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Facilitation and inhibition of larval attachment of the bryozoan *Bugula neritina* in association with mono-species and multi-species biofilms

Sergey Dobretsov, Pei-Yuan Qian *

Coastal Marine Laboratory and Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, PR China

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Abstract

In this study, we investigated the effect of mono-species and multi-species biofilms on larval attachment of the bryozoan *Bugula neritina*. The effect of biofilms was examined through a double-dish choice bioassay in which larvae were given the choice of attaching either to a clean surface of a container or to surfaces covered with biofilms. Larvae attached in response to mono-species biofilms of 5 out of 7 bacterial isolates from a subtidal region, but they avoided surfaces covered by biofilms of 7 out of 8 isolates obtained from an intertidal region. In the follow-up choice experiments with multi-species biofilms developed for 2 days, 7 days, 14 days, 28 days and 30 days, larvae preferentially attached to filmed surfaces over the unfilmed surfaces. When biofilms from 2 different tidal regions (intertidal and subtidal) were offered as choices in the double-dish bioassay, larvae in all cases attached on the subtidal biofilms. Two-day-old subtidal biofilms with low densities of bacteria induced significantly higher ($p<0.05$) attachment than did 30- day-old intertidal biofilms, which had high bacterial density. Terminal Restriction Fragment Polymorphism (T-RFLP) analysis revealed that the bacterial communities were substantially different in the subtidal and intertidal regions during all periods of the experiment. Attachment of *B. neritina* on subtidal biofilms did not depend on the bacterial density but rather was negatively correlated with diatom density, thickness of the exopolysaccharide layer and biofilm age. Our results suggest that the larvae of *B. neritina* can discriminate between biofilmed and clean surfaces and between biofilms developed under different tidal zones.

Keywords: Attachment; Bacteria; Biofilms; Bryozoan larvae; *Bugula neritina*; Recruitment; Tidal zone

1. Introduction

In the sea, all natural (macroalgae, stones, etc.) and artificial substrates are covered with biofilms consisting predominantly of bacteria and diatoms (Little, 1984; Wahl, 1989). Natural biofilms often promote (Meadows and Willams, 1963; Kirchman et al., 1982; Lau and Qian, 1997; Lau et al., 2002) or inhibit (Maki et al., 1988, 1989; Keough and Raimondi, 1995, 1996; Holmstrøm and Kjelleberg, 1999; Dobretsov and Qian, 2002) attachment of invertebrate larvae. Several studies have shown that bryozoa in general are relatively indiscriminate settlers (Ryland, 1974, 1976; Brancato and Woollacott, 1982; Maki et al., 1989; Walters et al., 1999; Cancino and Gallardo, 2004).

* Corresponding author. Tel.: +86 852 23587331; fax: +86 852 23581559.
E-mail address: boqianpy@ust.hk (P.-Y. Qian).

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The composition and amount of biofilm components along with biofilm age may all affect larval attachment (Maki et al., 1988; Le Tourneux and Bourget, 1988; Miron et al., 1999; Qian et al., 2003). For example, diatoms can guide larvae of the barnacle *Semibalanus balanoides* to attach along the intertidal region (Le Tourneux and Bourget, 1988; Minchinton and Scheibling, 1991). Similarly, specific bacterial and algal metabolites affect larval attachment of the blue mussel *Mytilus edulis* under natural conditions (Dobretsov, 1999; Dobretsov and Wahl, 2001). Changes in the bacterial community profile in the biofilm affect the attractiveness of the biofilm to the barnacles *Balanus amphitrite* (Qian et al., 2003) and *Balanus trigonus* (Thiyagarajan et al., in press). The relationship between biofilm composition and larval attachment of the bryozoan *Bugula neritina* (L.) has not been previously studied.

The bryozoan *B. neritina* is one of the dominant foulers in Hong Kong waters and around the world (Liu and Li, 1987). It has been previously demonstrated that larvae of this bryozoan will attach to clean substrata within several hours without special cues (Bryan et al., 1997; Dahms et al., 2004a,b). Previous studies have documented either stimulatory effects of biofilms on larval attachment of *B. neritina* (Brancato and Woollacott, 1982; Kitamura and Hirayama, 1987; Walters et al., 1999), inhibitory effects (Perry et al., 2001), or no effects at all (Todd and Keough, 1994; Bryan et al., 1997).

In our previous investigations, we demonstrated that larvae of *B. neritina* respond differently to biofilms consisting of subtidal bacterial or diatoms species (Dahms et al., 2004a). On the contrary, we observed good attachment of larvae on mixed bacterial-diatom biofilms even though the mixture included 2 inhibitive species. In the field, we found that larvae of *B. neritina* predominantly attach to subtidal surfaces but not to intertidal surfaces (unpublished data). Therefore, we propose that communities of microorganisms associated specifically with subtidal and intertidal surfaces may guide larvae of *B. neritina* to attach preferentially and to metamorphose in subtidal habitats.

In this study, we isolated bacterial strains from intertidal and subtidal biofilms and tested their effects on larval attachment in bioassay choice experiments. Taking into account that less than 5% of bacteria from the natural environment are culturable (Eilers et al., 2000) we used multi-species intertidal and subtidal biofilms developed under natural conditions in the larval choice bioassays. Multi-species biofilms were characterized by a PCR-based molecular fingerprinting technique (t-RFLP) (Liu et al., 1997), which combined restriction fragment analyses of a phylogenetic marker with automated capillary electrophoresis. This fingerprinting method is a relatively fast and easy way to provide a semi-quantitative "snapshot" of community diversity (Kitts, 2001), which permits a quick comparison of different microbial communities. In our study the following questions were addressed: (1) Do the mono-species bacteria isolated from intertidal and subtidal biofilms affect the attachment of *B. neritina*? (2) Does the age, the thickness of exopolysaccharide layer (EPS) and the composition of multi-species biofilm affect the larval attachment of *B. neritina*? (3) Does a subtidal biofilm induce larval attachment if it is moved into an intertidal region?

2. Material and methods

2.1. Site description

Biofilms were developed at the Hong Kong University of Science and Technology (HKUST) pier (22°19′ N, 114°16′E) located in Port Shelter Bay (South China Sea). Port Shelter Bay is a shallow (around 10 m deep) semi-enclosed bay with semidiurnal tides with average amplitudes of 2 m. During the study period, the salinity ranged from 31 to 34 ppt and the temperature ranged from 16 to 20 °C.

2.2. Bacterial isolation and formation of mono-species biofilms

Petri dishes were exposed to fouling for 28 days (February–March 2004) in the intertidal zone (the maximal depth = 1 m at high tide) and the subtidal zone (the maximal depth = 3 m at high tide). The intertidal Petri dishes were exposed to the air for approximately half of the tidal cycle, while the subtidal dishes were not exposed to the air. The dishes were swabbed with sterile cotton balls to collect culturable bacteria. The swabs from five dishes were combined and suspended in autoclaved and filtered (0.22 μm) seawater (AFSW). Bacterial samples in suspension were diluted 100 times by AFSW. Aliquots of 200 μl of each suspension were streaked onto nutrient agar (composed of 1.5% agar in AFSW, 0.3% yeast extract, 0.5% peptone) with replication ($n = 3$), and incubated at 30 ± 1 °C under a 15 h light/9 h dark photoperiod for 5 days. The bacterial colonies were then examined, using a dissecting microscope, for conspicuous characteristics such as colour, shape, size, surface topography, and the presence of granules. Distinguishable colonies were isolated and purified. The bacterial isolates were stored

in 50% glycerol at −80 °C. The bacterial strains were identified by comparing their 16S rRNA gene sequences as described by Lau et al. (2002). The closest match to the 16S rRNA gene sequence of the respective bacterium was retrieved by comparison with data from GeneBank (www.ncbi.nlm.nih.gov). Isolated bacterial strains were grown to the stationary phase in tubes with 0.5% bacteriological peptone (Oxoid, USA) and 0.3% yeast extract (Oxoid, USA) in AFSW at 30± 1 °C, under a illumination cycle of 15 h light/9 h darkness and used in the bioassay experiments.

To prepare the bacterial films, the cell pellets were washed and re-suspended in AFSW to an optical density of 0.8 (Abs) at a wavelength of 610 nm (spectrophotometer, Shimatzu, China). Five milliliters of the bacterial suspension was added to polystyrene dishes and then incubated for 3 h at room temperature to allow the attachment of bacteria. After the incubation, unattached bacteria were decanted and the dishes were rinsed several times with AFSW to remove unattached bacteria and then used for the double-dish bioassays.

2.3. Formation multi-species biofilms

Biofilms were developed on polysteryne Petri dishes (Falcon #1006) exposed to fouling in the intertidal zone (the maximal depth = 1 m at high tide) or the subtidal zone (the maximal depth = 3 m at high tide) during February–March 2004. In experiment 1, 140 dishes were attached vertically to nylon ropes and the upper end of each rope was tied to the pier. A weight was attached to the lower end of the rope to keep the rope taut. There were 5 replicated ropes exposed to fouling in the intertidal zone and 5 replicated ropes exposed in the subtidal zone. Dishes were covered by a mesh (diameter of pour = 200 μm) in order to prevent larval recruitment. Twenty-one dishes were removed from the ropes at 3 different times (7 days, 14 days and 28 days) and used for bioassays and bacterial community analyses (see below).

In experiment 2, intertidal and subtidal biofilms were developed correspondingly for 30 days and 2 days on Petri dishes. Twenty-five dishes were attached vertically to 5 nylon ropes exposed to fouling in the intertidal zone and the same number of dishes were attached to 5 nylon ropes exposed to fouling in the subtidal zone. At the end of the exposure period, 21 Petri dishes from each of the 2 zones were used for bioassays and biofilm analyses.

In experiment 3, biofilms were developed on 52 Petri dishes in the intertidal and subtidal zones for 30 days. Then 5 Petri dishes from each zone were used for the analysis of microbial communities; the position of other dishes was then changed, i.e., the dishes with developed subtidal biofilms were moved into the intertidal zone and vice versa. After 5 days, 21 dishes from each zone were removed from 10 ropes and used for biofilm analyses and bioassays.

2.4. Biofilm analysis

2.4.1. Enumeration of bacteria and diatoms in biofilms

Bacterial and diatom abundances on the dishes were determined prior to the bioassays. First, the microbial film on the surface of dish was fixed with a 4% formalin solution in autoclaved filtered (0.22 μm) seawater (AFSW). Second, 5 dishes were rinsed with AFSW and stained with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Ltd, Switzerland) at 0.5 μg ml^{-1} for 15 min. The number of bacteria in 5 randomly selected fields of view was estimated using an epifluorescence microscope (Axiophot, Zeiss, Germany; magnification 1000×; λ_{Ex} = 359 nm, λ_{Em} = 441 nm).

2.4.2. Estimation of exopolysaccharide layer thickness

During each experiment 3 Petri dishes were used for the estimation of the exopolysaccharide (EPS) thickness of the biofilms. Prior to the analysis, the biofilms on the Petri dishes were stained with 1 mg ml^{-1} solution of FITC-labeled concanavalin A (ConA-FITC, Sigma, USA) in AFSW for 30 min in the dark. Then, the dishes were rinsed with AFSW and examined with a confocal scanning microscope (C1 plus, Nikon, Japan). We used the argon laser line at 488 nm for scanning-excitation and a 515–565 nm band-pass filter to detect green-fluorescence emission signals. The alignments of the laser and monitor set-up were chosen according to the manufacturer's instructions and the settings of the photomultiplier were kept constant for all samples. Samples were observed at 1500× magnification. The EZ-C1 software was used to generate pictures of the biofilms and to measure the thickness of EPS at 5 points within one Petri dish.

2.4.3. Investigation of the bacterial composition of biofilms

We compared bacterial communities using terminal restriction fragment length polymorphism (t-RFLP) analysis (Liu et al., 1997). For this purpose, the entire surface area (one Petri dish = 19.625 cm^2) of three Petri dishes was completely swabbed with sterile cotton. Swabs from each gel were individually suspended in

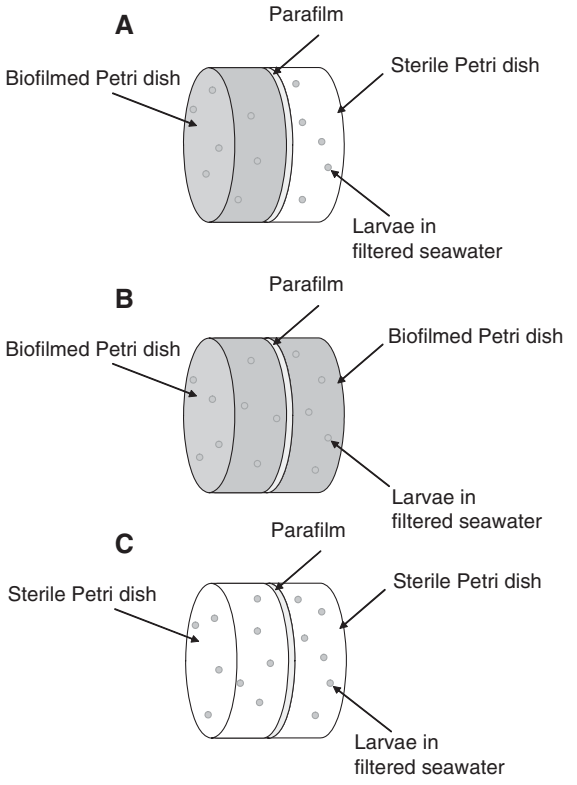

Fig. 1. Double-sided test containers used in our experiments. The containers consist of 2 Petri dishes joined by Parafilm® tape. Larvae and filtered seawater were injected by a syringe. Three different types of containers were used: (A) A sterile dish was paired with a dish with mono-species or multi-species biofilm. (B) Another container included a dish with intertidal biofilm paired with a dish containing subtidal biofilm. (C) For the control, two sterile dishes were attached to each other with the Parafilm®.

1ml extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M sodium chloride, 1% CTAB; at pH 8) in 2 ml micro-centrifuge tubes. For lysing, the samples were subjected to three cycles of freezing and thawing followed by 2 h incubation in 20% sodium dodecylsulfate (SDS) at 65 °C. The cotton swabs were removed and, after centrifugation (10,000 rpm × 5 min^{-1}), the total DNA in the supernatant was extracted and purified twice in a volume of 24:1 chloroform/isoamyl-alcohol, followed by precipitation in isopropanol at room temperature for 15 min. The precipitated DNA was washed with cold 70% ethanol and resuspended in 50 μl of autoclaved double-distilled water and frozen until use.

The PCR of the 16S rRNA genes (rDNA) of the bacterial community DNA was performed in a total volume of 25 μl containing 1 μl of DNA template, 250 μM each of desoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology, USA), 1 U of DNA taq polymerase (Amersham Biosciences, USA) and 0.8 μM of each universal primer: 341F forward (5′-CCTACGGGAGGCAGCAG-3′) and 926R reversal (5′-CCGTCAATTCCTTTRAGTTT-3′) (Amann et al., 1990; Lee et al., 1993). The 926R primer was labeled at the 5′end with 6-carboxy fluorescein (FAM) dye. PCR was performed at: 95 °C for 10 min; 35 cycles of 95 °C for 1 min; 55 °C for 1 min; 72 °C for 1 min; and 72 °C for 5 min. Amplification of DNA was verified by electrophoresis of 5 μl of PCR products in 1.5% agarose in TAE buffer.

Fluorescently labeled PCR products were purified with the Wizard® PCR preps DNA purification system (Promega, WI, USA) according to the manufacturer's protocol. Purified amplicons were digested with 20 U *Msp*I (Boehringer Mannheim Biochemicals, IN, USA) at 37 °C for 6 h. Ten microliters of digested products was mixed with 0.5 μL of internal size standard (ET550-R, Amersham Biosciences, UK). This mixture was denatured for 2 min at 95 °C and immediately chilled on ice

Table 1
Phylogenetic status of bacterial strains isolated from intertidal and subtidal biofilms

Code	Accession number in Genbank	Closest match in Genbank	Similarity (%)	Location
γ-Proteobacteria				
s1	AJ4211445	*Vibrio ichthyoenteri*	97	Subtidal
s2	AJ391203	*Vibrio* sp.	98	Subtidal
s3	AB049728	*Pseudoalteromonas atlantica*	97	Subtidal
i1	Y13830	*Vibrio pectenicida*	97	Intertidal
i2	AY373027	*Vibrio alginolyticus*	99	Intertidal
i3	AF007286	*Pseudoalteromonas peptidolytica*	97	Intertidal
i4	AF064637	*Vibrio* sp. NAP-4	98	Intertidal
i5	AY217772	*Vibrio* sp. LI-41	99	Intertidal
i6	AY245221	*Vibrio fluvialis*	96	Intertidal
Cytophaga–Flexibacter–Bacteroides				
i7	AB058905	*Flexibacter* sp.	96	Intertidal
s4	AB188791	Uncultured *Cytophaga* sp.	98	Subtidal
s5	AB032502	*Tenacibaculum mesophilum*	98	Subtidal
α-Proteobacteria				
s6	AB026194	Unidentified α-Proteobacterium	98	Subtidal
Firmicutes				
s7	X70316	*Exiguobacterium aurantiacum*	97	Subtidal
i8	AF447806	*Bacillus pumilus*	96	Intertidal

Table 2
Results of *G*-statistics used to investigate the preferences of *Bugula neritina* in attaching to mono-species biofilms in double dish bioassays

Bacteria tested	Treatment	Effect of biofilm	Log-likelihood ratio test	
			Total G_{adj}	*P* (*df*)
i1	Biofilm–sterile dish	Inhibitive	8.454	**<0.01 (1)**
i2	Biofilm–sterile dish	Inhibitive	39.215	**<0.001 (1)**
i3	Biofilm–sterile dish	Inhibitive	180.647	**<0.001 (1)**
i4	Biofilm–sterile dish	Inhibitive	95.988	**<0.001 (1)**
i5	Biofilm–sterile dish	Inhibitive	7.422	**<0.01 (1)**
i6	Biofilm–sterile dish	Inhibitive	219.233	**<0.001 (1)**
i7	Biofilm–sterile dish	No	0.278	>0.05 (1)
i8	Biofilm–sterile dish	Inhibitive	52.260	**<0.001 (1)**
s1	Biofilm–sterile dish	Inductive	54.356	**<0.001 (1)**
s2	Biofilm–sterile dish	No	3.109	>0.05 (1)
s3	Biofilm–sterile dish	Inductive	143.465	**<0.001 (1)**
s4	Biofilm–sterile dish	Inductive	31.622	**<0.001 (1)**
s5	Biofilm–sterile dish	Inductive	11.980	**<0.01 (1)**
s6	Biofilm–sterile dish	No	1.319	>0.05 (1)
s7	Biofilm–sterile dish	Inductive	28.450	**<0.001 (1)**
Control	Sterile dish–sterile dish	No	0.014	>0.05 (1)

The critical value for the log-likelihood test is G_{adj} (0.05, 1)=3.841. Data are pooled from 5 replicates. *P*-values are denoted with the degree of freedom (*df*). *G* is adjusted by Williams' correction. Values marked in bold mean that the attachment ratio was significantly different from the expected even distribution (1:1 ratio).

prior to capillary electrophoresis on a MegaBACE™ genetic analyzer (Amersham Biosciences, UK) operated in the genotyping mode. After the electrophoresis, the length of the fluorescently labeled terminal restriction fragments (t-RFs) was determined by comparison with internal size standards by using the "Fragment Profiler" software (Amersham Biosciences, UK). T-RFs that differed by less than 1 bp were considered identical.

2.5. Larval culture

Adult broodstocks of *B. neritina* were collected during February–March 2004 from pilings and floating rafts at

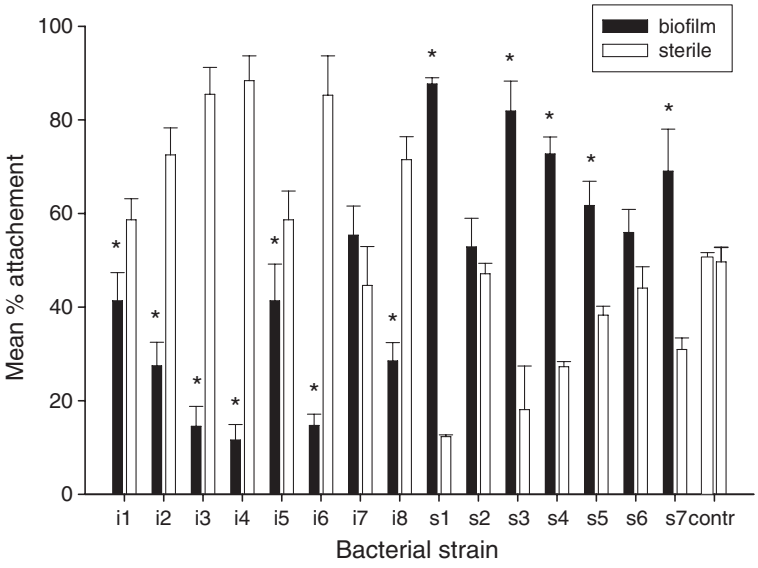

Fig. 2. The effect of mono-species bacterial films on larval attachment of *Bugula neritina*. Bacteria were isolated from an intertidal region (i1–i8) or from a subtidal region (s1–s7). Each test container contained biofilmed (dark bar) and sterile (open bar) polystyrene dishes. Data are means±S.E. of attachment in 5 replicated containers. Two sterile dishes attached to each other with the Parafilm® were used as a control (contr). Data that are significantly different according to a G-test ($p<0.05$; Table 2) are indicated by *.

Wong Shek Pier, Hong Kong (N22°25′, E114°20′). Larvae were obtained according to the method described by Bryan et al. (1997); and only newly (i.e., within 10 min) released larvae were included in the bioassays.

2.6. Bioassays

The choice bioassay was conducted in double-sided test containers as suggested by Harder et al. (2001), with the following modifications: each test container (Fig. 1) consisted of two Petri dishes (#1006, Falcon, USA) joined by the Parafilm®. One dish contained mono- or multi-species biofilms and the other dish was sterile, so that larvae in each container would have a choice of 2 surfaces types for attachment. Four treatments were tested. In 2 treatments, a sterile dish (referred to as "sterile") was paired with a biofilmed dish—either from the subtidal or the intertidal zone. For the control, we used two sterile dishes attached to each other with the Parafilm®. Finally, a fourth treatment included a dish with intertidal biofilm paired with a dish containing subtidal biofilm. Between 50 and 80 larvae were injected by a syringe into the container in 15 ml of AFSW. All containers were placed vertically and incubated for 1 h in darkness, as larval attachment has been shown to be induced by light (Wendt and Woollacott, 1999). After the incubation period, the test containers were opened and the seawater containing unattached larvae was transferred to a 25 ml container. The number of larvae attached to each side of 2 connected dishes, as well as the number of unattached larvae, was recorded using a dissecting microscope. All choice bioassays were performed with replicates ($n=5$).

2.7. Statistical analysis

The numbers of settled larvae were converted into percentages that were then arcsine-transformed. When no larvae attached, a value of $1/4n$ (n = number of larvae in a single replicate) was assigned to improve the arcsine transformation (Zar, 1999). The normality assumption was verified with the Shapiro–Wilk's test (Shapiro and Wilk, 1965). Attachment in test and control containers was compared to the null hypothesis of 50:50 distributions of attached larvae on either side of the container, using a replicated G-test for the goodness of fit (Zar, 1999). The G-value was calculated as a measure of heterogeneity among replicate containers within experimental type, as well as among experimental repeats. Homogeneous data sets were pooled and corresponding G-values were transformed by Willam's correction (Zar, 1999). The density of the bacteria was square-root transformed in order to ensure normality of variance. The linear correlations between the density of the bacteria, the density of the diatoms, the thickness of the biofilm, the age of the biofilm and larval attachment were analyzed by Pearsons correlation analysis. The t-RFLP patterns of different bacterial community DNA samples were used for the construction of the Bray–Curtis similarity matrix based on the total number of t-RFs observed in all samples compared with the presence or absence of these t-RFs in individual samples. The construction of a multi-dimensional scaling (MDS) plot to demarcate the similarity of microbial communities was performed using the PRIMER program (Plymouth Marine Laboratory, UK).

Table 3
Mean (±S.D., $n=3$) bacterial and diatom abundance and thickness of the biofilm exopolysaccharide (EPS) layer on Petri dishes used in the larval bioassay experiments

Treatment	Biofilm position	Biofilm age (day)	Bacterial density ($\times 10^3$ cells mm^{-2})	Diatom density (cells mm^{-2})	EPS thickness (μm)
Experiment 1	Intertidal	7	15.47±3.58	0	25.5±0.9
	Subtidal	7	19.78±3.29	69.5±19.0	85.4±7.3
	Intertidal	14	17.97±1.98	145.1±36.7	164.1±19.7
	Subtidal	14	23.37±5.08	436.7±78.9	216.8±15.4
	Intertidal	28	25.42±6.28	346.5±55.3	266.7±11.7
	Subtidal	28	25.92±2.65	695.9±125.8	388.0±7.2
Experiment 2	Intertidal	30	28.00±2.55	696.5±66.2	278.1±9.6
	Subtidal	2	2.53±0.30	2.9±2.9	31.6±1.5
Experiment 3	Intertidal—start	30	25.30±1.93	777.2±165.7	302.0±8.7
	Intertidal—end	30+ 5	25.53±1.89	772.1±94.8	360.3±7.6
	Subtidal—start	30	27.54±2.49	1179.9±176.1	379.7±14.1
	Subtidal—end	30+ 5	27.69±2.51	1128.7±150.0	325.0±9.7

In experiment 3, Petri dishes were exposed to fouling for 30 days and then the position of the dishes was changed for 5 days, i.e., the dishes with developed subtidal biofilms were moved into the intertidal zone and vice versa.

3. Results

3.1. Attachment of B. neritina on mono-species biofilms

Overall, 15 bacterial strains were isolated from the intertidal and subtidal biofilms (Table 1). In the tests with bacterial isolates from the subtidal biofilm, the larvae attached preferentially to the biofilm side of the dishes (Table 2; Fig. 2). In contrast, with the isolates from the intertidal biofilm, significantly more larvae attached to the "sterile" side than to the biofilmed side of the container. No such preferences were observed on the control unfilmed dishes with AFSW. The density of the bacterial strains varied from 20 to 30×10^3 cell mm^{-2} and did not affect larval attachment (data not shown).

3.2. Attachment of B. neritina on multi-species biofilms

3.2.1. Experiment 1

The biofilms used in this study were primarily composed of rod-shaped bacteria and diatoms, predominantly *Nitzschia* spp. and *Achnanthes* spp. The densities of bacteria on the plates from the intertidal zone increased from 1220 to 25,927 cells mm^{-2} during the period of the experiment (Table 3). The bacterial density was higher in the subtidal biofilms than in the intertidal biofilms. The density of the diatoms increased and reached about 500 cells mm^{-2} by the end of the experiments. There were 2–3 times more diatoms on the surfaces of the subtidal biofilms than on the surfaces of the intertidal biofilms. In our experiments, the lowest thickness of exopolysaccharide (EPS) layer in biofilms was found on 7-day-old intertidal biofilms and an EPS that was 15-fold thicker was observed in biofilms from the subtidal regions after 28 days from the start of the experiment. Generally, the thickness of EPS increased and reached its maximum by the end of the experiments.

After 7 days, 14 days and 28 days, the attachment of larvae in the test containers with biofilms differed significantly from a 50:50 distribution; larvae attached preferentially to biofilmed surfaces (Table 4; Fig. 3). Larvae attached primarily to the dishes with the subtidal bacterial film when intertidal and subtidal biofilms were tested together in the same containers (Fig. 3). Larvae attached in equal numbers to both sides of the control container, indicating that larvae had no preference for attaching to the right or left side of the container.

In both intertidal and subtidal biofilms, positive linear correlations were found between the diatoms abundance and the thickness of EPS, between the biofilm age and diatom densities, as well as between the biofilm age and EPS thickness (Fig. 4). In the intertidal biofilms, diatom density, EPS thickness, and biofilm age were positively correlated with larval attachment, while in the subtidal biofilms, all of these factors were negatively correlated with larval attachment. There was no correlation between bacterial abundance in the subtidal biofilms and the percentage of larval attachment.

Bacterial communities from the intertidal and subtidal zones differed from each other, as characterized by the absence of certain terminal restriction fragments (t-RFs) (i.e., ribotypes) and the presence of others (Fig. 5). For example, the presence of a t-RF at 314 bp was specific to all subtidal biofilms, while t-RFs at 315 bp

Table 4
Results of *G*-statistics used to investigate the preference of *Bugula neritina* in attaching to intertidal and subtidal biofilms in double dish bioassays

	Treatment	Log-likelihood ratio test	
		Total G_{adj}	*P* (*df*)
Experiment 1			
Day 7	Intertidal–sterile dish	121.785	**<0.001 (1)**
	Subtidal–sterile dish	573.763	**<0.001 (1)**
	Intertidal–subtidal	158.277	**<0.001 (1)**
	Sterile dish–sterile dish	0.065	>0.05 (1)
Day 14	Intertidal–sterile dish	89.436	**<0.001 (1)**
	Subtidal–sterile dish	99.754	**<0.001 (1)**
	Intertidal–subtidal	51.565	**<0.001 (1)**
	Sterile dish–sterile dish	0.522	>0.05 (1)
Day 28	Intertidal–sterile dish	60.970	**<0.001 (1)**
	Subtidal–sterile dish	149.833	**<0.001 (1)**
	Intertidal–Subtidal	32.889	**<0.001 (1)**
	Sterile dish–sterile dish	0.082	>0.05 (1)
	Sterile dish–sterile dish	0.073	>0.05 (1)
Experiment 2	Intertidal–sterile dish	102.183	**<0.001 (1)**
	Subtidal–sterile dish	252.334	**<0.001 (1)**
	Intertidal–subtidal	14.681	**<0.01 (1)**
	Sterile dish–sterile dish	0.252	>0.05 (1)
Experiment 3	Intertidal–sterile dish	89.295	**<0.001 (1)**
	Subtidal–sterile dish	180.478	**<0.001 (1)**
	Intertidal–subtidal	32.349	**<0.001 (1)**
	Sterile dish–sterile dish	0.623	>0.05 (1)

In experiment 1, biofilms were formed for 7 days, 14 days and 28 days, and then dishes were used for larval bioassay experiments. In experiment 2, Petri dishes with 30-day-old intertidal biofilms and 2-day-old subtidal biofilms were used. In the experiment 3, Petri dishes were exposed to fouling for 30 days and then the position of the dishes was changed, i.e., the dishes with developed subtidal biofilms were moved into the intertidal zone and vice versa. After 5 days, the dishes were used for bioassay experiments. The critical value for a log-likelihood test is G_{adj} (0.05, 1)=3.841. Data were pooled from the 5 replicates. *P*-values are denoted with the degree of freedom (*df*) and *G* is adjusted by Williams' correction. Values marked in bold mean that the attachment ratio was significantly different from the expected even distribution (1:1 ratio).

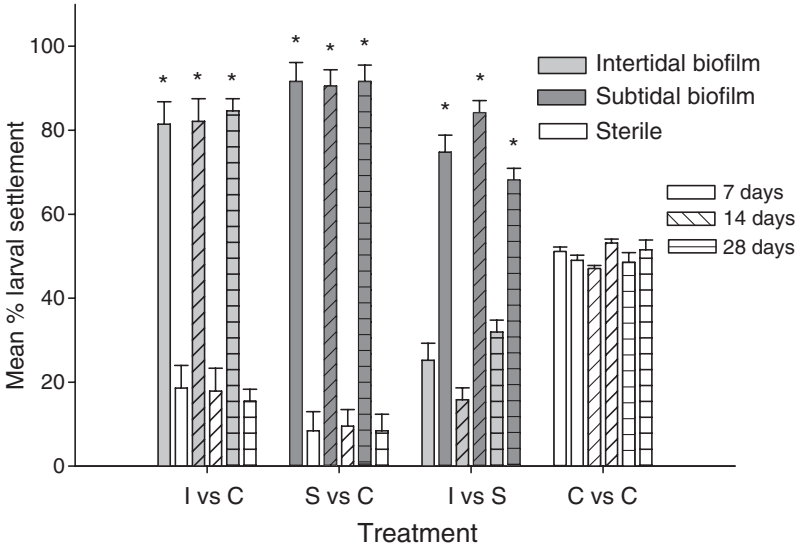

Fig. 3. The effect of multi-species biofilms on the larval attachment of *Bugula neritina*. Experiment 1. In two treatments, a sterile dish (open bar) was paired with a biofilmed dish for 7 days, 14 days or 28 days dish—either from the subtidal (S, dark grey bar) or the intertidal zone (I, grey bar). For the control (C vs. C), two sterile dishes were attached to each other with the Parafilm®. A fourth treatment (I vs. S) included a dish with an intertidal biofilm paired with a dish containing a subtidal biofilm. Data that are significantly different according to a *G*-test ($p<0.05$; Table 4) are indicated by *.

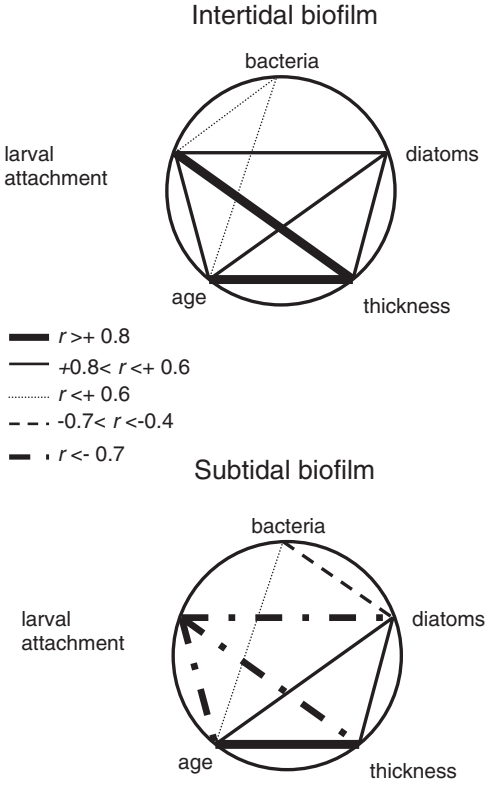

Fig. 4. Correlative relationships between attachments of larvae, biofilm quality (bacterial abundance, diatom abundance, and biofilm EPS thickness), and biofilm age. Data were obtained from experiment 1. Only statistically significant ($p<0.05$) correlations are shown.

and 423 bp were present almost in all intertidal biofilms except the 30-day-old biofilms. Some bacteria specifically were present only on Petri dishes at a certain tidal height at certain times during the incubation process. The t-RF at 199 bp was specific to 7-day-old subtidal biofilm and t-RFs at 259 bp and 261 bp were specific to 7-day-old intertidal biofilm. The t-RFs at 127 bp and 88 bp represented bacteria that were distributed in all the intertidal and subtidal biofilms.

The least number of bacterial ribotypes (20–28) were found in 7-day-old intertidal biofilms, while higher numbers of ribotypes (52–56) were found in 28-day-old intertidal and subtidal samples. Based on the presence (indicated by 1) or absence (indicated by 0) of a given t-RF in a pattern, the bacterial communities were separated into five groups (Fig. 5). The intertidal bacterial communities differed significantly from the subtidal bacterial communities during all periods of the experiment. Replicated bacterial communities from the same sampling date formed separate clusters in the MDS plot, suggesting that bacterial communities changed over time during our experiments (Fig. 5).

The combination of the t-RFLP technique with the TAP-t-RFLP program (RPD II Website http://rdp8.cme.msu.edu/html/TAP-trflp.html#program) provides preliminary information about relative identities of ribotypes within each bacterial community t-RF. In all treatments, the most abundant ribotypes (t-RFs of 126 and 88 bp lengths) were likely Planctomycetes (mostly

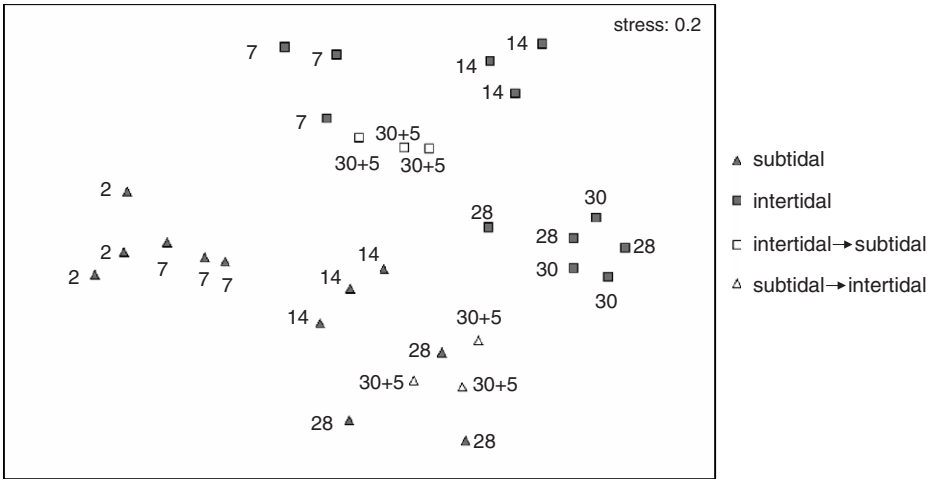

Fig. 5. Multi-dimensional scaling (MDS) ordination results of presence and absence of terminal restriction fragment in t-RFLP profiles of bacterial communities developed on Petri dishes ($n=3$) that were incubated in intertidal (inter) and subtidal (sub) regions for 2 days, 7 days, 14 days, 28 days and 30 days. In experiment 3 (marked as 30+5), Petri dishes were exposed to microfouling for 30 days and then the position of the dishes was changed, i.e. the dishes with developed subtidal biofilm were moved into the intertidal zone (inter-r) and *vice versa* (sub-r); the bioassays were conduced 5 days later. Numbers refer to the age of the biofilms in days.

Planctomycetes spp.), α-Proteobacteria (mostly *Roseobacter* spp., *Paracoccus* spp.), and Firmicutes (mostly *Halobacteroides* spp., *Haloanaerobacter* spp.), as well as Bacteroides (*Cytophaga* spp.). In the intertidal biofilm the most common ribotypes with t-RFs of 315 bp and 423 bp were probably Actinobacteria (mostly *Microbacterium* spp., *Corynebacterium* spp., *Brachybacterium* spp.), Firmicutes (mostly *Paenibacillus* spp.) and γ-Proteobacteria (mostly *Achromatium* spp.). The subtidal biofilms were dominated by a ribotype with a t-RFs length of 138 bp, which was likely α-Proteobacteria (*Blastochloris* spp.).

3.2.2. Experiment 2

The attachment of larvae in response to 30 days intertidal biofilms differed from the attachment of larvae in response to 2-day-old subtidal biofilms (Table 4, Fig. 6). Larvae predominantly attached to the biofilmed side of the Petri dishes compared to the clean side. Two-day-old subtidal biofilms attracted more larvae than did 30-day-old intertidal biofilms.

The least number of bacterial ribotypes (15–19) were found in 2-day-old subtidal biofilm communities. The highest numbers of ribotypes (37–41) were found in 30-day-old samples from both intertidal and subtidal zones. The lowest densities of bacteria and diatoms were found in 2-day-old subtidal biofilms, while in 30-day-old intertidal biofilm, their densities were 10 times higher (Table 3). The composition of 2-day-old subtidal communities was similar to that of 7-day-old subtidal bacterial communities; the composition of 30-day-old intertidal biofilms was similar to that of 28-day-old intertidal biofilms (Fig. 5).

3.2.3. Experiment 3

In the 3 days experiment, we tested the inductiveness of intertidal biofilms transferred into the subtidal zone and vice versa. The resulting biofilms influenced larval

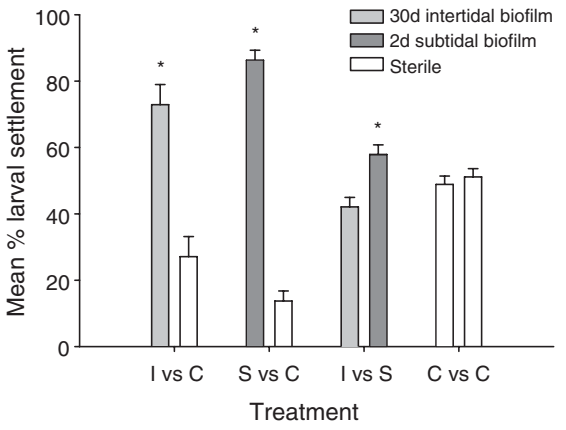

Fig. 6. The effect of multi-species biofilms on the larval attachment of *Bugula neritina*. Experiment 2: In two treatments, a sterile dish (open bar) was paired with a 30-day-old biofilm dish from the intertidal zone (I, grey bar) or with a 2-day-old biofilm dish from the subtidal zone (S, dark grey bar). For the control (C vs. C), two sterile dishes were attached to each other with the Parafilm®. A fourth treatment (I vs. S) included a dish with an intertidal biofilm paired with a dish containing a subtidal biofilm. Data are means ± S.E. of 5 replicates. Data that are significantly different according to a *G*-test ($p<0.05$; Table 4) are indicated by *.

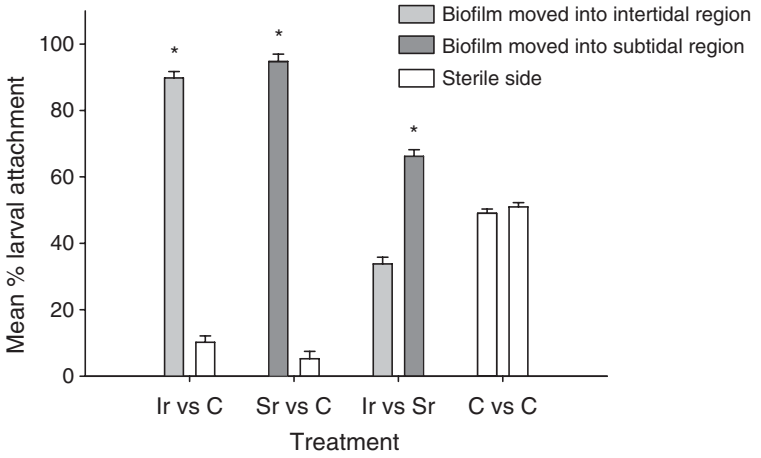

Fig. 7. The effect of multi-species biofilms on the larval attachment of *Bugula neritina*. Experiment 3: Petri dishes were exposed to microfouling for 30 days in both intertidal and subtidal habitats and then the position of the dishes was changed, i.e., the dishes with fully developed subtidal biofilms were moved into the intertidal zone (Ir) and vice versa (Sr). After 5 days, the dishes were removed from the ropes and used in bioassay for larval attachment. Each test container contained a biofilmed and a sterile dish attached to each other with the Parafilm®. Two attached sterile dishes were used as a control. The preference of larvae towards intertidal or subtidal biofilms was tested in the test container containing both of these biofilms (Ir vs. Sr). Data are means±S.E. of 5 replicates. Data that are significantly different according to a *G*-test ($p<0.05$; Table 4) are indicated by *.

attachment of *B. neritina* differently (Table 4; Fig. 7). As before, larvae predominantly settled on the biofilmed sides of the Petri dishes compared to the "sterile" sides. When intertidal and subtidal biofilms were combined together, larvae preferred to settle on the intertidal biofilms that have been moved into the subtidal region.

The bacterial and diatom densities, the dominant diatoms species, as well as the thickness of biofilms did not change much during this experiment (Table 3). After changing the position of the dishes to different tidal heights, the number of t-RFs decreased in both the intertidal and subtidal biofilms by 140% and 190%, respectively. The bacterial community profiles also changed dramatically, as indicated by the MDS plot (Fig. 5). T-RFLP profiles of the resulting intertidal (formerly subtidal) biofilms became similar to those of 28-day-old and 30-day-old intertidal biofilms. Analogously, the position of the resulting subtidal (formerly intertidal) biofilms became similar to those of 28-day-old subtidal biofilms.

4. Discussion

In this study, we found that bacterial isolates from intertidal and subtidal biofilms differentially affected larval attachment of *B. neritina*. Most of the isolates from intertidal biofilms inhibited attachment of larvae, while most of the isolates from subtidal biofilms induced larval attachment (Fig. 2). Previous studies also reported that different subtidal bacterial and diatom species affect larval attachment of *B. neritina* differentially (Brancato and Woollacott, 1982; Kitamura and Hirayama, 1987; Perry et al., 2001; Dahms et al., 2004a). These and our results suggest that the larvae of *B. neretina* can differentiate mono-species biofilms of bacteria.

In this study, we developed multi-species biofilms in intertidal and subtidal regions and tested their effects on larval settlement. At the start of the experiment, the thickness of EPS and the bacterial and diatom densities in the biofilms were low and reached their maximum values by the end of the experiments. Similar results have been demonstrated by other authors (Wieczorek and Todd, 1997; Oliver et al., 2000, Webster et al., 2004; Neu et al., 2005) and in our previous experiments (Qian et al., 2003; Lau et al., 2005).

The difference in environmental conditions between intertidal and subtidal regions may cause the formation of completely different microbial communities. The present study revealed that microbial communities from the intertidal zone were significantly different from those of subtidal communities during all time of the experiment (Fig. 5). In the intertidal biofilms, we commonly found ribotypes that likely are bacteria belonging to Actinobacteria, Firmicutes and γ-Proteobacteria groups, while a ribotype that likely indicated α-Proteobacteria was dominant in the subtidal biofilms. The presence of unique t-RFs in a particular tidal zone was not permanent during the experiments, suggesting that some bacterial species appeared only in young intertidal and subtidal biofilms, while others resided only in well-developed biofilms.

In our experiments, *B. neritina* larvae preferentially attached to multi-species biofilmed dishes in comparison with unfilmed ("sterile") dishes. This result was consistent with prior observations (Walters et al., 1999; Marshall and Keough, 2003; Dahms et al., 2004a). When given choices of biofilms from two tidal heights, the larvae in all cases primarily attached to subtidal biofilms. Our experiments demonstrated that bacteria but not diatoms drive larval attachment of *B. neretina* in subtidal biofilms. In experiment 1, larvae mostly attached to the biofilms with high diatom density, while in experiment 2, the attachment was higher on the subtidal biofilms even though the diatom density was very low. Our results suggest that larvae of the bryozoan *B. neritina* can differentiate multi-species biofilms from different habitats and prefer to attach to subtidal biofilms with specific bacterial composition.

Results of experiment 3, in which we developed intertidal and subtidal biofilms and then reversed their positions, suggested that bacterial and diatom density did not play an important role in *B. neretina* larval attachment, because densities of microorganisms did not change much with the change in location, while the composition of the bacterial communities and the inductivity of biofilms changed dramatically. For example, non-inductive intertidal biofilms that we then exposed to additional fouling in the subtidal zone became inductive to *B. neritina*. In contrast, subtidal biofilms that had been inductive did not induce the attachment of larvae after they were transferred to the intertidal zone. This experiment suggests that the biofilm composition plays an important role in the induction of larval settlement and inductive cues are not incorporated into the matrix of subtidal biofilms or are rapidly degradable.

How did the *B. neritina* larvae recognize subtidal biofilms in our experiments? It seems likely that the presence of specific microorganisms in subtidal biofilms, which produce compounds that can attract larvae or induce their settlement, play an important role in the substrate choice by *B. neritina*. Moreover, our experiments showed that changes in bacterial rather than in diatom composition led to the subsequent changes in the inductiveness of biofilms. This may be the case for invertebrate larvae from different taxonomic groups. For example, it has been shown that the presence of specific bacterial species in intertidal and subtidal biofilms determine larval attachment of the acorn barnacles *B. amphitrite* (Qian et al., 2003) and *B. trigonus* (Thiyagarajan et al., in press), which settled predominantly on intertidal and subtidal biofilms, respectively. Moreover, the presence of α-Proteobacteria and γ-Proteobacteria in biofilms stimulated larval settlement and metamorphosis of the hard coral *Acropora microphthalma* (Webster et al., 2004).

Overall, our experiments demonstrated that different microbial communities are associated with subtidal and intertidal surfaces. The differential response of *B. neritina* larvae to key components of biofilms, such as bacteria, may allow larvae to evaluate substrata and select subtidal surfaces.

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