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The effect of bacterial and diatom biofilms on the settlement of the bryozoan *Bugula neritina*

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Abstract

Biofilms of marine bacteria and diatoms and their combinations were examined in laboratory choice assays to determine their effects on the attachment and successful metamorphosis of the larvae of the bryozoan *Bugula neritina* (Linnéus). The larval settlement in response to unfilmed surfaces, a natural biofilm (NBF) and adsorbed cells of three strains of bacteria, five strains of pennate diatoms and combinations of the two at different densities. Bacterial and diatom strains showed different effects on the larval settlement of *B. neritina*. Bacterial monospecific strains of an unidentified α-*Proteobacterium* and *Vibrio* sp. mediated the same percentage of settlement as a filtered seawater control. Biofilms of *Pseudoalteromonas* sp. caused significantly lower larval settlement. Larval settlement of *B. neritina* was negatively correlated with increasing densities of *Pseudoalteromonas* sp. The highest percentages of settlement were mediated by the biofilms of the diatom species *Achnanthes* sp., *Amphora cofeaeformis*, *Amphora tenerrima*, *Nitzschia constricta* and a 5-day-old natural biofilm, while the lowest settlement was found on a *N. frustulum* film. A three-way analysis of variance demonstrated that the density of bacteria and the presence of particular species of diatoms and bacteria in combined biofilms, significantly affected the settlement of *B. neritina* larvae. High settlement of larvae (50–90%) at all treatments indicated that *B. neritina* larvae are much more indiscriminate settlers than previously expected. Hence, using this species as a monitoring

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organism to trace ecologically relevant subtle changes of settlement cues in the natural environment should be carefully re-examined.

Keywords: Settlement; *Bugula neritina*; Bacteria; Diatoms; Bryozoan larvae; Attachment; Monospecies biofilms; Two-species biofilms

1. Introduction

The early life cycle of most sedentary marine invertebrates includes a planktonic larval phase. At competence—a physiological state where dispersing larvae are able to attach and subsequently metamorphose into sedentary juvenile individuals—larvae start to explore and choose attractive substrata to attach and transform into juvenile organisms (Chia, 1978). Suitable substrates for larval settlement are known to be mediated by environmental cues including stimuli produced by microbial biofilms.

Bryozoans are important marine fouling organisms (Woollacott et al., 1989). They prevent themselves from being fouled by gardening symbiotic bacteria (Walls et al., 1993; Shellenberger and Ross, 1998; McGovern and Hellberg, 2003). The substratum preferences exhibited by their larvae range from very specific to very opportunistic (Ryland, 1974; Bryan et al., 1998; Abgrall and Walters, 2000). Most bryozoans require a substratum that provides firm support for attachment (Woollacott, 1984) and many prefer surfaces that have a smooth or glossy finish (Ryland, 1976). Most laboratory-based studies of settlement responses have focussed on the physico-chemical characteristics of substrata, such as surface free energy and roughness (Rittschoff and Costlow, 1989), rather than the composition, density and physiological activity of substratum-associated biofilms (cf. Wieczorek and Todd, 1997). In fact, all natural biotic or abiotic (here, natural as well as anthropogenic) marine surfaces are usually covered with biofilms, consisting predominantly of bacteria and diatoms (Wahl, 1989). Natural biofilms (NBF) influence the substratum selections of many, if not most, invertebrate larvae (for reviews, see: Pawlik, 1992; Rodriguez et al., 1993; Keough and Raimondi, 1995; Wieczorek and Todd, 1998; Hadfield and Paul, 2001). Although microbial films seem to be not essential for bryozoan larval attachment (Ryland, 1976)—several studies show that bryozoa in general are relatively indiscriminant settlers (Walters et al., 1999). Microbial films on substrata have been shown to influence the attachment of larvae of a number of bryozoan species. Brancato and Woollacott (1982) showed that certain bacterial monospecific strains facilitated settlement in three bryozoan species (among them *Bugula neritina*), while bacteria inhibited the larval settlement of the bryozoan *Bugula flabellatae* (Crisp and Ryland, 1960; Wieczorek and Todd, 1997).

The facilitating effects of marine biofilms on bryozoan larval settlement have been observed both in the laboratory (Mihm et al., 1981; Brancato and Woollacott, 1982; Mitchell and Maki, 1988; Maki et al., 1989; Szewzyk et al., 1991; Hölmström et al., 1992; Paull, 1995) and in the field (Roberts et al., 1991; Todd and Keough, 1994; Keough and Raimondi, 1996; Paull, 1995).

However, few studies have focused on the role of marine benthic diatoms in mediating or inhibiting the settlement of invertebrate larvae although their relative abundance in marine biofilms can be high, especially in the euphotic zone. Diatoms induce larval settlement of various invertebrate phyla, such as Crustacea, Mollusca, Annelida and Echinodermata (e.g. Harder et al., 2002). Even the larval settlement of the bryozoan *B. neritina* was shown to be mediated by pennate diatoms (Kitamura and Hirayama, 1987).

Most laboratory experiments have been performed with monospecies biofilms of bacteria or diatoms (Wieczorek and Todd, 1998), but natural biofilms comprise numerous species of bacteria and diatoms besides other groups of microorganisms, some of which may facilitate larval settlement while others may be inhibitory or have no effect on larval settlement. Changes in either bacterial and diatom species or the ratio of those in multispecies biofilms affect the larval settlement of the tubeworm *Hydroides elegans* (Lau and Qian, 1997). The effects of bacteria and diatoms, mixed at different ratios in order to mimic potential variability and patchiness of natural biofilms, on the larval settlement of *H. elegans* were investigated by Harder et al. (2002). In the latter study, most of the treatments showed higher percentages of settlement on a mixed diatom–bacteria biofilm than on a monospecies biofilm.

B. neritina is one of the dominant fouling species in tropical waters (Walters, 1992). Its post-settlement success or failure is largely dependent on the structural complexity of its substrata (Walters and Wethey, 1996) as well as on the swimming experience of its larvae (Wendt, 1996a,b, 1998, 2000). It has been previously demonstrated that this species settled on the substratum within only hours of release (Bryan et al., 1997; Walters et al., 1999).

The present study was designed to elucidate the effects of bacteria and benthic pennate diatoms, separately and in combination, on the larval settlement of *B. neritina*. We tried to mimic the complexity of natural biofilms as a first approximation by mixing different proportions of monospecies strains of bacteria and diatoms that had previously been isolated from natural biofilms to develop artificial biofilms and to investigate their effects on settling larvae. The objective was to test in vitro the hypothesis that larval settlement of *B. neritina* on two-species bacteria–diatom biofilms is determined by the properties and the densities of the constituent species that form artificial biofilms.

2. Material and methods

2.1. Preparation of monospecies and two-species bacterial and diatom biofilms

2.1.1. Development of bacterial films

Stock cultures of bacteria isolated from a “natural biofilm”, from a site where also adults of *B. neritina* had settled, were obtained from the Marine Bacterial Culture Collection at the Hong Kong University of Science and Technology. All bacteria were inoculated into sterile culture broth [0.5% (w/v) peptone, 0.3% (w/v) yeast extract in sterile-filtered (0.22 μm filter) (FSW)] and grown at 30 °C for 48 h to the stationary phase. Suspended bacteria were harvested by centrifugation and diluted in autoclaved FSW to an optical density of 0.1 units at a wavelength of 610 nm (using a spectrophotometer of TechComp, China). Polystyrene Petri dishes (#1006, Falcon, USA) were charged with 4 ml of the bacterial suspension and incubated for 3 h at 22 °C to allow bacteria to attach and form consolidated biofilms. Thereafter, the dishes were dip-rinsed in FSW to remove unattached cells. The remaining solid bacterial film was topped with 4 ml of FSW. The filmed dishes (seven replicates) were then used for larval settlement assays. Dishes treated with FSW (0.22 μm) and a NBF were used as controls. Three dishes per treatment were diverted for the enumeration of bacteria on the dish surface. Both monospecific and two-species biofilms (see below) were used in larval settlement assays.

2.1.2. Development of diatom biofilms

Diatoms from the HKUST culture collection were cultivated in aerated 400-ml Erlenmeyer culture flasks according to the procedure of Harder et al. (2002). When visible diatom films had developed in the flask, the monospecific diatom suspensions were transferred to experimental Petri dishes (Falcon, USA: eight replicates) according to the method of Harder et al. (2002) and incubated for 24 h to allow diatoms to attach and develop films on the substrata. The filmed Petri dishes were then washed carefully in FSW and five replicates were filled with FSW or bacterial suspension (two-species biofilms). The remaining three dishes were fixed with a 5% solution of formalin in seawater and used for diatom counts. Sterile-filtered seawater (0.2 μm) and a natural biofilm were used as controls.

2.1.3. Development of two-species biofilms

In order to generate multispecies biofilms, bacteria and diatoms (formation of diatom biofilms see below) were mixed together in 1:3 and 2:3 ratios (e.g. S8 1/3—means that one-third of a total of 4 ml of bacterial strain *Pseudoalteromonas* sp. (S8) suspension at 0.1 optical density is added to two-thirds of FSW; e.g. NF C10 2/3—means that two-thirds of a total of 4 ml bacterial strain C10 suspension (at 0.1 optical density) was added to a Falcon dish pre-filmed with the diatom *Nitzschia frustulum* (NF)—see the species and strain names in the Table 1). Thereafter, the dishes were dip-rinsed in FSW to remove unattached cells. The remaining bacterial film was topped with 4 ml of FSW. The filmed dishes (five

Table 1
The coding of benthic bacteria and diatoms used for the experiments

Bacteria

Abbreviation	Strain designation	Closest match at the Genbank (www.ncbi.nlm.nih.gov)	Similarity (%)
S8	UST991130-053	*Pseudoalteromonas* sp. PB-2 (access no. AF482707)	98
C16	UST991130-048	*Vibrio* sp. AS-42 (access no. AJ391203)	98
C10	UST991130-043	Unidentified α-*Proteobacterium* MBIC1876 (Mar. Biotech. Japan) (access no. AB026194)	98

Diatoms

Abbreviation	Taxon identity
9B	*Achnanthes* sp.
AC	*Amphora cofeaeformis*
29D	*Amphora tenerrima*
NF	*Nitzschia frustulum*
NC	*Nitzschia constricta*

replicates) were then used for larval settlement assays. Three dishes per treatment were used for the enumeration of bacteria and diatoms on the dish surface. Dishes treated with sterile-filtered seawater (0.22 μm filter) and a natural biofilm were used as controls.

2.1.4. Development of natural biofilms

The positive control "natural biofilm" was obtained by exposing Falcon dishes to the surface waters at the pier of the Hong Kong University of Science and Technology (HKUST), next to the Coastal Marine Laboratory (CML) (N22°21′ , E114°16′) for 5 days before using them for larval bioassays as positive controls.

2.1.5. Enumeration of bacterial and diatom surface densities

Attached bacteria and diatoms were visualized by the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI) (Fluka Chemie, Switzerland) at 0.5 μg ml^{-1} (Daley and Hobbie, 1975). Formalin-fixed (4% in FSW) Falcon dishes (n=3) were rinsed with distilled water and stained with DAPI for 5 min. Stained substrata were wet-mounted on a fluorescence microscope (Olympus, Japan) and bacterial and diatom cells recorded at 1000× magnification in five fields of view chosen 'ad hoc' (λ_{Ex}=359 nm, λ_{Em}=441 nm). Densities of bacteria and diatoms were recalculated to cells mm^{-2}.

2.2. Larval culture and bioassays

Adult broodstocks of *B. neritina* were collected from pilings and floating rafts at Wong Shek Pier, Hong Kong (N22°25′ , E114°20′) (cf. Liu and Li, 1987). Larvae were obtained according to the method described in Bryan et al. (1997), and only newly (i.e. within 15 min) released larvae were included in the bioassays. Still water laboratory bioassays were performed with seven replicates (n=7) in sterile polystyrene Falcon dishes containing 20 larvae and monospecific as well as two-species biofilms in 4 ml of sterile-filtered (0.22 μm) seawater. Previous studies showed that larvae did not interfere negatively with each other at this density (Bryan et al., 1997) but were very sensitive to light (Wendt and Woollacott, 1999). Therefore, the experiments were carried out in the dark. Settlement assays were run for 1 h. After 1 h, the number of attached juveniles was enumerated and the settlement was expressed in percentages. In all bioassays, FSW (seawater filtered through a 0.22-μm filter) served as a negative control while NBF (5-day-old natural biofilms formed on Falcon dishes) were used as a positive control.

2.3. Statistical analysis

The percentage of larval attachment in response to each experimental treatment was arc-sine transformed. To improve the arc-sine transformation, those replicates with no attachment were given the value of 1/4n (n=number of larvae in a single replicate) (Zar, 1996). The homogeneity and normality of the data sets were analysed with Levene's test and the Shapiro–Wilk test at a confidence level of 95%. The differences between treatments and control were determined by a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (HSD—see Zar, 1999). Also multiple comparisons were analyzed by Fisher's LSD test with the Bonferroni correction, which was employed for adjusting the significance level to control type I error rates (Quinn and Keough, 2002). The density data were square root transformed in order to ensure the normality of their

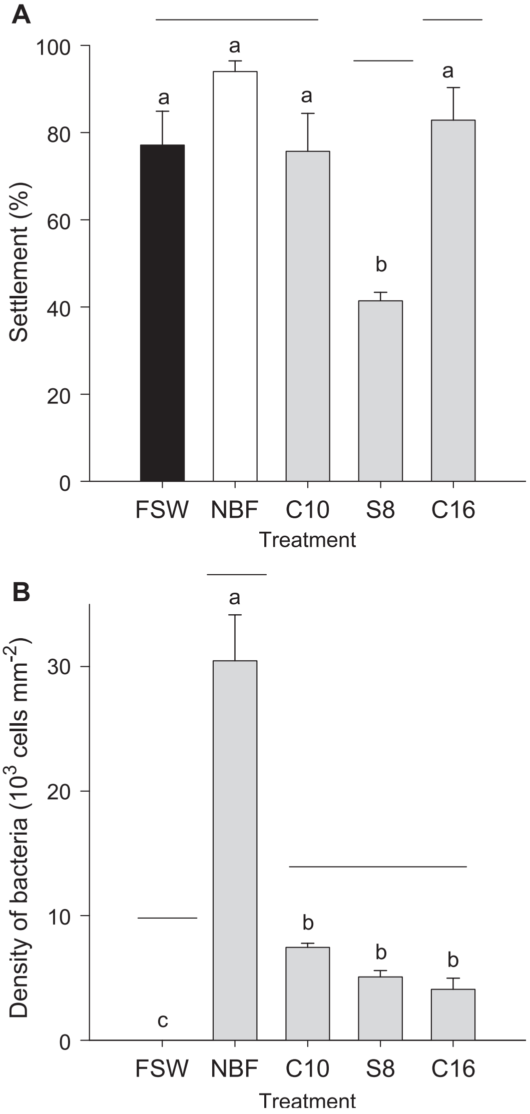

Fig. 1. (A) The effect of monospecies bacterial biofilms on the larval settlement of the bryozoan *B. neritina*. (B) Bacterial densities after the experiment. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bars; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to an LSD test with Bonferroni correction. The strains are described in Table 1.

variance. The densities of diatoms and bacteria in bioassays were compared by one-way ANOVA followed by a Tukey HSD post-hoc test. The significance level of an additional Fisher's LSD test was adjusted by Bonferroni correction. The correlation between larval settlement and bacterial density were analysed by using regression analysis. Each data

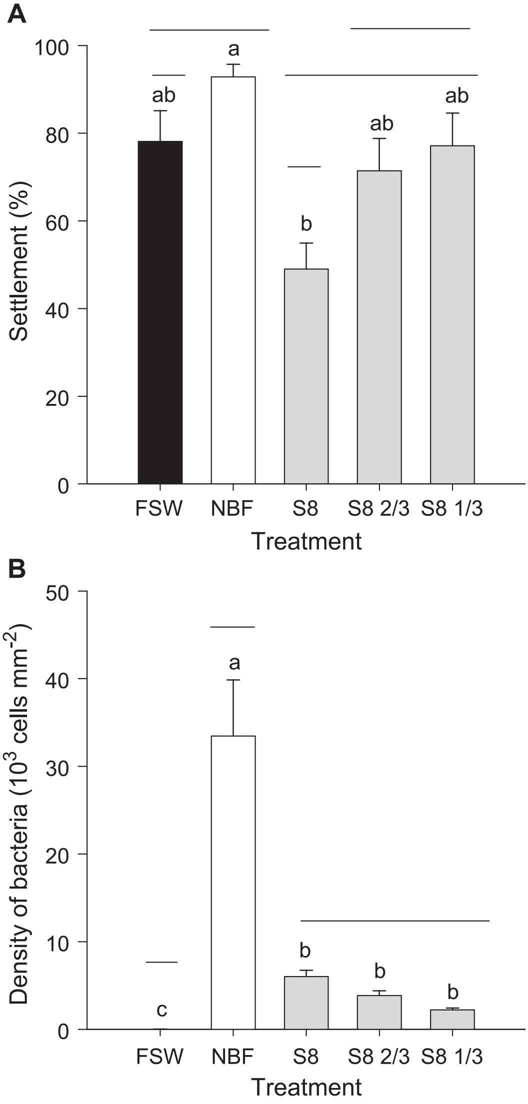

Fig. 2. (A) The effect of *Pseudoalteromonas* sp. (strain S8) biofilm density on larval settlement of the bryozoan *B. neritina*. (B) Bacterial densities after the experiment (e.g. S8 1/3—means that one-third of a total of 4mL of bacterial strain S8 suspension at 0.1 optical density is added to two thirds of FSW)—at S8 only bacterial suspension is added. The strains are described in Table 1. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bars; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to an LSD test with Bonferroni correction.

point on the resulting curve indicates the bacterial cell density and the corresponding percentage of settlement on a given dish after 1 h of incubation. The combined effect of diatoms and bacteria as well as their densities on larval settlement was analysed using a three-way ANOVA. In all cases, the threshold for significance was 5%. The data presented in the figures were not transformed.

3. Results

3.1. The effect of monospecies bacterial films on larval settlement

Monospecies bacterial films caused significantly different settlement of *B. neritina* larvae (Fig. 1A, ANOVA: F=7.12, p<0.001). HSD and LSD tests results categorized all tested bacterial strains into the following two groups. NBF, negative control (FSW) and bacterial strains C10 and C16 mediated the same percentage of larval settlement. The larval settlement on the biofilms of S8 was significantly (p>0.05) lower than that in the controls (FSW and NBF). The highest bacterial densities were found in dishes filmed with NBF (Fig. 1B). Bacterial strains C10, S8 and C16 showed significantly lower (p<0.05) bacterial densities than dishes with NBF but a higher density than Falcon dishes with FSW (Fig. 1B).

3.2. The effect of different monospecific bacterial densities on larval settlement

The density of the bacterium *Pseudoalteromonas* sp. (strain S8) affected the settlement of *B. neritina* larvae (Figs. 2A,B and 3, ANOVA: F=4.32, p<0.001). The highest density of bacteria (about 30,000 cells mm^{-2}) was detected on a NBF (Fig. 2B). The cell density of S8 monospecies biofilms varied between 2000 and 6000 cells mm^{-2}. The percentage of settlement varied between 49% and 90%. The highest density of bacteria led to the lowest

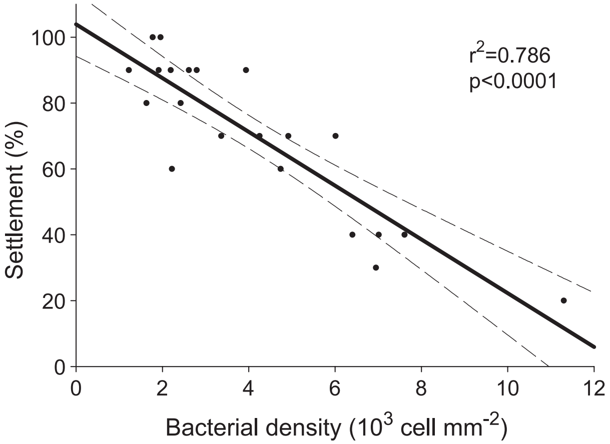

Fig. 3. The effect of *Pseudoalteromonas* sp. (strain S8) density on larval settlement of *B. neritina.* The relationship between bacterial density and larval settlement is indicated by the coefficient of correlation, r^2.

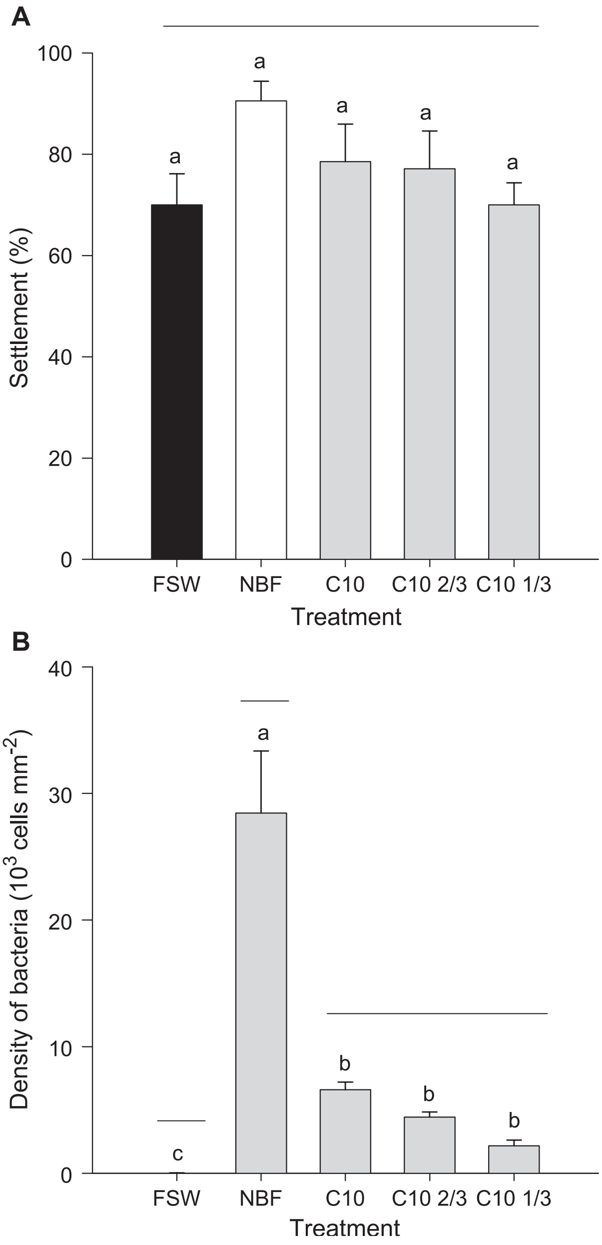

Fig. 4. (A) The effect of the biofilm density of an unidentified α-*Proteobacterium* (strain C10) on the larval settlement of the bryozoan *B. neritina*. (B) Bacterial densities after the experiment (e.g. C10 1/3—means that one-third of a total of 4 ml of bacterial strain C10 suspension at 0.1 optical density is added to two-thirds of FSW). The strains are described in Table 1. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bars; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to an LSD test with Bonferroni correction.

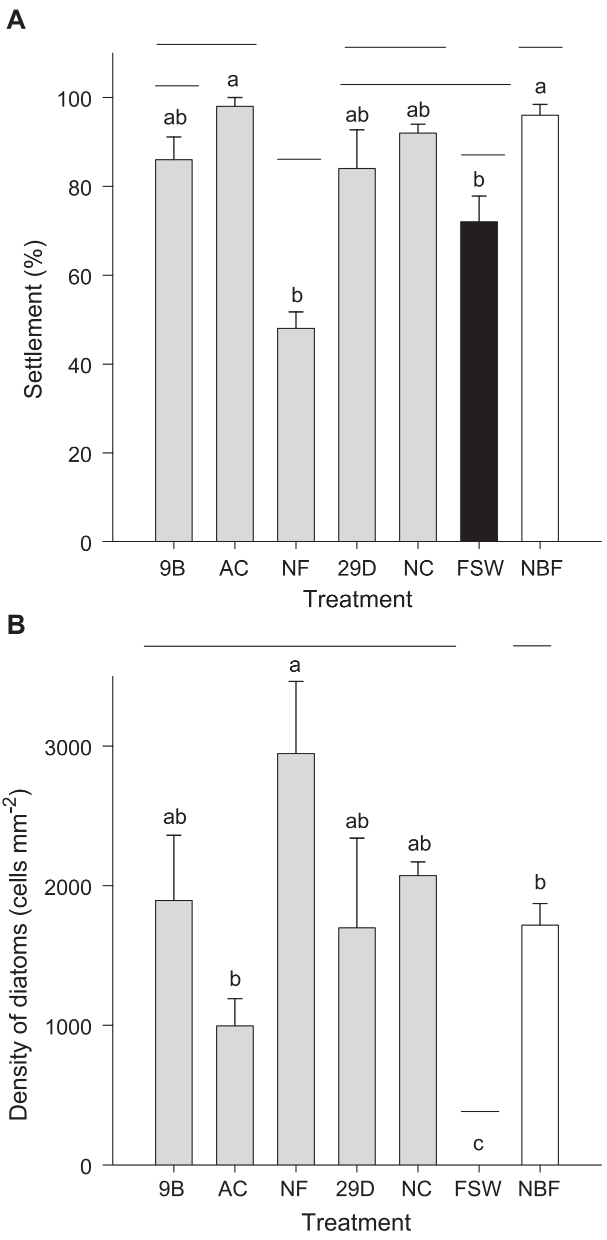

Fig. 5. (A) The effect of monospecies diatom biofilms on the larval settlement of the bryozoan *B. neritina*. (B) Diatom densities after the experiment. The species are described in Table 1. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bars; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to an LSD test with Bonferroni correction.

percentage of the larval settlement of *B. neritina*, which was significantly different ($p<0.05$) from the positive control (NBF) (Fig. 2A,B). Therefore, the settlement of *B. neritina* was negatively correlated ($r^2>0.786$, $p<0.0001$) with the density of the bacterium S8 (Fig. 3).

In the experiments with an unidentified α-*Proteobacterium* (strain C10), the highest percentage of settlement was observed on dishes filmed with NBF and the lowest settlement was found on the control dishes without biofilm (FSW—negative control), while all changes were not significantly different ($p<0.05$) (Fig. 4). There was no significant effect of the density of strain C10 on the larval settlement of *B. neritina*. The highest density of bacteria was recorded on surfaces filmed with NBF (positive control). The cell density of monospecies biofilms varied between 2000 and 6000 cells mm^{-2}. There was no correlation between the density of bacterial strain C10 and larval settlement ($r^2=0.102$, $p=0.157$).

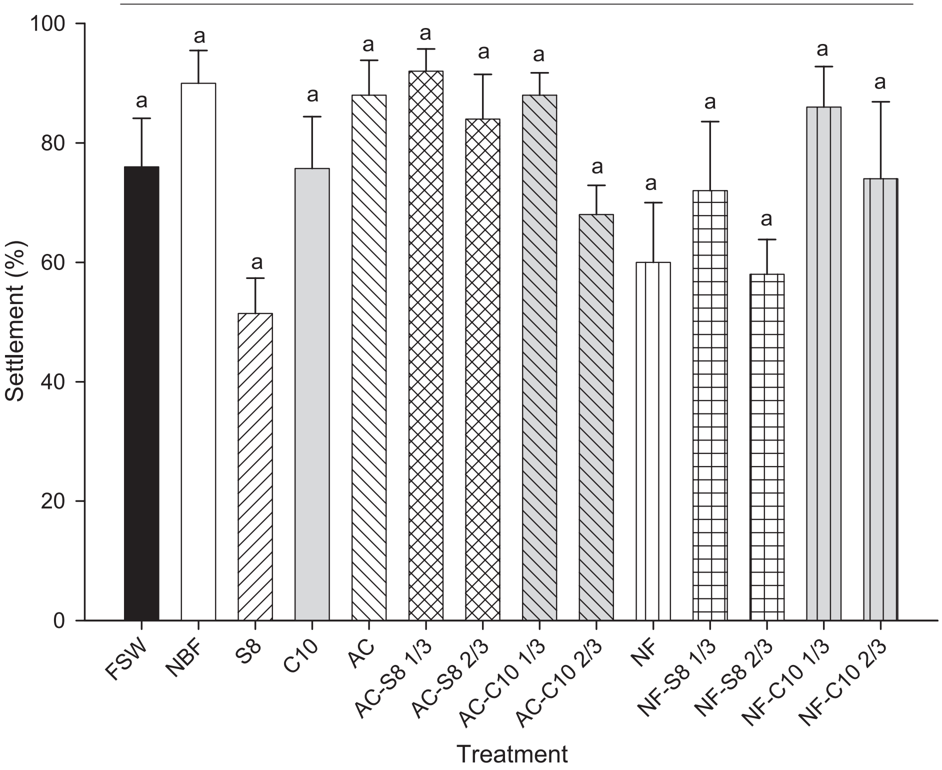

Fig. 6. The effect of two-species biofilms consisting of the bacterium *Pseudoalteromonas* sp. (S8) or an unidentified α-*Proteobacterium* (C10) with the diatom *A. cofeaeformis* (AC) or diatom *N. frustulum* (NF) on the larval settlement of *B. neritina* (e.g. NF-C10 2/3 means that two-thirds of a total of 4 ml of bacterial strain C10 suspension at 0.1 optical density is added to one-third of FSW to a dish filmed with the diatom *N. frustulum*). The strains are described in Table 1. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bars; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to an LSD test with Bonferroni correction.

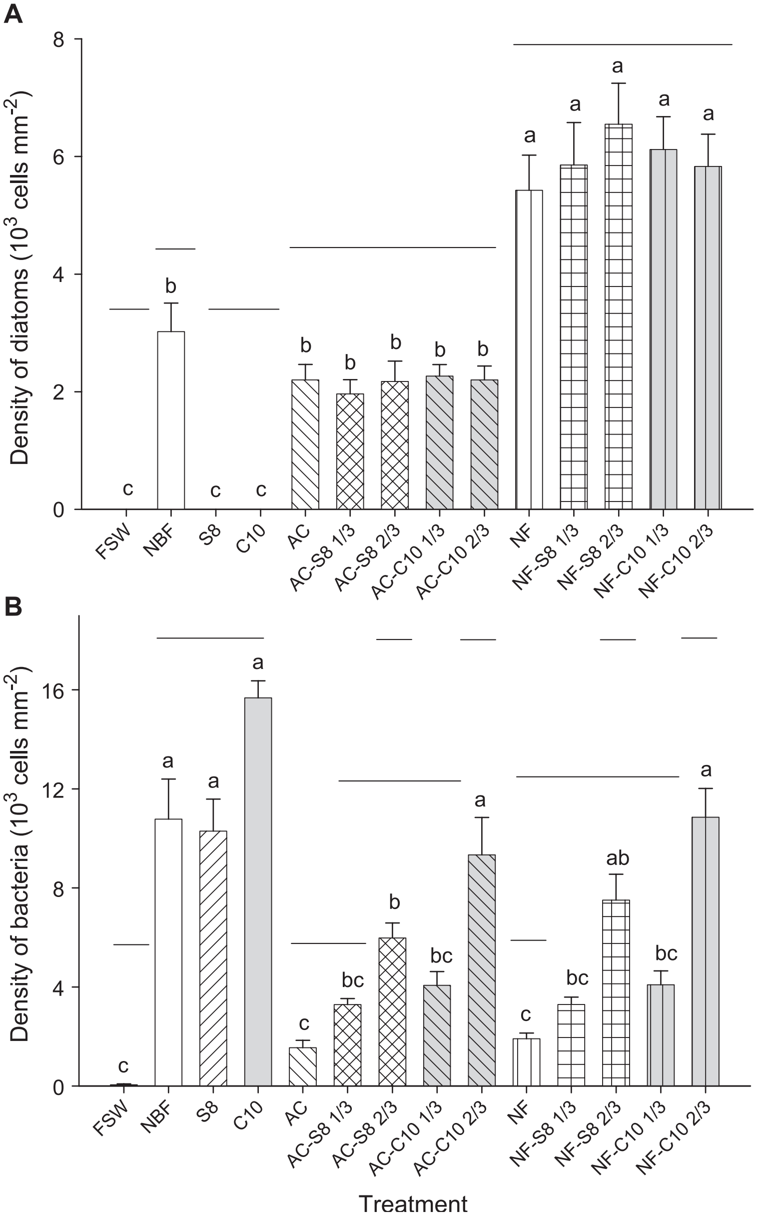

Fig. 7. The densities of diatoms (A) and bacteria (B) after experiments with two-species biofilms of the bacterium *Pseudoalteromonas* sp. (S8) or an unidentified α-*Proteobacterium* (C10) with the diatoms *A. cofeaeformis* (AC) or *N. frustulum* (NF) (e.g. NF-C10 2/3 means that two-thirds of a total of 4 ml of bacterial strain C10 suspension at 0.1 optical density is added with one-third of FSW to a dish filmed with the diatoms *A. cofeaeformis* and *N. frustulum*, respectively). The strains are described in Table 1. Data that are significantly different according to a HSD test (ANOVA: $p<0.05$) are indicated by different letters above the bar; means not joined by a horizontal line at the same level differed significantly ($p<0.05$) according to LSD test with Bonferroni correction.

3.3. The effect of diatom monospecies biofilms on the settlement of B. neritina

The biofilms of diatoms affected the larval settlement of *B. neritina* (Fig. 5A, ANOVA: F=8.89, p<0.0004). The highest percentages of settlement were mediated by biofilms of the diatom species *Achnanthes* sp. (9B), *Amphora cofeaeformis* (AC), *Amphora tenerrima* (29D), *Nitzschia constricta* (NC) (see Table 1) and NBF. The lowest settlement (about 48%) was observed in the dishes with the diatom NF and the control dishes with FSW. In these dishes, the settlement of *B. neritina* was significantly lower than that in NBF and AC (HSD, LSD Bonferroni, p<0.05).

The density of diatoms varied from 2800 to 1000 cells mm^{-2} (Fig. 5B). The highest density of diatoms was observed in Falcon dishes with monospecies diatom films of NF, 9B, 29D and NC. The densities in these Petri dishes were not significantly different from the density of the diatoms in the NBF (HSD, p>0.05). The lowest density of diatoms was found in dishes filmed with the diatom AC and in the FSW control. The diatoms 9B, NF and 29D formed clumps, which resulted in higher standard deviations than in other diatom biofilm treatments (Fig. 5B).

3.4. The effect of two-species biofilms on the settlement of B. neritina

Two-species biofilms were prepared by mixing different proportions of diatom and bacterial species and subsequently tested for their ability to suppress or enhance the larval settlement of *B. neritina*. The experimental results demonstrated that certain two-species biofilms affected larval settlement (Fig. 6, ANOVA: F=2.34, p<0.04). In treatments where the diatom AC and NF was mixed with an unidentified α-*Proteobacterium* (C10) or *Pseudomonas* sp. (S8), the highest percentage of settlement was observed on biofilms with low densities of bacterial strain C10 and with high as well as low densities of bacterial strain S8 (Figs. 6 and 7). The lowest settlement was observed in dishes filmed with bacterium S8 as well as in dishes filmed with the diatom NF and the bacterium S8 at

Table 2
The results of a three-way ANOVA examining the significance of the effect of two-species biofilms with different bacterial densities (an unidentified α-*Proteobacterium* or *Pseudoalteromonas* sp.) and diatom species (*A. cofeaeformis* or *N. frustulum*) on the larval settlement of *B. neritina*

	MS	F-value	p-value
Main factors	1709.636		
Diatoms	530.552	2.300	>0.13
Bacteria	11.855	0.051	>0.82
Bac. density	1167.229	5.060	<0.03*
Combined effect of main factors	1483.885		
Diatoms and bacteria	1343.661	5.82	<0.02*
Diatoms and bac. density	0.293	0.001	>0.97
Bacteria and bac. density	43.347	0.187	>0.66
Diatoms, bacteria and bac. density	96.584	0.418	>0.52
Error	230.673		

In the experiment, the main factors were the presence of a particular species of diatoms (diatoms) or bacteria (bacteria) as well as the density of bacteria (bac. density) in the two-species biofilms. An asterisk (*) denotes significant p-values at α=0.05.

bacterial cell concentrations of about 7500 cells mm^{-2}. In all cases, the settlement in the treatments and control were not significantly different from each other (HSD, LSD, Bonferroni, $p<0.05$).

Three-way ANOVA indicated that the densities of bacteria in two-species biofilms significantly ($p<0.05$) affected the settlement of *B. neritina* larvae (Table 2). The presence of particular species of diatoms and bacteria in the biofilms influenced larval settlement as larval settlement was influenced by the combined effect of bacteria and diatoms.

4. Discussion

In the marine environment, competent bryozoan larvae are influenced by a variety of physical, chemical and biological cues, such as the hydrodynamic regime during settlement processes (Walters et al., 1999; Qian et al., 2000). In an experiment by Walters et al. (1999), larvae of *B. neritina* settled only on 2-week-old microfouled surfaces. On the contrary, Todd and Keough (1994) found no effect of biofilms on the larval settlement of *B. neritina*. The major outcome of the present study confirmed that *B. neritina* seems to be a much more indiscriminate settler than previously expected. This behavioural trait is possibly fostered by its short planktonic retention time—the bulk of larvae settled within 1 h after their release on the different substrates during the present study. Nevertheless, pre-settlement behavior plays a critical role in the recruitment of bryozoans. A study by Paull (1995) shows the bryozoan *Bugula turrita* enhanced recruitment on the crusting alga *Peyssonnelia* sp., but reduced recruitment on the coralline algae *Lithothamnion* sp. and *Phymatolithon* sp. Hence, biological cues of either algal or bacterial origin have been considered as mediators of settlement patterns observed in laboratory and field experiments (Paull, 1995).

The results of the experiments presented here indicate that bacterial and diatom species composition played only a moderate role in the larval settlement of *B. neritina*. Out of eight species of diatoms and bacteria tested, only the bacterium *Pseudoalteromonas* sp.—S8—and the diatom NF were associated with a low settlement (about 50%) of *B. neritina*. Other species (the bacterium *Vibrio* sp.—C16, an unidentified α-*Proteobacterium*—C10, and the diatoms *Achnanthes* sp.—9B, *A. cofeaeformis*—AC, *A. tenerrima*—29D, *N. constricta*—NC) induced the same or higher levels of larval settlement than the control. Kitamura and Hirayama (1987) showed that the pennate diatoms *Nitzschia* spp., *Amphora* spp. and *Navicula* spp. stimulated the settlement of *B. neritina*. The marine bacterium *Halomonas marina* (American Type Culture Collection, access number: ATCC 27129) inhibited larval settlement and development of the sessile invertebrates *Balanus amphitrite* and *B. neritina* (Perry et al., 2001). Whole cells, culture filtrate and lysed cells embedded in a polyurethane coating also suppressed barnacle settlement as well as the settlement and maturation of *B. neritina*.

However, in other investigations, the effect of a monospecific bacterial film on the settlement of *B. neritina* was not substantiated (Bryan et al., 1997). Instead, it was suggested that larval settlement in *B. neritina* can be facilitated only by a natural biofilm (Walters et al., 1999). In our experiments, 5-day-old natural biofilms stimulated larval settlement, but only in one repeat the differences were statistically significant (HSD, $p<0.05$). It has also been demonstrated that the diatoms *Nitzschia* spp., *Amphora* spp. and *Navicula* spp., but not bacteria, stimulate larval settlement and attachment of *B. neritina* in a density-dependent fashion (Kitamura and Hirayama, 1987).

Kirchman et al. (1982) suggested that lectins on the surface of bryozoan larvae might mediate their choice of an attachment site when bacterial films are involved. However, the use of an adhesive for the temporary attachment of bryozoan larvae (Loeb and Walker, 1977) may provoke a situation similar to that during the attachment of barnacle cyprids. Crisp et al. (1985) suggested that cyprid larvae might avoid substrata to which a temporary adhesive secreted by their second antennae does not bind strongly. If bryozoan larvae select suitable substrata for the same reason, then the results of our experiments may be interpreted in terms of the temporary adhesives binding less strongly to the extracellular material of some bacteria than to the exopolysaccharides (EPS) of others.

In this study, the larval settlement of *B. neritina* on monospecific biofilms of *Pseudomonas* sp.—S8 was negatively correlated with total bacterial density. At the highest densities of the S8 strain, the lowest settlement of bryozoan larvae was observed whereas the percentage of larval settlement at lower bacterial densities was higher. Similar results have been demonstrated for the larval attachment of the barnacle *B. amphitrite* (Oliver et al., 2000; Lau et al., 2003). There may be three alternative explanations. Firstly, the differences in bacterial densities may lead to differences in the concentration of chemical repellents exuded by the bacteria, which may in turn affect larval settlement. Secondly, larval settlement may be affected by the amount of free space among the bacterial cells, which is usually covered by bacteria-derived exopolysaccharides. Thirdly, different densities of bacteria may lead to a different wettability of the surface, which can in turn affect bryozoan settlement. Mihm et al. (1981) demonstrated that bacterial films on polystyrene made the surface more wettable and also decreased the magnitude of larval settlement of *B. neritina*. While the first explanation (concentration of repellents) cannot be entirely excluded, the second (bacteria-free space on biofilmed dishes) and the third (different wettability) explanations better explain the present results and the findings of other authors. Although bacteria-free space has not been explicitly measured during this study, it is expected that a high density of bacteria will provide a higher coverage by EPS on the surface. The wettability of the experimental dish surfaces associated with the different treatments was not explicitly determined neither. It was probably very high, though, due to the extracellular glucopolysaccharide (EPS) production of the microbiota forming the existing biofilms, even at the lowest densities of the various monospecies studied. Mihm et al. (1981) pointed out that a reduction in larval settlement could not be attributed to changes in surface free energy alone and suggested that *Bugula* larvae respond to two sensory stimuli: one being surface free energy, the second some constituent of the bacterial-organic film. The latter cue could override the response to surface free energy alone (Eiben, 1976; Mihm et al., 1981). The data of the present investigation support the hypothesis of Mihm et al. (1981) and Maki et al. (1989) that bryozoan larvae possess a detection mechanism for a characteristic of the bacterial-organic film other than its elevated wettability (i.e. surface free energy).

The results of three way ANOVA revealed that both qualitative and quantitative changes in the bacterial and diatom composition of the two-species films affected the larval settlement of *B. neritina*. Diatoms are difficult to maintain without bacterial contamination (cf. Harder et al., 2002), but contamination was relatively low in the present study (see Fig. 7B—AC, NF). Bacterial effects were therefore only discernable in the combined treatment (Fig. 6). Contrary to our previous experiments with monospecies biofilms of the diatom NF and the bacterium S8 in which we observed the lower settlement of larvae the resultant bacterial–diatom film of these bacteria and diatoms induced the same proportion of settlement as the FSW and NBF controls (compare results of Figs. 1, 2, 5 and 6). This shows that larval settlement response to mixed biofilms was not a simple sum of the parts. The maximal, but significant, inhibitive and inductive effect, were observed only when the bacterial densities in the two-species biofilm were comparatively high (approximately 6000–10,000 cells mm^{-2}) (Figs. 6 and 7). The complicated and sometimes unpredictable response of larvae to multispecies biofilms relative to monospecies films is certainly worthwhile of being highlighted. The simplified assumption that results from monospecific film experiments can adequately predict the settlement of larvae in response to mixed-species biofilms was not substantiated by the present study.

In conclusion, qualitative and quantitative changes in bacterial and diatom taxon composition and density affected the larval settlement of the bryozoan *B. neritina*. However, *B. neritina* happened to be a quite indiscriminate settler. Therefore, using this species as a monitoring organism to trace ecologically relevant subtle changes in the natural environment should be carefully considered. On the other hand, larval bioassays with *B. neritina* are highly recommended where strong inhibitory effects are to be expected. This holds particularly for anti-fouling assays where the observation period can be restricted to 1 h.

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