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Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues

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ABSTRACT: Larval settlement in the fouling polychaete *Hydroides elegans* Haswell (1883) is mediated by natural biofilms, a complex consortium of marine bacteria, benthic diatoms, protozoa, fungi and adsorbed organic matter. Currently, the inductive effect of natural biofilms on larval settlement is attributed to the bacterial component. In this paper, we present results of an investigation of the diatom component of natural biofilms with respect to induction of larval settlement in *H. elegans*. Thirty-two diatom strains were isolated from natural biofilms, identified and individually investigated in still-water laboratory settlement assays. A multiple pairwise comparison of larval settlement rates grouped the diatoms into 3 statistical categories: (1) 'inductive'; same percentage of larval settlement as in the positive control (glass slide with natural biofilm); (2) 'no effect'; same percentage of larval settlement as in the negative control (clean glass slide); and (3) 'weakly inductive'; percentage of larval settlement significantly higher and lower than in the negative and the positive control, respectively. Out of 32 diatom strains, 3 (10%) fell into the first, 19 (59%) into the second and 10 (31%) into the third category. The variability in larval settlement was not correlated with the density of diatoms in the monospecific films. Despite sterile measures and the use of antibiotics, diatom films under investigation were not axenic, with bacterial abundances ranging from 200 to 8000 cells mm^{-2}. No quantitative correlation between the bacterial abundance in diatom films and the percentage of larval settlement was observed. To evaluate the qualitative impact of the bacterial fraction in the mixed diatom-bacteria films on the induction of larval settlement, 6 representative diatom strains, comprising the 3 categories stated above, were selected to create diatom-free microbial films with the bacterial contaminants of each culture. Whilst the bacterial abundance in the non-axenic diatom films and the diatom-free control films was statistically the same, the percentage of larval settlement towards these treatments differed significantly for the diatom strains belonging to category (1) and (3). These categories comprised 4 different genera of diatoms, indicating that the observed inductive effect on larval settlement was not restricted to a certain diatom genus. Our results suggest that in addition to microbial films, benthic diatoms may significantly contribute to the mediation of larval settlement behaviour in *H. elegans* by marine biofilms.

KEY WORDS: Biofilm · Diatoms · Larval settlement · Bacteria · *Hydroides elegans*

INTRODUCTION

The early life cycle of the majority of marine invertebrates includes a larval phase. A critical stage in the larval life history occurs at competence, a physiological state wherein dispersing larvae are able to attach and subsequently metamorphose (in the following referred to as 'settlement') into sedentary juvenile individuals (Crisp 1974, Scheltema 1974, Chia 1978). The onset of larval surface exploration and substratum choice for permanent attachment is influenced by the presence of bioorganic films on marine surfaces (Zobell & Allen 1935). Marine bioorganic films (in short 'natural bio-

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films' or 'NBF') are highly variable over time and heterogeneous in composition. They are mainly comprised of adsorbed macromolecules, attached bacteria and diatoms, with all of these components enmeshed in a matrix of extracellular polymers (Mihm et al. 1981). Biofilm formation on a marine substratum modifies its surface chemistry (Characklis & Cooksey 1983) and strongly affects recruitment of larval invertebrates. The relevant literature reports numerous examples of marine invertebrate larvae being attracted to, or repelled by NBF (reviewed by Maki et al. 1992, Pawlik 1992, Rodriguez et al. 1993, Todd & Keough 1994, Wieczorek & Todd 1998, Qian 1999). In these studies, the inductive or inhibitive effect of NBF on larval settlement was attributed to the bacterial component of biofilms, more specifically bacterial glycoproteinacious exopolymers (e.g. Zobell & Allen 1935, Kirchmann et al. 1982, Johnson et al. 1991, Szewzyk et al. 1991, Leitz & Wagner 1993, Maki 1999).

Surprisingly few studies have addressed the role of marine benthic diatoms in mediating the settlement response of invertebrate larvae although their proportion in marine biofilms can be high, especially in the case of illuminated surfaces. In the barnacle *Balanus balanoides*, an alga and a diatom mediate larval surface exploration and substratum choice. On a small scale, these biological cues sharply define an area of suitability for permanent attachment and metamorphosis in *B. balanoides* (Le Tourneux & Bourget 1988). Apart from this example, previous studies were mainly directed towards the potential effects of marine benthic diatoms on larval settlement of invertebrate species with grazing juveniles, such as abalones (Ebert & Houk 1984, Kawamura & Kikuchi 1992, Slattery 1992, Bryan & Qian 1998), sea urchins (Tani & Ito 1979) and sea cucumbers (Ito & Kitamura 1997). The nature of settlement cues involved has not been elucidated but was correlated with the diatom cell density on the settlement substratum and associated with appropriate post-larval dietary requirements (Slattery 1992, Daume et al. 1999).

In this study, we investigated the role of benthic diatoms as mediators of larval settlement in the serpulid polychaete *Hydroides elegans* Haswell (1883), a filter-feeding fouling invertebrate, abundant in tropical and subtropical waters around the world. This species was chosen as a test organism owing to its well investigated larval settlement behaviour. Physical surface parameters are of minor importance in the induction of larval settlement of *H. elegans*. Although settlement patterns are passively influenced by larval capture in current traps and eddies (Walters et al. 1997), it was demonstrated that water flow (Qian et al. 1999) and surface free energy (Qian et al. 2000) can affect the larval settlement pattern but may not act as physical settlement cues. Instead, larval settlement in *H. elegans* is strongly induced by NBF (Hadfield et al. 1994), making this species an early colonist of newly submerged substrata. The bacterial component in particular has so far been reported to be responsible for the overall inductive effect of natural biofilms on larval settlement of *H. elegans*. Only a small fraction of bacterial species within the complex biofilm community is reported to be inductive, whilst the majority of tested strains showed no or a weak effect on larval settlement (Lau & Qian 1997, Unabia & Hadfield 1999).

In summary, previous investigations have clearly demonstrated that the microbial proportion in marine biofilms can act as a larval settlement cue for *Hydroides elegans*, which is consistent with reports on larval settlement induction in other invertebrate taxa by marine bacteria. However, in the marine environment, NBF generally comprise a variety of additional components. Surface exploring larvae will therefore encounter a highly diverse conglomerate of further cues that may regulate the settlement behaviour.

In this study, we investigated the induction of larval settlement in *Hydroides elegans* by NBF, focusing on the potential role of the diatom component in NBF as a mediator of larval settlement behaviour. A variety of benthic diatoms were isolated from NBF, purified, identified and individually tested for their inductive effect on larval settlement in still-water laboratory assays. Our goal was to test the hypothesis that monospecific diatom films generate a similar range of settlement responses in *H. elegans* as shown with monospecific films of bacteria from NBF.

MATERIALS AND METHODS

Larval culture. Adult *Hydroides elegans* were obtained from PVC settling panels (100 cm^2) that had been deployed underneath fish farm rafts at 1 m depth at Long Harbour, Hong Kong between July and August 2000. After this period, except for some encrusting bryozoans, barnacles and spirorbid tubeworms, panels were almost uniformly covered with a 1 cm thick layer of sexually mature *H. elegans*. Panels were brought to the laboratory where the calcareous tubes were scraped with forceps. Panels were placed in glass dishes with seawater and were removed after 5 min when a sufficient amount of gametes had been released. The gamete containing seawater was filtered through 70 µm mesh to remove debris and subsequently through 25 µm mesh to retain fertilised eggs. Eggs were gently rinsed in 0.22 µm filtered seawater (FSW) and transferred into aerated 2 l plastic containers containing FSW and the flagellate *Isochrysis galbana* (Tahitian strain) at 10^5 cells ml^{-1}. Larval cultures

were incubated at 24°C under a 15 h light : 9 h dark photo cycle. After Day 4, the larval culture was filtered through 90 µm mesh and provided with new FSW. Larval competence was determined according to morphology as described in Wisely (1958) and by a quick test with 3-isobutyl-1-methylxanthine (IBMX) (Qian & Pechenik 1998). After 5 to 6 d, when the culture contained mostly competent larvae, it was filtered through 110 µm mesh. The larvae retained on the mesh were immediately transferred into FSW and used for larval settlement assays within 60 min.

Isolation and culture of benthic diatoms. From May to June 2000, sterile glass microscope slips were suspended vertically for 5 d underneath fish farm rafts at 1.5 m depth at the same location given above. The slips were dip-rinsed in FSW and the biofilm was brushed off with sterile paintbrushes into 50 ml CORNING tubes containing FSW. A dilution series of the biofilm suspension was inoculated onto 1% agar prepared with f/2 nutrient medium (Guillard & Ryther 1962). Agar plates were incubated at 24°C with overhead fluorescent illumination (0.3×10^{16} quanta s^{-1} cm^{-1}) under a 15 h light : 9 h dark photo cycle. Distinguishable diatom colonies formed within 1 wk of incubation and were successively sub-cultured until monospecific diatoms were obtained. Diatoms were cultured in aerated 400 ml Erlenmeyer flasks under the above conditions. The nutrient medium was changed weekly.

Development of diatom films and diatom-free microbial control films. ***Expt 1:*** When a visible diatom film had formed in the culture flasks, monospecific diatom suspensions were prepared by brushing the culture flasks with sterile paintbrushes. A concentrated stock solution of the antibiotics streptomycin and penicillin was added to the suspensions at a final concentration of 2.5×10^{-4} M streptomycin and 1.0×10^{-4} M penicillin. After 6 h, 4 ml aliquots of the diatom suspensions were transferred into sterile petri dishes (FALCON, #3001; 8 replicates) containing autoclaved glass microscope cover slips (24 × 24 mm) and incubated for 24 h under gentle shaking at the growth conditions stated above. Diatoms attached and proliferated during this period and formed thin algal films on the glass slips. Subsequently, filmed cover slips were dip-rinsed in autoclaved FSW and transferred into sterile petri dishes (FALCON, #1006). Five replicate dishes were filled with FSW for larval bioassays, the remaining 3 slips were fixed with 4% formaldehyde in FSW for enumeration of diatom and bacterial cell densities.

Expt 2: In addition to the procedure described above, the diatom strains 5, 23, 17, 27, 29 and 30 were selected to create diatom-free microbial films with the bacterial contaminants of each culture. Suspensions of diatoms and bacteria were filtered through 1 µm mesh. After washing the diatom containing filter residue with autoclaved FSW, diatom cells were re-suspended in 50 ml of FSW. The volume of the bacteria containing filtrate was reduced to 50 ml on a 0.22 µm filter membrane. The bacteria containing diatom suspensions and the corresponding diatom-free bacterial samples were used to treat cover slips according to the procedure described above. To evaluate the extent of a systematic bacterial contamination of samples due to experimental handling procedures, a control was prepared according to the procedures described above, but without the inoculation of a diatom strain.

Enumeration of diatom and bacterial cell densities. Diatoms were enumerated by chlorophyll fluorescence and direct light microscopy. Bacteria were visualised with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie). The formalin-fixed cover slips (3 per diatom strain) were rinsed with distilled water and stained with DAPI at a concentration of 0.5 µg ml^{-1} for 5 min at room temperature. Cover slips were individually wet mounted and diatom/bacterial cells were recorded at a magnification of 100× (for diatoms) and 1250× (for bacteria) in 5 randomly chosen fields of view (ZEISS Axiophot fluorescence microscope, λ_{Ex} = 359 nm, λ_{Em} = 441 nm). The enumeration of cell densities of diatoms and bacteria prior to the bioassay served to describe the film characteristics encountered by surface exploring larvae at the commencement of the assay.

Experimental protocol. Still water larval settlement assays were performed with replication (n = 5). Sterile glass cover slips and slips filmed with 5 d old NBF served as the negative and positive controls, respectively. Twenty competent larvae of *Hydroides elegans* were pipetted in a small volume (≤100 µl) from larval batches of high density and transferred into sterile petri dishes with treatment slips in 4.5 ml of FSW each. After 24 h incubation at 24°C, the number of settled juveniles in experimental dishes was determined under a dissecting microscope.

Identification of diatoms. Diatoms were taxonomically described where possible by visualisation and comparison of their fine structure with references. Scanning electron micrographs of diatoms were prepared according to Stosch's method (Tomas 1996). After neutralisation and several washing steps with distilled water, diatom frustules were pipetted onto glass slips and dried overnight at 80°C. Dried samples were sputter-coated with gold before visualisation by a JOEL 6300f scanning electron microscope at 10 kV acceleration voltage.

Statistical analysis. Statistical analyses were carried out using the STATISTICA (StatSoft) and SPSS (SPSS) computer packages. The data format percentage of larval settlement was arcsine-transformed prior to sta-

tistical analysis. To improve the transformation, a value of 1/(4 n) (n = number of larvae used in a replicate) was given to the replicates containing no settled larvae (Zar 1996). Cell densities of bacteria and diatoms were not transformed. The normality assumption of data was checked with Shapiro-Wilk's *W*-test. Larval settlement rates on diatom-filmed cover slips were individually evaluated versus the positive and the negative control by multiple pairwise comparison (Dunnett's test). Bacterial densities in mixed diatom-bacteria films and diatom-free bacterial films originating from the same diatom culture, as well as the corresponding larval settlement rates towards these films, were compared by Student's *t*-test for independent samples. Relationships among larval settlement rates, and diatom and bacterial densities on cover slips were analysed by multiple regression analysis. The data presented in the figures are not transformed.

RESULTS

Identification of diatoms

Diatoms were identified to species level where possible. The identified species comprised 9 different genera; *Nitzschia* spp. (12 strains), *Amphora* spp. (3 strains), *Achnanthes* spp. (4 strains), *Synedra* spp. (6 strains), *Navicula* sp. (1 strain), *Denticula* sp. (1 strain), *Scoliopleura* sp. (1 strain) and *Tropidoneis* sp. (1 strain). Three strains remained unidentified. No electron micrographs were obtained for diatoms of the genus *Synedra*. Identification numbers were assigned to individual diatom strains in ascending order according to their effect on induction of larval settlement (Table 1).

Table 1. *Hydroides elegans*. Taxonomic assignment of diatom strains. Monospecific strains are enumerated in ascending order according to their effect on induction of larval settlement (see Fig. 1)

Strain ID	Species
1	Unidentified
2	Unidentified
3	Unidentified
4	*Nitzschia fasciculata*
5	*Achnantes longipes*
6	*Nitzschia frustulum*
7	*Amphora tenerrima*
8	*Synedra* sp.
9	*Nitzschia amplectens*
10	*Amphora castellata*
11	*Synedra* sp.
12	*Synedra* sp.
13	*Nitzschia kuetzingiana*
14	*Nitzschia* sp.
15	*Tropidoneis* sp.
16	*Nitzschia* sp.
17	*Synedra* sp.
18	*Navicula retrocurvata*
19	*Nitzschia* sp.
20	*Achnanthes* sp.
21	*Nitzschia* sp.
22	*Scoliopleura* sp.
23	*Nitzschia* sp.
24	*Synedra* sp.
25	*Achnantes petersenii*
26	*Synedra* sp.
27	*Navicula* sp.
28	*Nitzschia* sp.
29	*Amphora coffeaeformis*
30	*Nitzschia paleacea*
31	*Denticula* sp.
32	*Nitzschia constricta*

Expt 1: Larval settlement

Larval settlement rates in treatments with monospecific diatom films ranged from 1 to 71% and were ranked in ascending order of percentage of larval settlement (Fig. 1). A multiple pairwise comparison of larval settlement rates grouped the diatoms into 3 statistical categories: (1) 'inductive'; same percentage of larval settlement as in the positive control (glass slide with natural biofilm); (2) 'no effect'; same percentage of larval settlement as in the negative control (clean glass slide); and (3) 'weakly inductive'; percentage of larval settlement significantly higher and lower than in the negative and the positive control, respectively. Out of 32 diatom strains, 3 (10%) fell into the first, 19 (59%) into the second and 10 (31%) into the third category (Dunnett's test, $\alpha = 0.05$; 1-way ANOVA, $p < 0.001$).

Expt 1: Quantitative relationships between larval settlement rates and cell densities of diatoms and bacteria

Diatom densities on monospecific films ranged from 200 to 90 000 cells mm^{-2} (Fig. 2). Despite the application of sterile measures during the set-up of the filming procedure, bacteria were observed on diatom-filmed cover slips at densities ranging from 100 to 10 000 cells mm^{-2} (Fig. 3). Cell densities of bacteria and diatoms on 5 d old NBF were enumerated to 38 000 ± 11 000 and 4000 ± 250 cells mm^{-2}, respectively (Figs 2 & 3). The relationship between larval settlement rates and cell densities of diatoms or bacteria on cover slips was analysed by multiple regression analysis. The analysis over all treatments resulted in no significant correlation among larval settlement rates, and cell densities of diatoms and bacteria ($R^2 = 0.078$; $p = 0.308$; $\beta_{Bacteria} = -0.17$, $p = 0.345$; $\beta_{Diatom} = 0.216$, $p = 0.234$).

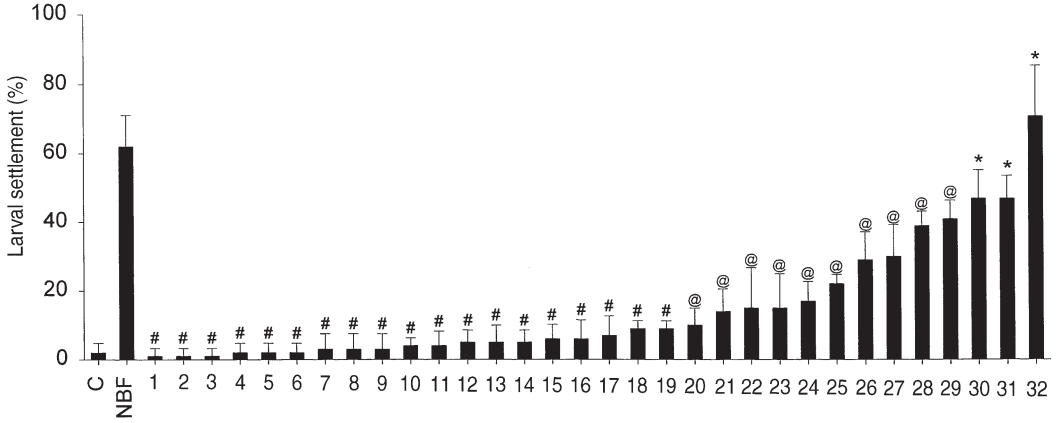

Fig. 1. *Hydroides elegans*. Mean percentage of larval settlement after 24 h in response to 32 individual monospecific diatom films. A clean glass slip represented the negative control (C); a glass slip filmed with natural biofilm represented the positive control (NBF). Bars are denoted with symbols indicating the assignment to one of the following statistical categories (multiple pairwise comparison of settlement rates on individual diatom-filmed slips with both controls; Dunnett's test, $\alpha = 0.05$; 1-way ANOVA, $p < 0.001$): #, no effect (same percentage of larval settlement as in the negative control); *, inductive (same percentage of larval settlement as in the positive control); @, weakly inductive (percentage of larval settlement significantly higher and lower than in the negative and the positive control, respectively). Data plotted are means ± SD of 5 replicates

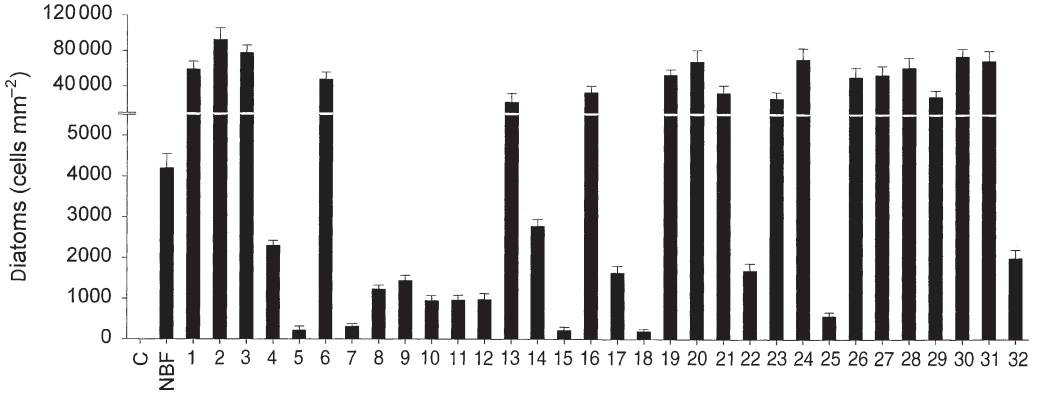

Fig. 2. *Hydroides elegans*. Mean density of diatom cells mm^{-2} in 32 experimental diatom films, the positive (NBF) and the negative control (C). Data plotted are means ± SD of 3 replicates based on counts of 5 fields at 100× magnification

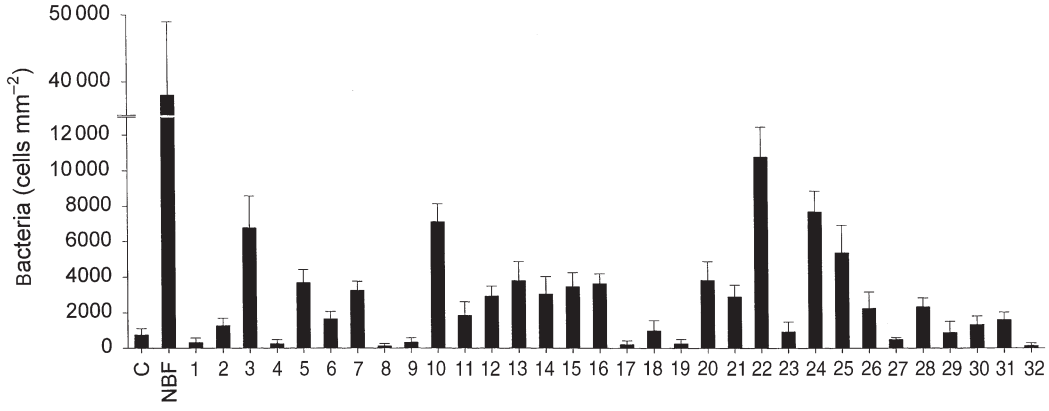

Fig. 3. *Hydroides elegans*. Mean density of bacterial cells mm^{-2} in 32 experimental diatom films, the positive (NBF) and the negative control (C). Data plotted are means ± SD of 3 replicates based on counts of 5 fields at 1250× magnification

Expt 2: Qualitative evaluation of bacterial contaminants in diatom films on induction of larval settlement

Diatom densities in the mixed diatom-bacteria films, generated with diatom cultures 5, 17, 23, 27, 29 and 30, ranged between 2000 and 7000 cells mm^{-2} (Fig. 4C). The bacterial abundance in diatom-free microbial films and mixed diatom-bacteria films originating from the same algal cultures was statistically the same (Student's *t*-test; Fig. 4B). With the exception of the experimental films made from diatom cultures 5 and 17, the percentage of larval settlement in response to mixed diatom-bacteria films was significantly higher than to the corresponding diatom-free microbial films (Student's *t*-test; Fig. 4A). The observation of 2500 ± 1540 bacteria cells mm^{-2} in the control indicated a systematic bacterial contamination due to experimental handling procedures despite the application of sterile measures. The effect of this contamination on the mean percentage of larval settlement induction was comparatively weak with 5 ± 5%.

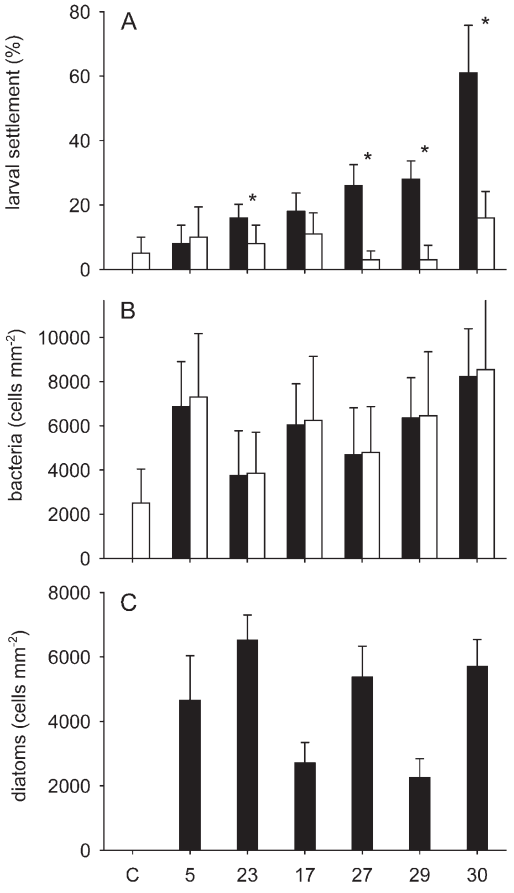

Fig. 4. *Hydroides elegans*. (A) Mean percentage of larval settlement after 24 h in response to mixed diatom-bacteria films (black bars) and diatom-free microbial films (white bars) originating from non-axenic diatom cultures. A microbial control film prepared from diatom nutrient medium served as a control (C). Significant differences between larval settlement rates on the 2 film types are asterisked (Student's *t*-test, $\alpha = 0.05$). Numbers indicate diatom strains under investigation. Data plotted are means ± SD of 5 replicates. (B) Mean density of bacterial cells mm^{-2} in mixed diatom-bacteria films (black bars) and diatom-free microbial films (white bars). Data plotted are means ± SD of 3 replicates based on counts of 5 fields at 1250× magnification. (C) Mean density of diatom cells mm^{-2} in mixed diatom-bacteria films. Data plotted are means ± SD of 3 replicates based on counts of 5 fields at 100× magnification

DISCUSSION

This study is an extension of our investigations on the relative roles of the various components of marine natural biofilms (NBF) in stimulating larval settlement in the serpulid polychaete *Hydroides elegans*. In addition to previous studies on the significance of the bacterial component in NBF (Lau & Qian 1997, Beckmann et al. 1999, Harder & Qian 1999), we tested a variety of monospecific diatoms isolated from NBF with respect to their role in mediating larval settlement in *H. elegans*.

The statistical evaluation of test results of 32 monospecific diatom films in comparison to the controls of NBF and clean glass substratum categorised the settlement response into 3 distinct groups, i.e. (1) the same as in the positive control (glass slip with natural biofilm); (2) the same as the negative control (clean glass slip); and (3) in-between the positive and negative control. Out of 32 diatom strains under investigation, 13 fell into the categories (1) and (3) with an inductive effect on larval settlement of *Hydroides elegans*. These strains comprised 7 different diatom genera, indicating that the inductive effect on larval settlement was not genus specific.

However, an unambiguous ascription of the obtained test results to the presence of diatoms turned out to be impossible as bacterial contamination was observed on the filmed cover slips (see Fig. 3). The observed bacterial densities were at least 3 times lower than in the NBF control, and thus may have played only a minor effect on larval settlement. Moreover, no significant linear relationship between larval settlement rates and cell densities of diatoms or bacteria was observed over all strains of diatoms. However, a mere quantitative evaluation of the impact of the bacterial contamination would have neglected the significant qualitative effect of some bacterial strains on the induction of larval settlement. Therefore, the bacterial contamination in the diatom films under investigation confounded the interpretation of our test results.

Considering the experimental difficulties to achieve total axenicity of monospecific diatom cultures (Bhosle et al. 1995), an alternative approach was taken in order to decouple the larval response to the bacterial component from that to non-axenic diatom films. The bacterial communities associated with diatom cultures were separated by filtration to create diatom-free microbial films, representing the respective bacterial contaminants of each culture. Under the assumption that the separated bacteria reflect the community structure in the mixed diatom-bacteria films, a comparison of the 2 film types allowed the evaluation of the qualitative effect of bacterial contaminants in non-axenic, monospecific diatom films on the induction of larval settlement. For this investigation, 6 diatom cultures were selected. Whilst the observed bacterial densities in diatom-free microbial films and non-axenic diatom films were statistically the same, larval settlement on diatom films was significantly higher than on the microbial control films. Importantly, this was the case for those diatoms belonging to the categories (1) and (3). These results indicate that the induction of larval settlement by these treatments was due to the presence of diatoms and not confounded by the presence of bacteria. The systematic bacterial contamination due to experimental handling procedures, despite the application of sterile measures, was comparatively low and posed only a minor effect on the induction of larval settlement. The bacterial abundance in the diatom films was at least twice as high as in the control, indicating that these bacteria were associated with the diatoms rather than being accidentally introduced into experimental treatments. Despite the limitations in achieving total axenicity in single diatom cultures, our results suggest that the underlying causal mechanism for differences in observed larval settlement rates is due to qualitative characteristics of the individual diatom strains.

Previous investigations of larval settlement induction by marine benthic diatoms were mainly focused on invertebrate species with grazing juveniles, such as abalones (Ebert & Houk 1984, Kawamura & Kikuchi 1992, Slattery 1992, Bryan & Qian 1998, Daume et al. 1999), sea urchins (Tani & Ito 1979) and sea cucumbers (Ito & Kitamura 1997). Marked differences both in the time for completion of settlement and the settlement rate were correlated with the quality of diatoms and their densities on the settlement substratum. The nature of the settlement cues in all of the above cases has not been elucidated but was hypothesised to be associated with post-larval dietary requirements (Slattery 1992, Daume et al. 1999). In contrast to these studies, post-larval survival in *Hydroides elegans* does not depend on surface-associated food availability as juveniles are efficient filter feeders.

The induction of larval settlement in *Hydroides elegans* is most likely mediated by chemical cues associated with a suitable settling substratum. In the case of bacteria, the inductive activity has been attributed to bacterial secondary metabolites and glycoproteinacious bacterial exopolymers (Unabia & Hadfield 1999, unpubl. data). Similar conclusions were drawn with respect to the induction of larval settlement by NBF in other invertebrate taxa (Zobell & Allen 1935, Kirchmann et al. 1982, Johnson et al. 1991, Szewzyk et al. 1991, Leitz & Wagner 1993, Maki 1999). As 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 compounds.

The objective of this investigation was to test the hypothesis that individual monospecific diatom films generate a similar range of settlement responses in *Hydroides elegans* as has been shown for monospecific films of bacteria from NBF. Despite the potentially confounding effects of bacteria, our results suggest that this hypothesis cannot be rejected.

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