

## Effect of temperature, salinity and biofilm on the zoospores settlement of *Enteromorpha flexuosa* (Wulfen) J. Agardh

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Present study consists the, effect of temperature (10-30°C), salinity (15-35 psu) and biofilm on the settlement of *Enteromorpha flexuosa* zoospores. Settlement of zoospores was significantly ( $p < 0.05$ ) influenced by both temperature and salinity. Highest percentage of settlement occurred at 25°C and 30 psu respectively. Subsequently the influence of biofilm was evaluated at the optimum 25°C and 30 psu culture conditions. Among these, the glass cover slip substratum preconditioned with biofilm facilitated higher settlement. Highest spore settlement occurred in the first 30 minutes of incubation and further increase was gradual with time until it reached the optimum.

[**Keywords:** *Enteromorpha flexuosa*, Biofilm, Salinity, Substratum, Temperature]

### Introduction

Fouling organisms (algae, mussels and barnacles) in marine environment entails a serious economic and ecological consequence. These organisms employ a broad range of settlement strategies and bioadhesives for attachment to the hard surface<sup>1,2</sup>. The colonization and establishment of a benthic macroalgal community from a motile, planktonic life form to an attached, sedentary state and this phase is considered crucial for benthic organism including macroalgae which last for minutes, hours or days depending on the species<sup>3</sup>. This is followed by permanent adhesion through rapid secretion and curing of an N-linked, self-aggregating glycoprotein that anchors the spore to the substratum surface<sup>4</sup>.

A variety of factors influence settlement of zoospores (biological<sup>4</sup>; topography of surface<sup>5-6</sup>; charge<sup>2</sup>; chemical patterns<sup>7</sup>). Studies<sup>8</sup> have shown there is a positive correlation between an uncharacterized assemblage of bacteria and attached spores of *Enteromorpha*. However, biofilm of some bacterial strain are reported to inhibit settlement and growth of other microorganism including spores of *Enteromorpha*<sup>9</sup>. Beside this, abiotic factors like salinity and temperature are known to determine period of motility and ability of spores to settle<sup>10</sup>.

*Enteromorpha flexuosa* (Wulfen) J. Agardh (syn. *Ulva flexuosa* Wulfen<sup>11</sup>, is a green alga known to colonizes variety of substrata in intertidal region<sup>12</sup>. *Enteromorpha*'s are cosmopolitan with a worldwide

distribution ([http://hawaii.edu/reefalgae/invasive\\_algae](http://hawaii.edu/reefalgae/invasive_algae)) and *E. prolifera*, a congeneric species is known to cause huge macroalgal bloom in recent times<sup>13,14</sup>. *Enteromorpha* sp. has implication in bioinvasion and ships ballast water management due to high reproductive potential and ability to proliferate at a rapid rate. These spores have greater sensitivity to the influence of abiotic factors than adult. The present study attempts to indicate how an environmental and biological factor influences the settlement process of *Enteromorpha* zoospores.

### Materials and Methods

Fertile mature thalli were collected from Dias beach, Dona Paula bay, Goa, India (15°27'N, 73°48'E). It was washed with filtered seawater and wrapped with absorbent to remove the excess water. They were stress desiccated overnight in dark at 4°C for early zoospore release and zoospores were released by transferring the fronds to a 5 mL Petri dish containing filtered seawater (0.22 µm) under fluorescent light (40 µmol m<sup>-2</sup>s<sup>-1</sup>) at room temperature. Hemocytometer was used to count the number of zoospores in a suspension.

The glass cover slips were used for settlement and were prepared by soaking in 1 M HCl overnight and rinsed with sterile distilled water. Another set was immersed in sterile filtered (0.45 µM) sea water for 15 min to condition the substratum with biofilm. Glass slide were placed in a 5 cm diameter sterile

Petri dish followed by incubation at  $25\pm 1^\circ\text{C}$  in dark by adding 2 mL spore suspension ( $1\times 10^4$  spores,  $\text{mL}^{-1}$ ). Incubation was stopped at 0.15, 0.30, 1.0, 6.0, 12, 24 and 48 hour (h) of time interval. Triplicates of each treatment was set up and maintained. Slides were then washed by passing back and forth in filtered seawater to remove the nonadhered or loosely attached zoospores before fixing in 2% glutaraldehyde (v/v) for 20 minutes. This was followed by rinsing in 1:1 (v/v) seawater/distilled water and allowed to dry. The number of zoospores settled was counted under light microscope at 40x and a mean of 20 count  $\pm$  standard error (SE) of three replicates were calculated.

#### Effect of salinity and temperature on spore adhesion

In the present study, influence of temperature ( $10\text{-}30^\circ\text{C}$ ) and salinity (15-35 psu) on the spore settlement of *Enteromorpha* was tested under laboratory culture conditions. Filtered sea water ( $0.22\ \mu\text{m}$ ) was adjusted to different levels of salinity viz. 15, 20, 25, 30 and 35 practical salinity unit (psu). Salinity was adjusted by diluting in double distilled water. Dishes were filled by 2 mL each of salinity adjusted seawater and a glass cover slip was placed into it. Zoospore suspension ( $1\times 10^5$  spores.  $\text{mL}^{-1}$ ) of 1 mL was dispensed and dishes were incubated at  $25\pm 1^\circ\text{C}$  for 30 min in dark.

Similarly, dishes containing 2 mL of filtered seawater ( $0.22\ \mu\text{m}$ ) was conditioned at different levels of temperature ranging from  $10\text{-}30^\circ\text{C}$  for 30 minutes before the addition of 1 mL spore suspension ( $1\times 10^5$  spores.  $\text{mL}^{-1}$ ). The dishes were consequently incubated at various temperatures for 30 minutes in dark. Three replicate for each treatment were maintained.

#### Statistical analysis

Means and standard error were calculated from the respective replicates. Significance testing within

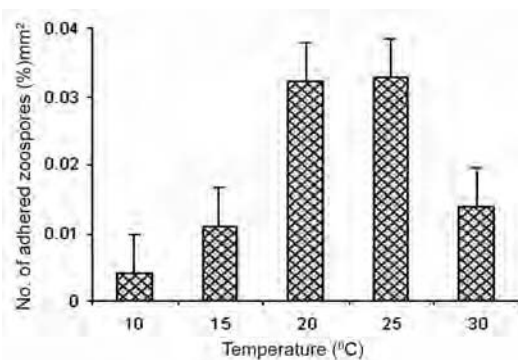


Fig. 1—Effect of temperature on settlement of zoospores. Standard error (SE) is indicated by bar.  $1\times 10^5$  spores.  $\text{mL}^{-1}$

experiments was performed by analysis of variance (ANOVA) (confidence level 95%) using Statistica 6.

#### Result and Discussions

The effects of temperature and salinity on the biological processes of living organisms are well documented, however the tolerance limit vary among species. Settlement of zoospores was strongly influenced by both temperature and salinity. Zoospores settlement witnessed an increment with increase in temperature and highest percentage of settlement was recorded at  $25^\circ\text{C}$  followed by  $20^\circ\text{C}$  (Figure 1), the settlement at this temperature was significantly different ( $P < 0.05$ ; Tukey test; Table 1a). In a similar study, Callow *et al.*<sup>12</sup> reported that spore adhesion was strongly temperature dependent at  $25^\circ\text{C}$ . *Enteromorpha*'s are known to have a wide range of tolerance of salinity but the effective level of salinity for a settlement process was not known. The result obtained revealed an upward trend in the rate of spore settlement with salinity until the maximal settlement at 30 psu (Figure 2) which were statistically significant ( $P < 0.05$ ; Tukey test; Table 1a) and further increase in salinity level showed a decreased rate of settlement. Laboratory study of green tide (bloom) forming algae showed that it grows rapidly at temperature of 15 or  $20^\circ\text{C}$ , tolerating up to  $30^\circ\text{C}$  while it showed a broad tolerance of salinity from 3.4 to 34 psu<sup>15</sup>. However, no consistent relationship to salinities is reported to exist<sup>16</sup> instead, marine algae tolerance to salinity is largely related to an ecological position they occupy in the intertidal region<sup>17</sup>.

The result from the present study showed that the zoospore settlement was significantly different between two substratums, and the substratum conditioned with biofilm prior to settlement facilitated

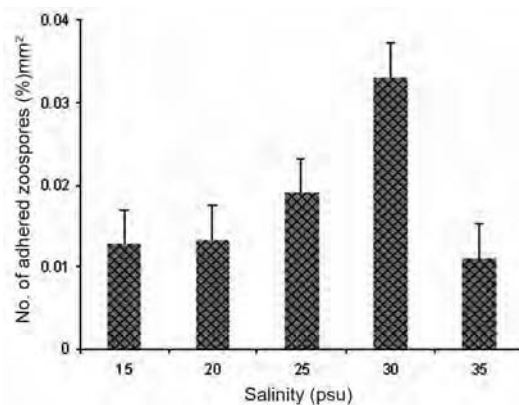


Fig. 2—Effect of salinity on settlement of zoospore. Bar indicates standard error (SE).  $1\times 10^5$  spores.  $\text{mL}^{-1}$

Table 1—Effect of temperature, salinity and biofilm on zoospore settlement

**a) One way ANOVA**

	SS	df	MS	F	p*
Temperature	341554.4	4	85388.6	5.29189	0.014946
Salinity	165632.9	4	41408.2	3.64747	0.044121

**b) Two way ANOVA**

Control	46707.0	6	7784.5	2.8550	0.049598
Biofilms	56411.6	6	9401.9	3.2765	0.031529
Control x Biofilms	103119	12	8593	3.0712	0.007048

\*indicates statistical significance

enhanced settlement compared to control. The influence of biofilm effect on the spore settlement was evaluated at the optimum temperature (25°C) and salinity 30 psu. The highest zoospore settlement occurred in the first 30 minutes of incubation and further increase was gradual until it reached optimum (Figure 3 a). The analysis by two-way ANOVA (Table 1b) on the effect of substratum's revealed that the substratum preconditioned with biofilm significantly facilitated higher spore settlement ( $P < 0.05$ ; Tukey test). In substratum preconditioned with biofilm, maximal settled zoospores (0.22%) was observed in 6 hours of incubation (Figure 3b), while, in control/unconditioned substratum, the number of settled spores (0.18%) was highest in 12 hours of

incubation (Figure 3b). The surface of an object, moreover, is known to rapidly adsorb the organic/inorganic matter present in the water column when an object is immersed in seawater, thereby conditioning its surface that assist colonization by various microorganisms<sup>18</sup>. Zoospores will preferentially settle on such surface due to availability of nutrients for its growth, and as early aged biofilms are believed to be chemically diverse and spatially dynamic from even a short period of immersion<sup>19</sup>. The presence of biofilm invariably provided the spores a favorable substratum for settlement. Similarly, enhanced settlement had been observed on mixed microbial biofilm of a congeneric species of *Enteromorpha*<sup>20</sup> and *Ulva fasciata* spore on a biofilm surface<sup>21</sup>.

This study shows that surface conditioning by biofilms prior to spore settlement implicitly facilitate settlement which probably might be due to chemical cues given out by the biochemical substances/biofilms. Further studies to asses and understand the mechanism responsible for release of chemical cues and the possible biochemical pathway would greatly provide better insight to enable the management of biofouling menace.

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**References**

- 1 Smith A M & Callow J A, Biological adhesives. Berlin. Springer. (2006).
- 2 Ederth T, Nygren P, Pettitt M E, Ostblom M, Du C X, Broo K, Callow M E, Callow J A & Liedberg B, Anomalous settlement behavior of *Ulva linza* zoospores on cationic oligopeptide surfaces. *Biofouling*, 24 (2008) 303–312.

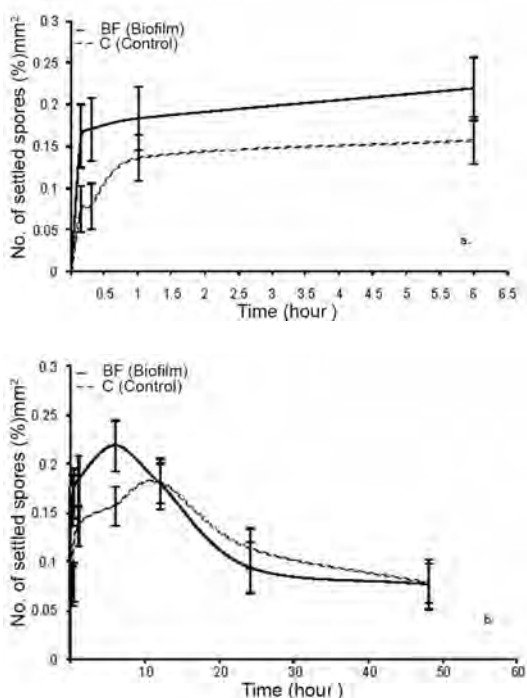


Figure 3a & b—Effect of substratum on the settlement of zoospores. Bar represents SE.  $1 \times 10^4$  spores. mL<sup>-1</sup>

- 3 Fletcher R L & Callow M E, The settlement, attachment and establishment of marine algal spores. *Br. Phycol. J.*, 27 (1992) 303 – 329.
- 4 Callow M E & Callow J A, Substratum location and zoospore behaviour in the fouling alga *Enteromorpha*. *Biofouling*, 15 (2000) 49–56.
- 5 Granhag L M, Finlay J A, Jonsson P R, Callow J A & Callow M E, Roughness-dependent removal of settled spores of the green alga *Ulva* (Syn. *Enteromorpha*) exposed to hydrodynamic forces from a water jet. *Biofouling*, 20 (2004) 117-122.
- 6 Long C J, Schumacher J F, Robinson II Paul A C, Finlay J A, Callow M E, Callow J A & Brennan A B, A model that predicts the attachment behavior of *Ulva linza* zoospores on surface topography. *Biofouling*, 26 (2010) 411-419.
- 7 Finlay J A, Krishnan S, Callow M E, Callow J A, Dong R, Asgill, N, Wong K, Kramer E J & Ober C K, Settlement of *Ulva* zoospores on patterned fluorinated and PEGylated monolayer surfaces. *Langmuir*, 24 (2008) 503–510.
- 8 Joint I, Callow M E, Callow J A & Clarke K R, The attachment of *Enteromorpha* zoospores to a bacterial biofilm assemblage. *Biofouling*, 16 (2000) 151–158.
- 9 Patel P, Callow M E, Joint I & Callow J A, Specificity in the settlement – modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. *Environmental Microbiol.*, 5 (2003) 338-349.
- 10 Christie A O, Spore settlement in relation to fouling by *Enteromorpha*. In *Proc. Third Int. Congr. Mar. Corrosion and Fouling* (Acker, R F, Floyd Brown, B., De Palma, J. R. & Iversion, W.P., editors),. Northwestern University, Evanston. (1973) 674-681.
- 11 Hayden H S, Blomster J, Maggs C A, Silva P C, Stanhope M J & Waaland J R, Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *Eur. J. Phycol.*, 38 (2003) 277-294.
- 12 Callow M E, Callow J A, Pickett-Heaps J D & Wetherbee R, Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules quantitative settlement studies and video microscopy. *J. Phycol.*, 33 (1997) 938-947.
- 13 Liu D Y, Keesing J K, Xing Q G & Shi P, World’s largest macroalgal bloom caused by expansion of seaweed aquaculture in China. *Mar. Poll. Bull.*, 58 (2009) 888-896. doi:10.1016/j.marpolbul.2009.01.013.
- 14 Gao S, Chen X, Yi Q, Wang G & Pan G A, Strategy for the Proliferation of *Ulva prolifera*, Main Causative Species of Green Tides, with Formation of Sporangia by Fragmentation. *PLoS ONE.*, 5 (2010) 85-71. doi:10.1371/journal.pone.0008571
- 15 Taylor R, Fletcher R L & Raven J A, Preliminary studies on the growth of selected green-tide algae in laboratory culture: Effects of irradiance, temperature, salinity and nutrients on growth rate. *Botanica Marina*, 44 (2001) 327–336.
- 16 Gessner F & Schramm W, Salinity: Plants. In: Kinne, O. (Ed.) *Marine Ecology*. Wiley and Sons, New York 1, 2 (1971) 705- 820.
- 17 Perkins E J, The biology of estuaries and coastal waters. Academic Press, London., (1974) 678.
- 18 Baker D P, Klijnstra J W, Busscher H J & Van der Mei H C, The effect of dissolved organic carbon on bacterial adhesion to conditioning films adsorbed on glass from natural seawater collected during different seasons. *Biofouling*, 19 (2003) 391 – 397.
- 19 Michael T & Smith C M, Lectins probe molecular films in biofouling: Characterization of early films on non-living and living surfaces. *Mar. Ecol. Progr. Ser.*, 119 (1995) 229-236.
- 20 Dillon P S, Maki J S, Mitchell R, Adhesion of *Enteromorpha* swimmers to microbial films. *Microb. Ecol.*, 17 (1989) 39–47.
- 21 Shin H W, Rapid attachment of spores of the fouling alga *Ulva fasciata* on biofilms. *J. Environ. Biol.*, 29 (2008) 613-619.