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# Induction of larval settlement in the polychaete *Hydroides elegans* by surface-associated settlement cues of marine benthic diatoms

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ABSTRACT: Larval settlement in the polychaete *Hydroides elegans* is mediated by marine biofilms, which are complex agglomerates of bacteria, diatoms, fungi and protozoa. The induction of *H. elegans* larval settlement by marine biofilms has been mainly attributed to bacteria and diatoms. In contrast to bacteria-derived settlement cues, the nature and origin of diatom-derived settlement cues is poorly understood. In this study, we present the first investigation on the nature and origin of larval settlement cues produced by marine diatoms. Diatoms with inductive (i.e. *Achnanthes* sp. and *Nitzschia constricta*) and non-inductive (i.e. *Amphora tenerrima* and *N. frustulum*) effects on larval settlement of *H. elegans* were selected as model strains in this investigation. Larval settlement bioassays with a choice between monospecies diatom films and unfilmed substratum revealed that the diatom-derived settlement cue was water-insoluble and associated with the film surface. There was a clear correlation between the surface coverage of diatoms in films and their inductive effect on larval settlement. In the case of *Achnanthes* sp., even the lowest surface coverage of 1.8% induced larval settlement significantly more than the control of filtered seawater. The interstitial distance between diatom cells at this low film density was within the body size range of *H. elegans* larvae (100 ± 30 µm). The inductive effect of diatom films on larval settlement prevailed even after heat treatments (121°C for 1 h) that completely killed the diatoms, as verified with the vital stain fluorescein diacetate. These results suggest that the induction of larval settlement by diatoms is not related to their viability, and that, contrary to marine bacteria, diatom-induced larval settlement cues are composed of heat-stable surface components, such as capsular extracellular polysaccharides. These results not only demonstrate that different components of biofilms play inductive and inhibitive roles on larval settlement, but also that their relative space occupation deserves consideration regarding their roles as mediators of larval settlement.

KEY WORDS: Diatom · Larvae · Settlement · Biofilm · *Hydroides elegans*



## INTRODUCTION

The life cycle of many sedentary marine invertebrates includes a pelagic larval stage. The exploration of marine substrata by larvae leads either to permanent attachment and metamorphosis (in the following referred to as settlement), or to rejection and dislodgement from the substratum. The larval recognition of suitable marine surfaces as permanent attachment sites is largely guided and mediated by marine biofilms (Zobell & Allen 1935, Pearce & Scheibling 1991, Slattery 1992, Tamburri et al. 1992, Keough & Raimondi 1996, Chan & Walker 1998). Marine biofilms develop on all submerged surfaces and comprise complex agglomerates of bacteria, diatoms, fungi, and protozoa, all of which are embedded in a matrix of extracellular polymers (Mihm et al. 1981, Wahl 1989, Holmström & Kjelleberg 1994). Biofilms have been intensively investigated with respect to their stimulatory or inhibitory role in larval settlement (Olivier et al. 2000). In the majority of these investigations, the biological role

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of marine biofilms was largely attributed to the bacterial component in biofilm matrices (Maki et al. 1988, Holmström et al. 1992, Wieczorek et al. 1995, Wieczorek & Todd 1997).

The proportion of benthic diatoms in marine biofilms can be significant, particularly in the photic zone. Under the consideration of relative space occupation of different components of marine biofilms, we previously investigated the role of various monospecies diatom films as potential mediators of larval settlement in *Hydroides elegans* Haswell (Harder et al. 2002a), a serpulid polychaete with a strong settlement preference for marine biofilms in general (Beckmann et al. 1999), and a particular preference towards the bacterial component of these films (Lau & Qian 2000). Out of 32 benthic diatoms isolated from marine biofilms in Hong Kong waters, 13 strains from 7 genera significantly evoked larval settlement of *H. elegans*, while the rest had no effect. Importantly, the effect of benthic diatoms on the mediation of larval settlement was not due to bacterial contaminants in non-axenic diatom films (Harder et al. 2002a).

Similarly, bacteria with an inductive or non-inductive effect on larval settlement of *Hydroides elegans* have been identified from marine natural biofilms (Lau et al. 2002). It was shown that bacterial exopolymers facilitate the mediation of bacteria-derived settlement cues to *H. elegans* larvae (Lau et al. 2003), yet the settlement signal is mainly comprised of small hydrophobic molecules (Harder et al. 2002b). Contrary to chemical cues, physical surface properties, such as wettability and surface roughness, are less important in the induction of larval settlement of *H. elegans* (Qian 1999, Qian et al. 1999, 2000).

Based on this body of knowledge, we investigated the induction of larval settlement in *Hydroides elegans* by marine benthic diatoms, with particular emphasis on the origin and nature of diatom-derived settlement cues in comparison with bacteria-derived counterparts. The objectives of this study were to (1) investigate potential origins of diatom-derived settlement cues, e.g. waterborne or cell-associated, and (2) investigate the effect of diatom cell viability and the inductive effect on larval settlement of selected diatom species. Based on our previous categorization of inductive and non-inductive diatoms (Harder et al. 2002a), the inductive species *Achnanthes* sp. and *Nitzschia constricta* and the non-inductive species *Amphora tenerrima* and *N. frustulum* were selected as model strains for this study.

## MATERIALS AND METHODS

**Larval culture.** From October 2002 to March 2003, white PVC settling panels (10 × 10 cm) were submerged for 4 wk at a fish farm in Yung Shue O, Hong Kong (22° 19′ N, 114° 16′ W) at 2 m depth. After this period, the panels were covered with a thick meshwork of the calcareous tubeworm *Hydroides elegans* and the ascidian *Styela plicata*. In the laboratory, adult *H. elegans* were dislodged from these panels and induced to spawn by gentle tapping on the posterior end of the tube with a dissecting needle. The resulting gamete-containing seawater was filtered through 70 µm mesh to remove debris and then washed in 0.22 µm filtered seawater (FSW) before being transferred into culture containers. The larval cultures were incubated at 24°C under a 15:9 h light:dark (L:D) photo cycle and maintained according to the methodology of Qiu & Qian (1997) and Harder et al. (2002a). Larval competence was determined according to larval morphology in Wisely (1958) and by a rapid test with 3-isobutyl-1-methylxanthine (IBMX) according to Qian & Pechenik (1998). Batches of competent larvae were sieved through a 110 µm nylon mesh. The larvae retained on the mesh were transferred into a container with FSW and used for bioassays within 1 h.

**Development of marine biofilms.** Sterile polystyrene petri dishes (∅ = 5 cm; FALCON #1006, Becton Dickinson) without lids were enclosed in 125 µm nylon bags and anchored at 1.5 m depth at the sampling site for 5 d. The dishes were retrieved and immediately immersed in FSW to avoid desiccation. In the laboratory, biofilmed dishes were rinsed in autoclaved FSW and used as positive controls in subsequent larval settlement bioassays.

**Diatom cultures and development of monospecies diatom films.** Based on our previous data (Harder et al. 2002a), the inductive diatoms *Achnanthes* sp. and *Nitzschia constricta* were chosen as model strains in this study, while the diatoms *Amphora tenerrima* and *N. frustulum* served as non-inductive controls. The criterion for the choice of these diatoms was their tenacity to attach to a substratum, which was a prerequisite for subsequent bioassays. Pure diatom stock cultures were inoculated into 400 ml Erlenmeyer flasks containing autoclaved *f*/2 nutrient medium in FSW (Guillard & Ryther 1962). These cultures were incubated for 1 wk at 24°C with overhead fluorescent illumination ($0.3 \times 10^{16}$ quanta $s^{-1}$ $cm^{-1}$) under a 15:9 h L:D photo cycle. When a visible diatom film had formed in the culture flasks, monospecies diatom suspensions were prepared by brushing the flask interior with a sterile paintbrush. Known volumes of non-axenic suspensions of diatoms and bacteria were filtered through 1 µm filter membranes (Osmonics) with the exception of *N. constricta*, where a 0.45 µm membrane was utilized. Filter residues were washed with autoclaved FSW to reduce the number of non-attached bacteria. Subsequently, diatoms were resuspended in 50 ml FSW. The diatom-free bacterial filtrate was reduced to the

original volume on a 0.22 µm filter membrane. The bacteria-containing diatom suspensions and the corresponding diatom-free bacterial samples were used to prepare diatom films and diatom-free bacteria films. Briefly, 5 ml aliquots of the suspensions were transferred into sterile petri dishes with replication (n = 8) and incubated for 24 h for the attachment of cells and development of films. Subsequently, filmed dishes were dip-rinsed in autoclaved FSW to remove unattached cells. Prior to the larval settlement bioassay, 5 ml autoclaved FSW was added to dishes.

**Larval settlement bioassays.** Still-water larval settlement assays were performed with replication (n = 8). Twenty competent *Hydroides elegans* larvae were pipetted from larval batches of high density in the smallest volume possible (ca. 200 µl), transferred into filmed petri dishes and incubated at 24°C under a 15:9 h L:D photo cycle. Clean and biofilmed dishes served as negative and positive controls, respectively. Assays were evaluated after 24 h by recording the number of settled juvenile worms with calcareous tubes under the dissecting microscope. Five replicate dishes were used for larval settlement assays, the remaining 3 dishes were fixed with 4% formaldehyde in FSW for the enumeration of diatoms and bacteria in experimental films.

**Enumeration of attached diatoms and bacteria.** Diatom cells were enumerated by light microscopy and chlorophyll fluorescence. Bacteria were visualized with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie). The formalin-fixed dishes were rinsed with distilled water and stained with DAPI at a concentration of 0.5 µg $ml^{-1}$ for 5 min at room temperature. Diatoms and bacteria were recorded at a magnification of 100× and 1250×, respectively, in 5 randomly chosen fields of view (Zeiss Axiophot; $\lambda_{Ex}$ = 359 nm, $\lambda_{Em}$ = 441 nm). The viability of bacteria in heat-treated diatom films was determined with acridine orange (Sigma) according to Daley & Hobbie (1975). Briefly, the formalin-fixed dishes were rinsed with distilled water and stained with acridine orange at a concentration of 1 mg $ml^{-1}$ for 5 min at room temperature.

**Experimental setups.** ***Expt 1:*** To investigate the effect of different surface wettability on diatom attachment and subsequent larval settlement, polystyrene and borosilicate glass (Pyrex) dishes were tested for their comparability of bioassay results. The wettability of polystyrene and borosilicate glass dishes was determined by surface contact angle measurements (Qian et al. 2000).

***Expt 2:*** To investigate the effect of diatom film density on the induction of larval settlement, concentrated suspensions of the diatoms *Achnanthes* sp. and *Nitzschia constricta* were diluted 0.1× and 0.01× prior to the filming procedure described above. The surface coverage of diatom cells in diatom films was determined with the Spot Insight software package (Diagnostic Instruments). Digital pictures of diatom films were taken under the light microscope at a magnification of 100×. An area of the diatom film was selected, measured with the software, and diatoms within the known area were enumerated. The surface coverage of single diatoms and their relative interstitial distances were measured in different fields of view with replication (n = 3). The surface coverage of diatom cells in diatom films under investigation was expressed as the percentage of cell coverage in a field of known size.

***Expt 3:*** To investigate the potential effect of waterborne diatom film-derived compounds on the induction of larval settlement, filmed polystyrene dishes were concurrently bioassayed with unfilmed counterparts (double-dish bioassay; Harder et al. 2001). These dishes were combined to form a test-vessel for the settlement assay. In detail, 20 larvae were pipetted into the unfilmed petri dish under the dissecting microscope. A PVC ring (inner diameter: 51 mm, width: 6 mm) was tightly fitted around the dish and subsequently filled with 13 ml FSW. From the top, a diatom-filmed dish was inserted into the open ring to close the test-vessel. Control-vessels were prepared accordingly by pairing unfilmed petri dishes (negative control) and clean dishes containing 5 d old biofilms (positive control). Bioassays were run in vertically mounted vessels on an orbital shaker (60 rpm; Thermolyne) under conditions otherwise as mentioned above. The underlying assumption in the design of this assay was that waterborne products could freely diffuse through the assay vessel. Provided that diatoms and bacteria did not significantly colonize the clean substratum during the assay period, a larval settlement response to water-soluble settlement cues (unfilmed side) could be distinguished from a response to surface-associated settlement signals (filmed side).

***Expt 4:*** To investigate the effect of diatom viability on the induction of larval settlement, diatom films on borosilicate glass petri-dishes were exposed to 121°C for 1 h. Dishes with and without 5 d old biofilms served as positive and negative controls, respectively. Prior to larval settlement assays, dishes were dip-rinsed in autoclaved FSW to wash off intracellular cell components that may have formed on the dish surface due to the rupture of cells. After the heat treatment, the viability of diatoms and bacteria was recorded by fluorescent microscopy (Zeiss Axiophot; $\lambda_{Ex}$ = 485 nm, $\lambda_{Em}$ = 540 nm) using fluorescein diacetate (Sigma Chemical) according to Dorsey et al. (1989), and acridine orange according to Daley & Hobbie (1975).

**Statistical analysis.** Larval settlement bioassays were repeated thrice to ensure the reproducibility of results. The software packages Statistica (StatSoft) and

SPSS were used for statistical analyses. The percentage of larval settlement was arcsine-transformed before analysis. To improve the transformation results, a value of $(4 \times n)^{-1}$ (n = number of larvae used in each replicate) was given to the replicates with no settled larvae (Zar 1996). The normality of the data was checked with Shapiro-Wilk's *W*-test (Shapiro & Wilk 1965). Data that failed to meet the homogeneity criterion were analyzed non-parametrically. This was achieved by transformation of values to ranks, followed by 1-way ANOVA and Tukey's multiple comparison test (Conover & Iman 1981). Pairwise comparisons were performed with Student's *t*-test. The effects of diatom quality and surface wettability on larval settlement were analyzed by 2-way ANOVA. The data presented in the figures are not transformed.

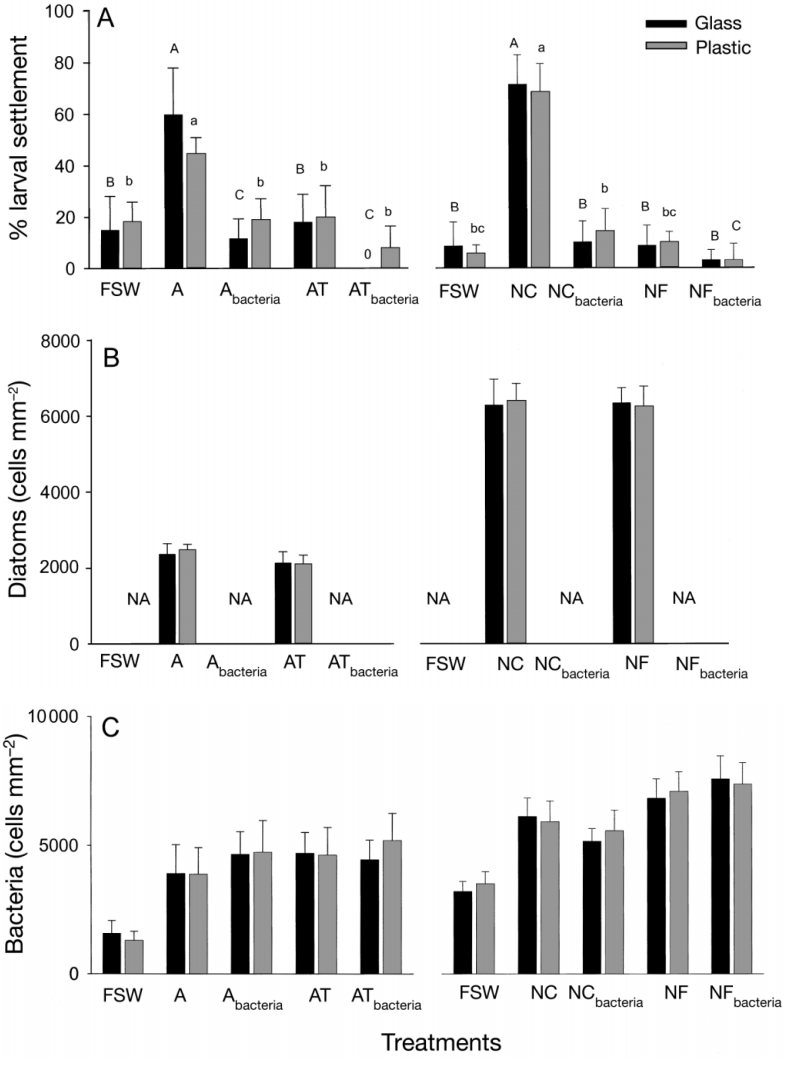


Fig. 1. *Hydroides elegans*. (A) Mean % larval settlement after 24 h in response to diatom films (A: *Achnanthes* sp.; AT: *Amphora tenerrima*; NC: *Nitzschia constricta*; NF: *N. frustulum*) and diatom-free bacterial films (e.g. $A_{bacteria}$) on glass (black) and polystyrene (shaded) together with a control of filtered seawater (FSW). Larval settlement rates on each type of substratum were individually analyzed by multiple comparison. Data that are significantly different (Tukey's test, $\alpha = 0.05$) are indicated by capital (glass dishes) and small (polystyrene dishes) letters. Data are means ± SD of 5 replicates. (B) Mean density of diatoms. Data are means ± SD of 3 replicates based on counts of 5 fields of view at 100× magnification. NA = not applicable. (C) Mean density of bacteria in diatom films and diatom-free bacterial films. Data are means ± SD of 3 replicates based on counts of 5 fields of view at 1250× magnification

## RESULTS

### Expt 1: Effect of surface wettability on diatom attachment

Since the diatom pairs *Achnanthes* sp./*Amphora tenerrima* and *Nitzschia constricta*/*N. frustulum* were investigated in separate bioassays with different batches of larvae, the respective results are presented individually. There was no effect of surface wettability of the dish-material (borosilicate glass and polystyrene) on larval settlement (2-way ANOVA, $p > 0.41$). Larval settlement on films of *Achnanthes* sp. and *N. constricta* was significantly higher than that in the FSW-treatments (Tukey's test, $p < 0.05$; Fig. 1A), whilst larval settlement on films of *A. tenerrima* and *N. frustulum* was the same as that in FSW (Tukey's test, $p = 1.00$; Fig. 1A). The abundance of diatoms in experimental films and 5 d old biofilms ranged from 2000 to 6000 cells $mm^{-2}$ and was roughly the same in individual bioassays with different diatom pairs (Fig. 1B). The cell densities in individual diatom films on polystyrene and glass surfaces were the same (Student's *t*-test, $p = 0.75$; Fig. 1B). The abundance of bacteria in diatom-free bacterial films ranged from 1340 ± 450 (SD) to 7600 ± 900 cells $mm^{-2}$ (Fig. 1C). Although bacterial abundance in non-axenic diatom films was the same as that in diatom-free bacterial films (Tukey's test, $p = 0.84$; Fig. 1C), larval settlement on non-axenic diatom films was significantly higher than that on diatom-free bacterial films (Tukey's test, $p < 0.05$). A significant difference between larval settlement and diatom quality was observed (2-way ANOVA, $p < 0.001$; Table 1), whilst no significant difference between larval settlement and surface quality was found (2-way ANOVA, $p = 0.41$ for *Achnanthes* sp./*A. tenerrima*; $p = 0.96$ for *N. constricta*/*N. frus-*

Table 1. *Hydroides elegans*. Effect of substratum type (glass or polystyrene) and diatom quality (inductive, i.e. *Achnanthes* sp. and *Nitzschia constricta*; non-inductive, i.e. *A. tenerrima* and *N. frustulum*) on the induction of larval settlement of *H. elegans* larvae (2-way ANOVA)

| Factor | df | MS | *F* | p-value |
|---|---|---|---|---|
| *Achnanthes* sp./*A. tenerrima* | | | | |
| Substratum (A) | 1 | 39.95 | 0.73 | 0.41 |
| Diatom (B) | 1 | 2119.30 | 38.68 | <0.05 |
| A × B | 1 | 93.65 | 1.71 | 0.21 |
| *Nitzschia constricta*/*N. frustulum* | | | | |
| Substratum (A) | 1 | 6.1E-02 | 0.003 | 0.95 |
| Diatom (B) | 1 | 4495.18 | 222.9 | <0.05 |
| A × B | 1 | 8.07 | 0.4 | 0.54 |

*tulum*). No statistical interaction was observed between the factors diatom and surface quality (2-way ANOVA, p = 0.21 for *Achnanthes* sp./*A. tenerrima*; p = 0.54 for *N. constricta*/*N. frustulum*; Table 1).

### Expt 2: Effect of diatom film density on the induction of larval settlement

Since there was no difference in larval settlement towards diatom films on glass or polystyrene, the effect of diatom abundance on the induction of larval settlement was investigated on polystyrene only. The dilution of diatom suspensions was paralleled by a reduced diatom abundance in experimental films made with these dilution series (Fig. 2A,B). For both inductive diatom strains, there was a positive linear relationship between diatom abundance and the percentage of larval settlement (*Achnanthes* sp.: $r^2 = 0.98$, p = 0.07; *Nitzschia constricta*: $r^2 = 0.99$, p < 0.05; Fig. 2A). Although the bacterial abundance in diatoms films originating from dilution experiments was slightly different, the bacterial densities in *Achnanthes* sp.- and *N. constricta*-treatments were either the same (Tukey's test, p = 0.72) or lower than that in the FSW control (Tukey's test, p < 0.05; Fig. 2C). The mean single-cell size of *Achnanthes* sp. and *N. constricta* was 48.8 ± 0.8 and 55.1 ± 3.2 µm, respectively. The surface coverage of diatoms in films made by dilution series of diatom suspensions at 1×, 0.1×, and 0.01×, and the mean interstitial distances between individual diatom cells, are presented in Fig. 3.

### Expt 3: Effect of waterborne diatom film-derived compounds on the induction of larval settlement

Although diatoms colonized the formerly clean counter dish during the double-dish bioassay, diatom densities on filmed surfaces were significantly higher than on unfilmed counterparts (Student's *t*-test, p < 0.05; Fig. 4B). In the *Achnanthes* sp.-treatment, bacterial densities on both sides of test vessel were the same (Student's *t*-test, p > 0.05; Fig. 4C). In the *Nitzschia constricta*-treatment they were slightly higher on the filmed dish (Student's *t*-test, p < 0.05; Fig. 4C). In both treatments, larval settlement on the filmed surface of the test vessel was significantly higher than on the unfilmed side (Student's *t*-test, p < 0.05; Fig. 4A).

### Expt 4: Effect of diatom viability on the induction of larval settlement

Heat treatment effectively killed diatoms and bacteria in experimental films. In contrast to green epi-

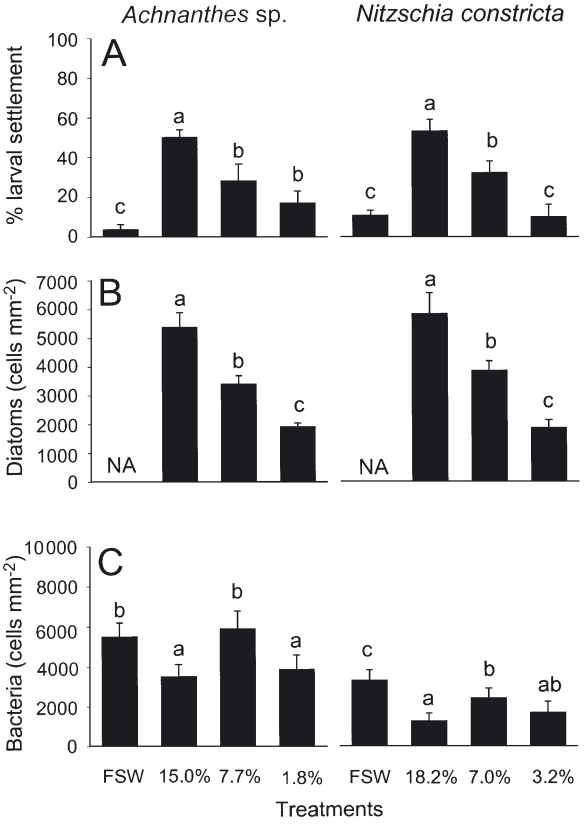


Fig. 2. *Hydroides elegans*. (A) Mean % larval settlement after 24 h in response to diatom films of *Achnanthes* sp. and *Nitzschia constricta* at different densities and a control of filtered seawater (FSW). Densities are expressed as % of 2D surface coverage of diatoms in these films. Data that are significantly different at α = 0.05 in Tukey's tests are indicated by different letters. Data are means ± SD of 5 replicates. (B) Mean density of diatoms at different surface coverage of diatoms. Data are means ± SD of 3 replicates based on counts of 5 fields of view at 100× magnification. NA = not applicable. (C) Mean density of bacteria in diatom films. Data are means ± SD of 3 replicates based on counts of 5 fields of view at 1250× magnification

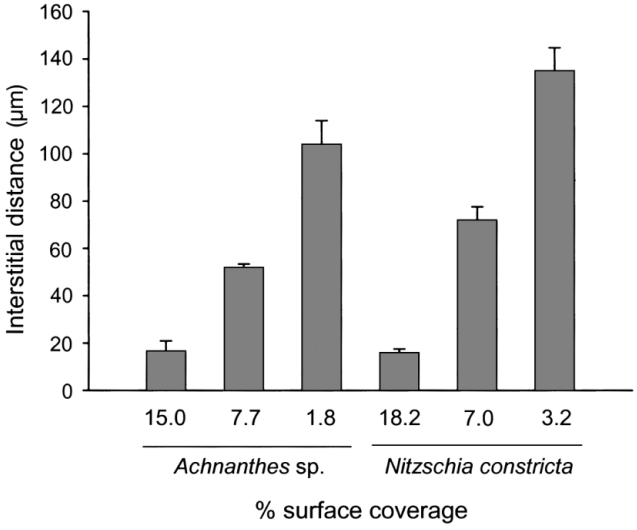


Fig. 3. *Hydroides elegans*. Mean single-cellular surface coverage of *Achnanthes* sp. and *Nitzschia constricta*. The surface coverage (%) of diatoms in films made by dilution series of diatom suspensions at 1×, 0.1×, and 0.01× and the interstitial distances between individual diatoms are presented as means of 3 replicates. Mean single-cellular size of *Achnanthes* sp. and *N. constricta* was 48.8 ± 0.8 and 55.1 ± 3.2 µm, respectively

fluorescence of diatoms and bacteria (indicating live and viable cells), red epifluorescence (indicating dead cells) was observed after the heat treatment. In the experimental treatments of *Achnanthes* sp. and *Nitzschia constricta*, the percentage of larval settlement on live diatom films was the same as that on dead films (Tukey's test, $p = 0.99$ for *Achnanthes* sp.; $p = 0.86$ for *N. constricta*; Fig. 5), but statistically different from the FSW-control (Tukey's test, $p < 0.05$), while the percentage of larval settlement on live films of *Amphora tenerrima*- and *N. frustulum*-treatments was statistically the same as that on dead films (Tukey's test, $p = 0.99$ for *A. tenerrima*; $p = 1.00$ for *N. frustulum*) and the FSW-control (Tukey's test, $p = 0.60$). The abundance of diatoms in heated and non-heated experimental films was the same, verifying that rinsing procedures did not falsify the bioassay procedure (1-way ANOVA, $p > 0.05$; Table 2).

## DISCUSSION

Until now, the role of diatoms as potential mediators of larval settlement was thought to be predominantly associated with grazing juvenile invertebrates, such as abalones (Ebert & Houk 1984, Kawamura & Kikuchi 1992, Slattery 1992, Bryan & Qian 1998, Roberts 2001), sea urchins (Tani & Ito 1979) and sea cucumbers (Ito & Kitamura 1997). This association was often linked to the appropriate post-larval dietary requirements (Slattery 1992, Daume et al. 1999). Although the inductive effect of certain diatoms on larval settlement was correlated with their surface coverage, the nature of settlement cues had not been elucidated. In this study, we firstly investigated the nature and origins of larval settlement cues from marine diatoms. While larval settlement in the polychaete *Hydroides elegans* is significantly mediated by marine biofilms, with bacteria (Lau & Qian 2001) and diatoms (Harder et al. 2002a) being major sources of settlement cues, all the stages during the life cycle of *H. elegans* (i.e. larvae, juveniles, adults) are strictly planktotrophic (Chia 1978).

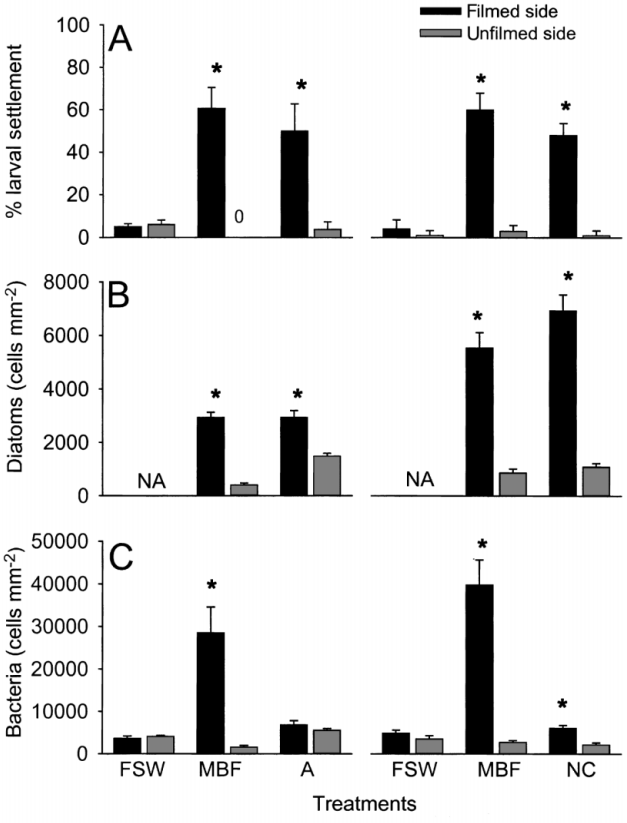


Fig. 4. *Hydroides elegans*. (A) Mean % larval settlement after 24 h in response to filmed (black) and unfilmed (shaded) surfaces in comparison to controls of marine biofilms (MBF) and filtered seawater (FSW). Significant differences between larval settlement rates on filmed and unfilmed surfaces are asterisked (Student's *t*-test, $\alpha = 0.05$). Data plotted are means ± SD of 5 replicates. (B) Mean density of diatoms on filmed and unfilmed surfaces. NC: *Nitzschia constricta*; A: *Achnanthes* sp. Significant differences between diatom densities are asterisked (Student's *t*-test, $\alpha = 0.05$). Data plotted are means ± SD of 3 replicates based on counts of 5 fields of view at 100× magnification. NA = not applicable. (C) Mean density of bacteria on filmed and unfilmed surfaces. Significant differences between bacterial densities are asterisked (Student's *t*-test, $\alpha = 0.05$). Data plotted are means ± SD of 3 replicates based on counts of 5 fields of view at 1250× magnification

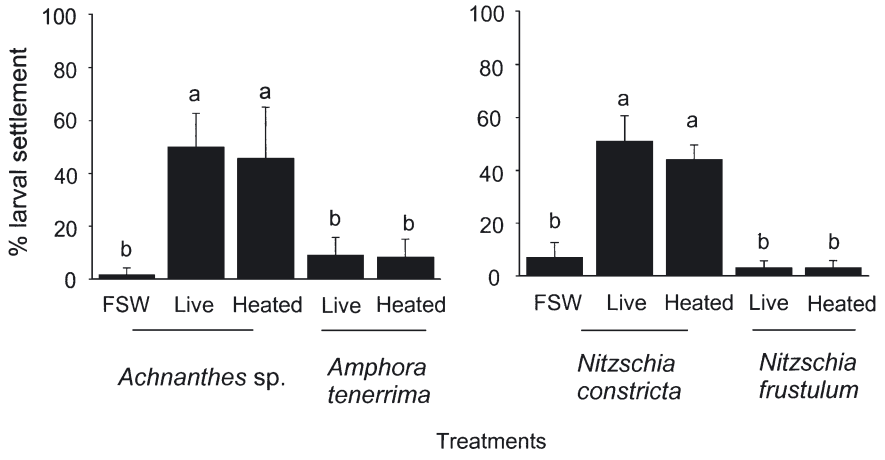


Fig. 5. *Hydroides elegans*. Mean % larval settlement after 24 h in response to live and dead diatom films in comparison to a control of filtered seawater (FSW). Data that are significantly different at $\alpha = 0.05$ in Tukey's test are indicated by different letters. Data plotted are means ± SD of 5 replicates

Hence, attached diatoms do not significantly contribute to the diet of *H. elegans*. The role of attached diatoms on *H. elegans* larvae is therefore likely restricted to the mediation of settlement and metamorphosis in this tubeworm. In this investigation, we attempted to further study the effects of 2 inductive (*Achnanthes* sp. and *Nitzschia constricta*) and 2 non-inductive diatoms (*Amphora tenerrima* and *N. frustulum*) on larval settlement of *H. elegans*. The first objective was to compare the origins of settlement cues derived from diatoms and bacteria. Secondly, we addressed the possible effect of substratum wettability on settlement induction activity of attached diatoms. Overall, our results showed that: (1) marine benthic diatoms played a similarly important role in larval settlement as marine bacteria in biofilms, and (2) the inductive cues from the diatoms tested in this study were surface-associated, and their activity was not affected by the surface wettability of the substratum. More importantly, our results indicated that different components of biofilms played inductive or inhibitive roles on larval settlement. In an earlier study (Beckman et al. 1999), we identified a systematic technical shortfall in the bioassay-based investigation of larval settlement cues of *H. elegans*, i.e. the unavoidable introduction of bacteria into assay vessels together with larvae (exemplified in Fig. 1C). Due to the potentially significant effect of bacteria on larval settlement (Lau et al. 2002), the effect of bacteria had to be decoupled from the effect of the diatoms under investigation. Next to a quantitative evaluation of bacterial densities in test vessels in comparison with controls, bacterial contaminants in non-axenic diatom cultures were separated by filtration, and qualitatively evaluated as diatom-free bacterial films representing the bacterial contaminants of each diatom culture (Fig. 1C). Under the assumption that community profiles in these bacterial films were comparable to those in mixed diatom-bacterial films, a comparison of larval settlement rates between mixed diatom-bacteria films and bacterial contaminant films allowed the evaluation of the qualitative effect of these bacteria. Larval settlement on non-axenic diatom films was significantly higher than on diatom-free microbial films (Fig. 1A), yet the bacterial abundance in these films was statistically the same (Fig. 1C). These results showed that the induction of larval settlement in these treatments was due to the presence of diatoms and not the presence of bacteria. Bacteria in the FSW-control posed a negligible effect on the induction of larval settlement. In spite of the above limitations of running larval settlement assays with *H. elegans* under sterile conditions, our results suggested that settlement results were predominantly associated with qualitative differences of diatoms under investigation, rather than bacterial contaminants.

Table 2. *Hydroides elegans*. Settlement cues of *H. elegans* in relation to cell abundance of diatoms and bacteria in mixed diatom-bacteria films after the 24 h larval settlement bioassay. Live: no heat treatment of films; dead: heat treatment of films at 121°C for 1 h; FSW control: clean petri dish with autoclaved seawater. Data plotted are means ± SD of 5 replicates based on counts of 5 fields of view at 100× (for diatoms) and 1250× (for bacteria) magnification. NA: not applicable

| | Diatom density (in cells $mm^{-2}$) | | Bacterial density (in cells $mm^{-2}$) | |
|---|---|---|---|---|
| | Live | Dead | Live | Dead |
| *Achnanthes* sp. | 2940 ± 250 | 3154 ± 338 | 4760 ± 681 | 3660 ± 763 |
| *Amphora tenerrima* | 3025 ± 245 | 3246 ± 268 | 2710 ± 551 | 3335 ± 540 |
| *Nitzschia constricta* | 5642 ± 422 | 4925 ± 597 | 7020 ± 931 | 6150 ± 1209 |
| *Nitzschia frustulum* | 5500 ± 291 | 5258 ± 692 | 6200 ± 941 | 8305 ± 1176 |
| FSW control | NA | NA | 5460 ± 671 | NA |

Previous studies have shown that bacterial attachment differed with respect to surface wettability (Fletcher & Pringle 1985, also see Fig. 1 in Qian et al. 2000). The different modification of the bacterial cell-surface chemistry due to molecular arrangements of extracellular polymers (Marshell & Cruickshank 1973) may affect larval settlement differently (Maki et al. 1992). Similar to bacteria, surface attachment of diatoms is established by secretion of extracellular polymers (Allan et al. 1972). Prior to the identification of origin or location of diatom-derived larval settlement cues, we performed a series of experiments with diatom strains that aimed at studying the importance of physical factors, such as the substratum wettability (Expt 1) and the effect of surface abundance of diatoms (Expt 2) on larval settlement. Our results showed not only that diatoms attached similarly well on glass and polystyrene, but also that larval settlement was statistically the same, irrespective of dish wettability (Fig. 1A,B). Similar results were reported in Qian et al. (2000), in which larval settlement of *Hydroides elegans* in response to bacterial film was not affected by surface wettability, although bacterial attachment on surfaces of different wettability varied. The dilution series of diatom suspensions, which served to generate diatom films of different surface abundance, was reflected in different percentages of surface coverage of diatoms ranging between 1.8 and 15% (*Achnanthes* sp.), and 3.2 and 18.2% (*Nitzschia constricta*) (Fig. 3). There was a clear density-dependent effect of diatom surface coverage on the induction of larval settlement (Fig. 2A,B). In the case of *Achnanthes* sp., even the lowest surface coverage of 1.8% induced larval settlement significantly more than the control of filtered seawater. The interstitial distance between diatom cells at this low film density was ca. 100 µm, which is similar to the body size of *H. elegans* larvae. Considering that extracellular polymers are invisible under conditions of light microscopy but are generally spread around individual cells (Allan et al. 1972), the actual space occupation by diatom cells and extracellular secretions might have been even higher. No linear relationship between the abundance of diatoms and bacteria in non-axenic diatom films resulted from the dilution of diatom suspensions (Fig. 2B,C). This observation confirmed the inherent limitations of running larval settlement assays with *H. elegans* under sterile conditions.

Larval settlement bioassays with a choice between filmed and unfilmed substrata (double-dish design, Expt 3) revealed that the diatom-derived settlement cue was either water-insoluble or waterborne, but with strong adsorption affinity to the filmed side. Both explanations involved a clear association of the diatom-derived settlement cue with the diatom film (Fig. 4A). These results were statistically significant, despite the fact that a certain proportion of diatoms and bacteria became dissociated from the test film during the assay period and attached on the adjacent, formerly clean side of the double-dish (Fig. 4B,C). The inductive effect of diatoms *Achnanthes* sp. and *Nitzschia constricta* on larval settlement prevailed, even after heat treatments that completely killed diatoms and bacteria in these films (Fig. 5). The same observation was made with the non-inductive diatoms *Amphora tenerrima* and *N. frustulum*, as in these experiments the inductive activity remained low after heat treatments.

In terms of these properties, the larval settlement cue of *Hydroides elegans* produced by the diatoms under investigation could be compared with bacterial settlement cues as follows: In analogy to diatoms, the induction of larval settlement by bacterial films did not differ with respect to the surface material, i.e. glass and polystyrene (Lau 2001). Given the choice between bacteria-filmed and a non-filmed substrata, larvae settled preferentially on the bacteria-filmed dish (Lau 2001). However, the settlement-induction effect of bacteria was effectively destroyed by methods that rendered bacterial cells non-viable, such as heat, formaldehyde and UV light (Lau & Qian 2001, Lau et al. 2003). Based on this comparison, we conclude that the diatom-induced larval settlement cue did not comprise diatom-produced secondary metabolites, but was more likely composed of a heat-stable surface component, such as capsular, extracellular polysaccharides. The involvement of polysaccharides as larval settlement cues has previously been addressed in other invertebrate phyla, such as polychaetes, tunicates, hydroids and barnacles (Kirchmann et al. 1982, Szewzyk et al. 1991, Leitz & Wagner 1993, Maki 1999). Since surface-attached bacteria and diatoms share the characteristic of extracellularly-secreted polymers, such as polysaccharides, proteins and glycoproteins (Cooksey et al. 1984), it is possible that larval settlement induction by certain marine benthic diatoms is also based on this class of compound. Overall, our study highlighted that not only different components of biofilms play inductive or inhibitive roles in larval settlement, but also that the relative surface coverage of biofilm components deserves consideration regarding their roles as mediators of larval settlement.

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LITERATURE CITED


Allan GG, Lewin J, Johnson PG (1972) Marine polymers. IV. Diatom polysaccharides. Bot Mar 15:102–108

Beckmann M, Harder T, Qian PY (1999) Induction of larval settlement and metamorphosis in the serpulid polychaete *Hydroides elegans* by dissolved amino acids: mode of action in laboratory bioassays. Mar Ecol Prog Ser 190:167–178

Bryan PJ, Qian PY (1998) Induction of larval attachment and metamorphosis in the abalone *Haliotis diversicolor* (Reeve). J Exp Mar Biol Ecol 223:39–51

Chan ALC, Walker G (1998) The settlement of *Pomatoceros lamarckii* larvae (Polychaeta: Sabellida: Serpulidae): a laboratory study. Biofouling 12:71–80

Chia FS (1978) Perspectives: settlement and metamorphosis of marine invertebrate larvae. In: Chia FS, Rice ME (eds) Settlement and metamorphosis of marine invertebrate larvae. Elsevier Science Publishers, New York, p 283–285

Conover WJ, Iman RL (1981) Rank transformations as a bridge between parametric and nonparametric statistics. Am Stat 35:124–129

Cooksey B, Cooksey KE, Miller CA, Paul JP, Webster D, Rubin RW (1984) The attachment of microfouling diatoms. In: Costlow JD, Tipper RD (eds) Marine biodeterioration: an interdisciplinary study. US Naval Institute Press, Annapolis, MD

Daley RJ, Hobbie JE (1975) Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol Oceanogr 29:875–882

Daume S, Brand-Gardner S, Woelkerling W (1999) Preferential settlement of abalone larvae: diatom films vs non-geniculate coralline red algae. Aquaculture 174:243–254

Dorsey J, Yentsch CM, Mayo S, McKenna C (1989) Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. Cytometry 10:622–628

Ebert E, Houk J (1984) Elements and innovations in the cultivation of red abalone *Haliotis rufescens*. Aquaculture 39:375–392

Fletcher M, Pringle JH (1985) The effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces. J Colloid Interface Sci 104:5–14

Guillard R, Ryther J (1962) Studies of marine planktonic diatoms. Can J Microbiol 8:229–239

Harder T, Vengatesen T, Qian P Y (2001) Effect of cyprid age on the settlement of *Balanus amphitrite* Darwin in response to natural biofilms. Biofouling 17:211–219

Harder T, Lam C, Qian PY (2002a) Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229:105–112

Harder T, Lau SCK, Dahms HU, Qian PY (2002b) Isolation of bacterial metabolites as natural inducers for larval settlement in the marine polychaete *Hydroides elegans* (Haswell). J Chem Ecol 28:2009–2023

Holmström C, Kjelleberg S (1994) The effect of external biological factors on settlement of marine invertebrate and new antifouling technology. Biofouling 8:147–160

Holmström C, Rittschof D, Kjelleberg S (1992) Inhibition of settlement by larvae of *Balanus amphritie* and *Ciona intestinalis* by a surface colonizing marine bacterium. Appl Environ Microbiol 58:2111–2115

Ito S, Kitamura H (1997) Induction of larval metamorphosis in the sea cucumber *Stichopus japonicus* by periphitic diatoms. Hydrobiologia 358:281–284

Kawamura T, Kikuchi S (1992) Effects of benthic diatoms on settlement and metamorphosis of abalone larvae. Suisanzoshoku 40:403–409

Keough MJ, Raimondi PT (1996) Responses of settling invertebrate larvae to bioorganic films: effects of large-scale variation in films. J Exp Mar Biol Ecol 207:59–78

Kirchmann D, Graham S, Reish D, Mitchell R (1982) Bacteria induce settlement and metamorphosis of *Janua* (*Dexiospira*) *brasiliensis* Grube (Polychaeta: Spirorbidae). J Exp Mar Biol Ecol 56:153–163

Lau SCK (2001) The chemical nature of bacteria-derived larval settlement cue(s) for the marine polychaete *Hydroides elegans* (Haswell). PhD thesis, Hong Kong University of Science and Technology

Lau SCK, Qian PY (2001) Larval settlement and metamorphosis in the serpulid polychaete *Hydroides elegans* in response to bacterial films: an investigation of the nature of putative larval settlement cue. Mar Biol 138:321–328

Lau SCK, Mak KKW, Chen F, Qian PY (2002) Bioactivity of bacterial strains from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. Mar Ecol Prog Ser 226: 301–310

Lau SCK, Harder T, Qian PY (2003) Induction of larval settlement in the serpulid polychaete *Hydroides elegans* (Haswell): role of bacterial extracellular polymers. Biofouling 19:197–204

Leitz T, Wagner T (1993) The marine bacterium *Alteromonas espejiana* induces metamorphosis of the hydroid *Hydractinia echinata*. Mar Biol 115:173–178

Maki JS (1999) The influence of marine microbes on biofouling. In: Fingermann M, Nagabhushanam R, Thompson M (eds) Recent advances in marine biotechnolgy. Science Publishers, Enfield, NH

Maki JS, Rittschof D, Costlow JD, Mitchell R (1988) Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. Mar Biol 97:199–206

Maki JS, Rittschof D, Mitchell R (1992) Inhibition of larval barnacle attachment by bacterial films: an investigation of physical properties. Microb Ecol 23:97–106

Marshell KC, Cruickshank RH (1973) Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Arch Mikrobiol 91:29–40

Mihm JW, Banta WC, Loeb GI (1981) Effect of adsorbed organic and primary fouling films on bryozoan settlement. J Exp Mar Biol Ecol 54:167–179

Olivier F, Tremblay R, Bourget E, Ritschoff D (2000) Barnacle settlement: field experiments on the influence of larval supply, tidal level, biofilm quality and age on *Balanus amphitrite* cyprids. Mar Ecol Prog Ser 199:185–204

Pearce JR, Scheibling RE (1991) Effect of macroalgae, microbial films, and conspecifics on the induction of metamorphosis of the green sea urchin, *Strongylocentrotus droebachiensis* (Müller). J Exp Mar Biol Ecol 147:147–162

Qian PY (1999) Larval settlement of polychaetes. Hydrobiologia 402:239–253

Qian PY, Pechenik JA (1998) Effects of larval starvation and delayed metamorphosis on juvenile survival and growth of the tube-dwelling polychaete *Hydroides elegans* (Haswell). J Exp Mar Biol Ecol 227:169–185

Qian PY, Rittschof D, Sreedhar B, Chia FS (1999) Macrofouling in unidirectional flow: miniature pipes as experimental models for studying the effects of hydrodynamics on invertebrate larval settlement. Mar Ecol Prog Ser 191: 141–151

Qian PY, Rittschof D, Sreedhar B (2000) Macrofouling in unidirectional flow: miniature pipes as experimental models for studying the interaction of flow and surface characteristics on the attachment of barnacle, bryozoan and polychaete larvae. Mar Ecol Prog Ser 207:109–121

Qiu JW, Qian PY (1997) Combined effects of salinity, temperature and food on early development of the polychaete *Hydroides elegans*. Mar Ecol Prog Ser 152:79–88

Roberts R (2001) A review of settlement cues for larval abalone (*Haliotis* spp.). J Shellfish Res 20:571–586

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611

Slattery M (1992) Larval settlement and juvenile survival in the red abalone (*Haliotis rufescens*): an examination of inductive cues and substrate selection. Aquaculture 102: 143–153

Szewzyk U, Holmström C, Wrangstadh M, Samuelsson MO, Maki JS, Kjelleberg S (1991) Relevance of the exopolysaccharide of marine *Pseudomonas* sp. strain S9 for the attachment of *Ciona intestinalis* larvae. Mar Ecol Prog Ser 75:259–265

Tamburri MN, Zimmer-Faust RK, Tamplin ML (1992) Natural sources and properties of chemical inducers mediating settlement of oyster larvae: a re-examination. Biol Bull 183:327–338

Tani Y, Ito Y (1979) Effects of benthic diatoms on settlement and metamorphosis of the sea urchin, *Pseudocentrotus depressus*. Suisanzoshoku 27:148–150

Wahl M (1989) Marine epibiosis. I. Fouling and antifouling: some basic aspects. Mar Ecol Prog Ser 58:175–189

Wieczorek SK, Todd CD (1997) Inhibition and facilitation of bryozoan and ascidian settlement by natural multi-species biofilms: effects of film age and the roles of active and passive larval attachment. Mar Biol 128:463–473

Wieczorek SK, Clare AS, Todd CD (1995) Inhibitory and facilitatory effects of microbial films on settlement of *Balanus amphitrite* larvae. Mar Ecol Prog Ser 119:221–226

Wisely B (1958) The development and settling of a serpulid worm, *Hydroides norvegica* Gunnerus (Polychaete). Aust J Mar Freshw Res 9:351–361

Zar JH (1996) Biostatistical analysis, 3rd edn. Prentice Hall, Upper Saddle River, NJ

Zobell CE, Allen EC (1935) The significance of marine bacteria in the fouling of submerged surfaces. J Bacteriol 29: 239–251

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