butyl phthalate under a range of concentrations from 0.1 to 100 µg mL⁻¹ on spore settlement in *U. tepida* are shown in Fig. 3. Exposure to 5 µg mL⁻¹ (i.e., 20.16 µM) of the fatty acid C16:4 significantly enhanced spore settlement compared with the control (0 µg mL⁻¹/0 µM) (Fig. 3a, *P* < 0.001). In the di-n-butyl phthalate assay, the biomass of the settled spores in each of the treatments of 0.1–10 µg mL⁻¹ (i.e., 0.36–35.97 µM) was significantly higher than that in the control (Fig. 3b, *P* < 0.001). Toxic effects of both compounds at 50–100 µg mL⁻¹ (i.e., 201.61–403.23 µM for C16:4 and 179.86–359.71 µM for di-n-butyl phthalate) were observed in the 48-h post-exposure period, as indicated by the lysis and death of the settled spores (Supplementary Fig. 1). In contrast, settled spores from the treatments of 0–10 µg mL⁻¹ of the two compounds (i.e., 0–40.32 µM for C16:4 and 0–35.97 µM for di-n-butyl phthalate) increased in size in the subsequent 48-h post-exposure period, indicating the initiation of germination.

C16:4 and di-n-butyl phthalate triggered gregarious settlement (Fig. 3d and e). Most of the spores in the control (Fig. 3c) attached as a single cell or 2–3 cells. In the presence of C16:4 and di-n-butyl phthalate, the spores aggregated and formed bigger rafts of spores.

3.2. Effects of fatty acids (C_{16} - C_{18}) with different saturation degrees on the settlement of U. tepida spores

Seven fatty acids with different carbon chains (C_{16} – C_{18}) and saturation degrees were tested for spore settlement activity in *U. tepida* (Fig. 4a). Spore settlement differed with different fatty acids. Treatments of the saturated fatty acids with different length carbon chains, namely C16:0, C17:0, and C18:0, were not significantly different from the control with regards to spore settlement. In contrast, treatments of the unsaturated fatty acids (C16:1, C16:4, and C18:2) all exhibited significant increases in spore settlement compared to the control, confirming the settlement-inducing activities of these unsaturated fatty acids. As observed with C16:4, the fatty acids C16:1 and C18:2 also caused the gregarious settlement of spores (Fig. 4c–e). However, it should be noted that the increased number of double bonds in the fatty acids did not guarantee higher settlement-inducing efficiency. In contrast with C16:0 (with no double bond), which showed no inducing

activity, C16:1 (with one double bond) and C16:4 (with four double bonds) both exhibited inducing activity. However, C16:1 was active at 0.5 and $5 \,\mu g \,m L^{-1}$ (i.e., 1.97 and 19.69 μ M), whereas C16:4 was only active at $5 \,\mu g \,m L^{-1}$ (i.e., 20.16 μ M), suggesting that four double bonds in C₁₆ did not increase the inducing activity compared with one double bond in C₁₆. The C₁₈ fatty acid results further confirmed this. While C18:0 (with no double bond) and C18:1 (with one double bond) showed no induction activity for spore settlement, C18:2 (with two double bonds) significantly induced spore settlement at $0.5 \,\mu g \,m L^{-1}$ (i.e., 1.79 μ M), but when the number of double bonds further increased to three (C18:3), there was no inducing activity. In addition, it was observed that the two unsaturated fatty acids with 18-atom carbons (C18:2 and C18:3) were toxic to spores at $5 \,\mu g \,m L^{-1}$ (i.e., 17.86 μ M for C18:2 and 17.99 μ M for C18:3) (Supplementary Fig. 2).

4. Discussion

U. tepida has been found in Japan (Masakiyo and Shimada, 2014), Australia (Carl et al., 2014), India (Bast et al., 2014) and the eastern Pacific (Chávez-Sánchez et al., 2019). Here we found this species in southern China. *U. tepida* is similar in phenotype with another biofouling and bloom-forming alga *U. intestinalis* (Masakiyo and Shimada, 2014). Chávez-Sánchez et al. (2019) found the morphotype *U. intestinalis* collected in Mexico, corresponded genetically to *U. tepida*, suggested there was a misidentification and proposed the nomenclature adjustment for the previous collections of *U. intestinalis* morphotypes in Mexico. Likewise, in previous publications which identified *U. intestinalis* based on morphological and anatomical characters, there is a possibility that *U. tepida* has been misidentified as *U. intestinalis* (the molecular analysis is needed to confirm that), and *U. tepida* might be distributed more widely than reported.

In the present study, the fatty acid C16:4 and di-n-butyl phthalate were isolated from the gregariously settling macroalga *U. tepida* and were found to significantly induce the settlement of conspecific spores, suggesting that compounds from conspecific fronds may be involved in mediating the gregarious settlement.

The polyunsaturated fatty acid C16:4 has been previously reported in Ulva and other Chlorophyta species (Alamsjah et al., 2005; Kendel et al., 2015). This compound is also produced and released by diatoms into the seawater as a deterrent signal to grazers (Jüttner, 2001). Here, C16:4 induced the settlement of Ulva spores, indicating that fatty acids in the fronds of Ulva could function as natural chemical cues for spore settlement. Furthermore, because Ulva contains high amounts of C16 and C₁₈ polyunsaturated fatty acids (Jamieson and Reid, 1972), it is important to explore the effects of C_{16} and C_{18} polyunsaturated fatty acids on spore settlement. This study showed that the two polyunsaturated fatty acids C16:4 and C18:2 induced the settlement of Ulva spores. Callow and Callow (2000) previously reported that C18:1 induced the settlement of Ulva spores, which is in contrast to the present findings in U. tepida. Although Callow and Callow (2000) did not report the species of *Ulva* tested, this raises the possibility that the settlement responses of Ulva spores to fatty acids might be species-specific.

The role of fatty acids as natural settlement cues has been reported in some invertebrates. Fatty acids C16:1, C18:3, and C20:6 present in the sand matrices of adults of the tubeworm *Phragmatopoma californica* induced the settlement of conspecific larvae (Pawlik and Faulkner, 1986; Qian, 1999). The settlement of sea urchin (*Pseudocentrotus depressus* and *Anthocidaris crassispina*) larvae was induced by the fatty acids C20:4 and C20:5 present in the foliose coralline red alga *Corallina pilulifera*, upon which they feed (Kitamura et al., 1993). The fatty acids C18:1, C18:2, and C16:4 present in *Ulvella lens* and *U. rigida* germlings significantly induced larval settlement of the abalone *Haliotis tuberculata* (Vicose et al., 2012).

In the present study, the fatty acids C16:1, C16:4, and C18:2 constitute potential settlement cues for *U. tepida* spores in the natural environment. These fatty acids have previously been reported to be







Fig. 3. Effects of the fatty acid C16:4 and di-n-butyl phthalate on the settlement of *U. tepida* spores. (A) Chemical structure of the fatty acid C16:4 and settlement of *U. tepida* spores under exposure to different concentrations of C16:4. (B) Chemical structure of di-n-butyl phthalate and settlement of *U. tepida* spores under exposure to different concentrations of C16:4. (B) Chemical structure of di-n-butyl phthalate and settlement of *U. tepida* spores under exposure to different concentrations of C16:4. (B) Chemical structure of di-n-butyl phthalate and settlement of *U. tepida* spores under exposure to different concentrations of C16:4. (B) Chemical structure of di-n-butyl phthalate and settlement of *U. tepida* spores under exposure to different concentrations of di-n-butyl phthalate. (C) Micrograph of spore settlement in the control (0 µg mL⁻¹). (D) Micrograph of gregarious spore settlement in response to di-n-butyl phthalates at 5 µg mL⁻¹. (E) Micrographs were taken using a Leica DM IL inverted microscope equipped with a DCIM camera. Significant differences compared with the control (0 µg mL⁻¹) are indicated by * (Tukey's post-hoc test, *P* < 0.05). Data are expressed as the mean ± standard deviation (n = 3). The toxic effects of C16:4 and di-n-butyl phthalate at 50 and 100 µg mL⁻¹ are indicated in Supplementary Fig. 1. RFU: relative fluorescent unit.

produced by *Ulva*. C16:1, C16:4, and C18:2 respectively constitute about 1.7%, 7.2%, and 11.7% of the total fatty acid content in *U. intestinalis* (Jamieson and Reid, 1972). *U. lactuca* also contains 1.1%, 16.2%, and 2.3% of the total fatty acid content for C16:1, C16:4, and C18:2, respectively (Khotimchenko et al., 2002). Furthermore, *U. tepida* contains 4.37–5.15%, 8.11–8.50%, and 12.12–14.47% of the total fatty acid content for C16:1, C16:4, and C18:2 (Carl et al., 2016).

The second settlement-inducing compound we isolated from *U. tepida* was di-n-butyl phthalate, which is a phthalate ester (PAE). PAE has been found to accumulate in jellyfish (Morris, 1970), fish (Melancon and Lech, 1976), and bivalves (Wofford et al., 1981). It has also been found in marine algae, including *Sargassum, Ishige okamurae* (Cho et al., 2005; Bazes et al., 2009), and *Ulva* (Chen, 2004). PAE, the most common plasticizer, is widely used in the production of plastics, cosmetics, textiles, food packaging materials, medical bags, and rubber (Zhang et al., 2017). Anthropogenic PAEs originate from the land and enter into the ocean via rivers (Paluselli et al., 2018). Our finding of the inductive effect of di-n-butyl phthalate on *Ulva* settlement indicates the possibility that PAE compounds from human activities might be

involved in the triggering of green tides.

It should be noted that although PAEs are usually thought of as anthropogenic, dibutyl phthalate can be naturally produced by organisms, including *Ulva*. It has been reported that dibutyl phthalate can be synthesized by fungi through shikimic acid pathway, which is assembled by phthalic acid with butyl alcohol through esterification (Tian et al., 2016). The marine macroalga *Bangia atropurpurea* synthesizes di-(2-ethylhexyl) phthalate and di-n-butyl phthalate (Chen, 2004). Namikoshi et al. (2006) analyzed the ¹⁴C natural abundance content of dibutyl phthalate in the macroalgae *Undaria pinnatifida, Laminaria japonica,* and *Ulva,* and reported that dibutyl phthalate is produced by *U. tepida,* or accumulated by *U. tepida* from seawater. Further investigation is required to elucidate the source of di-n-butyl phthalate found in *U. tepida.*

Interestingly, in contrast to the majority of spores settling alone or in small-sized groups in the control, most spores settled in large groups in C16:1, C16:4, C18:2, and di-n-butyl phthalate (Fig. 3 c-e and Fig. 4 b-e). This induced gregarious settlement of spores in response to fatty



different saturation degrees on the settlement of U. tepida spores. (A) Settlement of *U. tenida* spores under exposure to fatty acids (C16-C18) with different saturation degrees. (B) Micrograph of spore settlement in the control ($0 \mu g m L^{-1}$). (C) Micrograph of gregarious spore settlement in response to C16:1 at $0.5 \,\mu g \,m L^{-1}$. (D) Micrograph of gregarious spore settlement in response to C16:4 at $5 \mu g m L^{-1}$. (E) Micrograph of gregarious spore settlement in response to C18:2 at $0.5 \,\mu g \,m L^{-1}$. Micrographs were taken using a Leica DM IL inverted microscope equipped with a DCIM camera. Significant differences compared with the control $(0 \mu g m L^{-1})$ are indicated by * (Tukey's post-hoc test, P < 0.05). Data are expressed as the mean ± standard deviation (n = 3). The toxic effects of C18:2 and C18:3 at $5 \mu g m L^{-1}$ are indicated in Supplementary Fig. 2. RFU: relative fluorescent unit. Con: Control.

Fig. 4. Effects of fatty acids (C₁₆-C₁₈) with



acids is consistent with the findings of Callow and Callow (2000).

The settlement-inducing fatty acids and di-n-butyl phthalate are toxic to Ulva spores at high concentrations. This phenomenon that the settlement-inducing properties and toxic activities are proportional to concentration has also been found in other compounds active for the larvae of some invertebrates (Morse et al., 1979; Young et al., 2015). From an ecological perspective, it could be speculated that the toxic effect of fatty acids and di-n-butyl phthalate at high concentrations may be a mechanism for preventing the colonization of algal surfaces by spores, since algal fronds may contain high amounts of these compounds. However, when these compounds are released into the surrounding seawater and diluted, they could stimulate spores to settle nearby.

The active compounds discovered in our study all have low polarity. To function as chemical cues, these compounds are soluble in low concentrations and can diffuse in seawater. Fatty acids are released by U. mutabilis into its seawater-based culture medium and are suggested to act as communication signaling molecules between U. mutabilis and bacteria (Alsufyani, 2014). Similarly, female gametes of brown alga use low polarity hydrocarbons (unsaturated nonfunctionalized acyclic and/ or alicyclic C₁₁ hydrocarbons) as pheromones to attract conspecific male gametes in the seawater (Boland, 1995). These studies support the role of low-polarity compounds as signaling molecules.

The surface of algal fronds (sporangia) after the release of spores loses cellular integrity, and holes appear on the surface of the sporangia (Supplementary Fig. 3), which may benefit the release of compounds from algal cells into the seawater. Furthermore, Ulva can produce dimethylsulfoniopropionate (DMSP) (Dickson et al., 1980; Van Alstyne et al., 2003). DMSP production would increase when the frond is senescent or exposed to oxidative stress, or when the algal cells lose their

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Fig. 5. The hypothesized schematic of U. tepida aggregation induced by conspecific cues. (A) Mature fronds of U. tepida release actively swimming spores during lunar tides. (B) Actively swimming spores encounter conspecific cues, and are induced to approach the substratum near the conspecific fronds. (C) The spores explore the substratum near the conspecific fronds. (D) The spores withdraw their flagella and release adhesives for settlement, and will later germinate and grow into new fronds. (E) Holes are produced on the surface of the algal fronds after releasing spores (Supplementary Fig. 3), and the DMSP produced by algae can be transformed into DMSO (Sunda et al., 2002), both of which may help increase the release and diffusion of conspecific cues in the seawater.

integrity (Karsten et al., 1990; Wolfe, 2000; Jüttner, 2001). DMSP is transformed into DMSO through the dimethyl sulfide (DMS) pathway (Sunda et al., 2002; Yoch, 2002). Therefore, the process of spore release from the sporangia may also possibly cause an increase in DMSO (due to the increased production of DMSP), which may further promote the release and diffusion of fatty acids and di-n-butyl phthalate in the seawater.

Responding to inductive cues from conspecific fronds enables *Ulva* spores to settle in habitats that are already proved by their predecessors to be suitable for *Ulva* survival and growth (Fig. 5). Fig. 5 is the hypothesized schematic of *Ulva* aggregation induced by conspecific cues. This process may be complex in the marine environment. For example, di-n-butyl phthalate can be biodegraded by environmental microorganisms (Xu et al., 2005; Gu, 2007). It would be interesting to study the effects of its metabolites on *Ulva* aggregation is important for developing controls for this alga in marine biofouling and green tides.

Here, compounds were tested for activity by direct dosing into FSW. The design of a chemical gradient, such as emission of the chemicals from a point source in T-maze (Pila et al., 2017) or Y-maze (Nocchi et al., 2017; Marquet et al., 2018), in which the spores could move into the inductive region and aggregate together, may help to further confirm their functions as aggregation cues. In addition, it should be noted that besides the inducing compounds found in this study, the possibility of the presence of other active chemicals in *Ulva* cannot be excluded. Whether the inducing compounds in *Ulva* have synergistic effect also needs further investigation.

5. Conclusions

The present results show that *U. tepida* contains inducing compounds for settlement of its spores, which support our hypothesis.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2019.104807.

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