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Attachment of *Balanus amphitrite* larvae to biofilms originating from contrasting environments

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ABSTRACT: This study examined the attachment response of *Balanus amphitrite* larvae to bacteria-dominated biofilms originating from 4 sites of varying environmental conditions in the intertidal region of subtropical Hong Kong waters and during 2 seasons (winter and summer), under both laboratory and field conditions. Using multiple fingerprinting techniques (terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis and fluorescence *in situ* hybridization), we observed differences in the bacterial community composition of biofilms originating from the 4 sites. These biofilm samples were used to study the linkage between spatial changes in bacterial communities of biofilms and larval choice at the time of attachment. It was hypothesized that cyprids can distinguish biofilms originating from habitats that support higher recruitment. Both laboratory and field multiple-choice bioassays demonstrated that cyprids preferred to attach on biofilms originating from habitats where recruitment, juvenile growth and survival were the highest, thereby accepting the hypothesis proposed. This study did not identify particular bacterial species or groups in biofilms that attract or inhibit larval attachment, but we could correlate site-specific variations in bacterial community composition with larval choice, whereas bacterial abundance in biofilms was less important in this regard. Overall, this study highlights the significance of site-specific variation in biofilms on larval recruitment and demonstrated the discriminative behavior of barnacle larvae to biofilms originating from contrasting environments in the intertidal region. Thus, attachment cues from biofilms may also play a significant role in generating spatial variation in larval recruitment.

KEY WORDS: *Balanus amphitrite* · Biofilms · Larval attachment · Bacterial community · Barnacle recruitment

INTRODUCTION

The barnacle *Balanus amphitrite* (Darwin) is one of the dominant species of the intertidal zone on rocky shorelines in tropical and sub-tropical regions. This species has been extensively used as a model organism to understand the mechanisms of cue detection during larval attachment, as well as to test the efficacy of anti-fouling compounds/paints (Rittschof et al. 1992). The life cycle of this barnacle includes a planktonic larval phase, followed by a benthic adult phase. After development from the planktotrophic nauplius stage into non-feeding cypris larvae, individuals are able to attach and commence a sessile benthic life. The choice of larval attachment site has been recognized as one of the main determinants of spatial–temporal variations in natural populations (see Keough & Downes 1982, Caffey 1985, Connell 1985, Gaines & Roughgarden 1985, Bertness et al. 1992, Underwood & Keough 2001) because, once attached, the individual must live in that area for the rest of its life. Thus, understanding the determinants of larval attachment is integral to benthic ecology.

Larvae of many barnacle species, including *Balanus amphitrite*, actively select a site for permanent attachment using external cues that trigger their metamor-

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phosis (reviewed by Rittschof et al. 1998). Biofilms exist on virtually all hard substrata, and are agglomerates of macromolecules, bacteria, diatoms, protozoans and fungi (Decho 2000). They also serve as an important cue for larval attachment (reviewed by Holmström & Kjelleberg 1994, Wieczorek & Todd 1998, Maki et al. 2000, Hadfield & Paul 2001, Steinberg et al. 2001). Among different biofilm components, bacteria are primarily responsible for larval attachment, particularly for barnacles (e.g. Maki et al. 1990, Wieczorek et al. 1995). Thus, numerous studies have examined the interaction between the bacterial component of the biofilm and larval attachment (see Qian et al. 2003 and references therein). These studies revealed that barnacle larval response (i.e. attachment induction or inhibition) is primarily determined by the abundance and community composition of bacteria in biofilms. For example, barnacle larvae can distinguish between biofilms of varying composition and preferentially attach on biofilms characteristic of their adult habitat, suggesting that microorganisms therein serve as an important signpost for larvae seeking suitable habitat (e.g. Strathmann et al. 1981, Thompson et al. 1998, Miron et al. 1999, Olivier et al. 2000). Recent studies have highlighted the significance of the bacterial community composition in biofilms on the modulation of larval attachment in barnacles (Qian et al. 2003, Thiyagarajan et al. 2006), corals (Webster et al. 2004) and polychaetes (Huang & Hadfield 2003, Lau et al. 2005). However, the linkage between natural biofilms, especially bacteria, and barnacle larval attachment remains largely unexplored, partially due to the fact that only a small fraction of the bacteria found in environmental samples is culturable (Ward et al. 1990). To overcome this problem, in the present study, we used different culture-independent DNA fingerprinting techniques to evaluate the role of bacterial community composition in biofilms in the modulation of larval behavior at the time of attachment. Although fingerprinting techniques have various biases (Dorigo et al. 2005), for example, due to the differential extraction of DNA from different bacterial populations (Martin-Laurent et al. 2001) and the preferential PCR amplification of numerically dominant DNA templates (Polz & Cavanaugh 1998), they have been widely used to obtain new insights into microbial diversity, although their application to understanding larval–biofilm interactions has not been fully explored. In the present study, two 16S-based fingerprinting techniques (terminal restriction fragment length polymorphism and denaturing gradient gel electrophoresis) were used to reach a more reliable description of bacterial community composition (Hoffmann et al. 2002, Lau et al. 2005).

It has long been speculated that environmental conditions (e.g. spatial–temporal variations in water quality) can have a tremendous effect on biofilms (e.g. Wieczorek et al. 1996, Chiu et al. 2005). However, the attachment response of larvae to spatial–temporal variations in biofilms has received little attention. For instance, field experiments by Wieczorek et al. (1996) highlighted changes in the larval attachment response to temporal variations in biofilms in subtidal habitats in Scottish waters. A series of field studies by Keough and co-workers showed that spatial changes in biofilms can affect larval recruitment in Australian waters (e.g. Todd & Keough 1994, Keough & Raimondi 1995, 1996). These pioneering works suggest that a range of marine invertebrate species respond to spatial–temporal changes in biofilms and, consequently, that the changes in biofilms can alter the recruitment pattern. Until now, the influence of spatial heterogeneity in water quality on biofilms and then on larval attachment in intertidal regions has not been explored. On the other hand, several studies have examined various physical and biological (excluding biofilms) determinants of the spatial distribution of barnacles (e.g. Wethey 1986, Pineda 1994, Sanford & Menge 2001, Thiyagarajan et al. 2005). In order to develop a complete understanding of the mechanisms of barnacle spatial population dynamics, it is important to examine the interaction among environmental heterogeneity, biofilms and larval attachment.

This study examined the attachment response of *Balanus amphitrite* larvae to biofilms developed at 4 sites of varying environmental conditions and during 2 seasons (winter and summer) under both laboratory and field conditions. Biofilms were characterized in terms of bacterial abundance and community composition. Specifically, this study tested the hypothesis that barnacle larvae can distinguish biofilms originating from habitats that support higher larval recruitment, juvenile growth and survival. In addition, we employed different fingerprinting techniques: (1) terminal restriction fragment length polymorphism (T-RFLP); (2) denaturing gradient gel electrophoresis (DGGE); and (3) fluorescence *in situ* hybridization (FISH) to understand the relationship between bacterial community composition in biofilms and larval attachment.

MATERIALS AND METHODS

Expt 1. Recruitment of barnacles on artificial substratum. The recruitment of barnacles at 4 sites in Hong Kong (PC: Peng Chau; OA: Old Airport; GI: Green Island; HKUST: The Hong Kong University of Science and Technology) in January 2005 (winter) and 3 sites (PC, OA and HKUST) in July 2005 (summer)

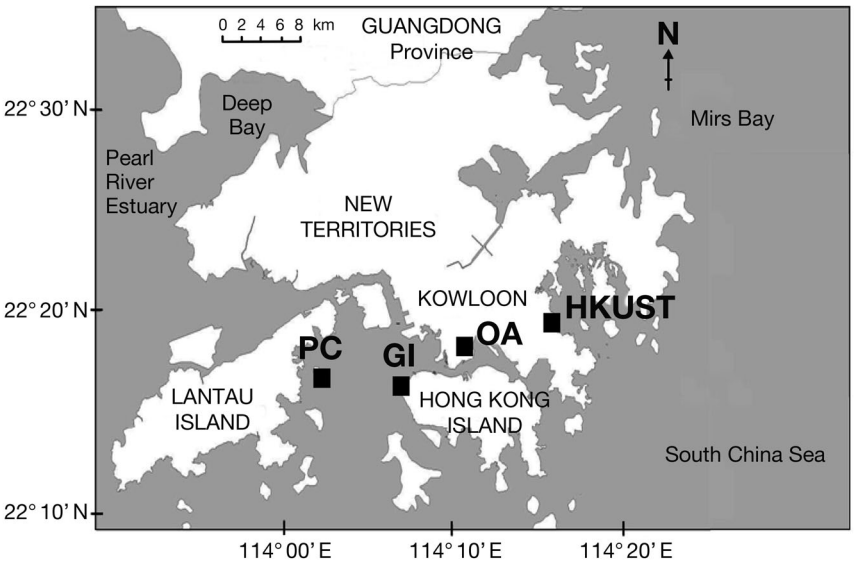

Fig. 1. A map of Hong Kong showing the sites used in this study to determine the recruitment of barnacles, develop biofilms and field larval attachment bioassay (PC: Peng Chau; GI: Green Island; OA: Old Airport; HKUST: The Hong Kong University of Science and Technology)

was monitored (Fig. 1). Replicate (n = 3), initially clean PVC plates (6 × 6 cm) were attached to a square mounting frame and submerged to mid-intertidal height (~1 m above the mean low water level) for 10 d. At each site, study locations with similar (for all sites) wave exposure and current speeds were chosen. The majority of barnacles recruited on the plates were *Balanus amphitrite*, and they were counted using a dissecting microscope.

Expt 2. Attachment response of barnacle larvae to biofilms originating from different habitats. In this experiment, biofilms were developed at 4 sites that differed in environmental conditions. Biofilms were characterized in terms of bacterial abundance and community composition. Subsequently, the attachment response of barnacle larvae to those biofilms was examined using laboratory and field bioassays. Experiments were repeated 2 times in both winter and summer.

Biofilm development sites: Biofilms were developed at 4 sites (PC, GI, OA and HKUST) during January to March 2005 (winter) and at 3 sites (PC, OA and HKUST) during July to August 2005 (summer) (see Fig. 1). A description for each site is available elsewhere (Thiyagarajan et al. 2005). Briefly, these sites are located along Victoria Harbor, Hong Kong (Fig. 1). The hydrography in the study area varies at spatial–temporal scales (for details see Shin & Ellingsen 2004). As a result of freshwater outflow from the Pearl River on the western side, an increasing trend in salinity concentration occurs from west to east during summer, but only minimal spatial variation occurs in winter. Human pollution (i.e. sewage discharge) is serious in the study area, particularly at OA. During the experimental period, the mean (±SD) water temperatures at the 4 sites were 18 ± 3 and 27 ± 2°C in winter and summer, respectively. In contrast, salinities at these 4 sites were markedly different only in summer. There was an oceanic zone at HKUST (~30 psu), a transition zone at OA (~25 psu) and an estuarine zone at PC (~20 psu). According to phytoplankton productivity (measured as chlorophyll *a*), the sites were ranked as follows: OA = PC (1.2 to 1.4 µg l^{-1}) > HKUST (0.6 µg l^{-1}), during summer. In contrast, according to the phytoplankton quality (measured as C:N ratio), the sites were ranked as follows: OA (~5) > HKUST (~6) > PC (~9.8), during summer. The same trend was also observed in winter. Most importantly, growth and survival of juvenile barnacles *Balanus amphitrite* differed significantly among these sites due to varying levels of food quality and quantity (see Thiyagarajan et al. 2005). According to the growth rates and survival of early juvenile barnacles, the sites were ranked as follows: OA > PC > HKUST. Therefore, this study area was chosen to test the hypothesis that the cypris larvae of barnacles can distinguish biofilms developed in habitats (sites) that support higher juvenile growth and survival.

Development of biofilms: Natural biofilms were developed on PVC plates (6 × 6 cm) according to Todd & Keough (1994). Plates were placed in a nylon mesh bag (mesh pore size = 110 µm) in order to prevent the attachment of invertebrate larvae. The bag was submerged for 10 d at mid-intertidal height (~1 m above the mean low water level) at 4 sites (PC, OA, GI, HKUST) and 3 sites (PC, OA, HKUST) in Hong Kong during winter and summer, respectively. Prior to bioassays, plates were dip-rinsed 10 times in 0.22 µm filtered seawater (FSW) to remove loosely attached bacteria. Bacteria remaining on the plate surface were regarded as an attached bacterial film.

Enumeration of bacterial abundance in biofilms: Before initiation of the larval bioassay, the abundance of bacteria in the biofilm was enumerated at a magnification of 1000× under an epifluorescent microscope after staining with DNA-binding fluorochrome 4′, 6-diamidino-2-phenylindole (DAPI). For each of the 3 replicates, 5 randomly chosen fields, corresponding to at least 1000 DAPI-stained cells, were counted.

Bacterial community composition in biofilms analyzed by T-RFLP: The bacterial community composition of biofilms used in Expt 2 (both winter and

summer) was analyzed by T-RFLP according to the procedures described in Qian et al. (2003). Briefly, the bacterial community DNA of biofilm samples (3 replicate biofilm plates per site) was extracted according to Zhou et al. (1996). The 16S rRNA genes (rDNA) of bacteria were amplified by PCR using the universal primers 968F and 1346R (Lau et al. 2005). Fluorescently labeled PCR products were digested with 20 U Msp I. The digested amplicons were mixed with an internal size standard (ET-550, Amersham). This mixture was denatured at 95°C and immediately chilled on ice before electrophoresis on a MegaBace genetic analyzer (Amersham). The lengths of the fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison with internal standards, using fragment profiler software (Amersham).

Bacterial community composition in biofilms analyzed by DGGE: The bacterial community composition in biofilms used in Expt 2 during summer was analyzed by DGGE according to the procedures described by Lau et al. (2005). Briefly, the 16S rRNA genes (rDNA) of bacteria were amplified by PCR using the universal primers 341F and 907R. DGGE of PCR products was performed in a DGGE-1001 system (C.B.S. Scientific). The PCR products were resolved in a 1 mm thick vertical gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of 45 to 75% denaturants. Electrophoresis (125 V for 18 h) was performed in 1× TAE buffer (Tris-acetate EDTA) maintained at 60°C. After that, DNA was visualized using Sybr Gold staining.

Laboratory bioassay: Adults of *Balanus amphitrite* Darwin were collected from the intertidal zone in Hong Kong (22° 19′ N, 114° 16′ E). Nauplius larvae, obtained from >100 adults, were reared to cypris stage in batch culture on a diet of *Chaetoceros gracilis* Schutt according to Thiyagarajan et al. (2003). Newly transformed cyprids were used immediately for bioassays. Six replicate multiple-choice chambers (polypropylene tanks: cyprids rarely attached on the container wall) were used. Each chamber accommodated a single replicate biofilm plate from each site, including the control plate (clean plate). Plates were secured on the walls using adhesive tapes. Then, 2 to 3 cyprids ml^{-1} of water were added. Chambers were kept at 28°C under a 15 h light:9 h dark photoperiod. The number of larvae attached on each plate was scored after 24 h.

Field bioassay: Field larval attachment bioassays were performed in summer at Old Airport (OA), which is located in the central region of Victoria Harbor (Fig. 1). In general, water at OA is organically polluted and has a high quantity of superior quality phytoplankton. The macro-invertebrate community in the intertidal region at OA is dominated by *Balanus amphitrite*. Biofilm plates were fixed to square mounting frames. Each frame held 16 plates in a 4 × 4 array according to the orthogonal Latin-square design, leaving 3 cm between adjacent plates. Initially clean plates served as controls. Each of the 4 plates (1 biofilm plate per site [PC, OA, HKUST] plus control) appeared exactly once in each of the 4 rows and in each of the 4 columns. Two replicate frames were used, which were retrieved after 18 h of immersion in the intertidal zone. The number of attached cyprids on each plate was scored under a dissecting microscope. Based on our visual examination, it was confirmed that the attached barnacles on experimental plates were *B. amphitrite*. The bacterial community composition in biofilms before and after the field larval attachment bioassay was analyzed by T-RFLP to understand the extent to which the native bacterial community of biofilms was altered at the end of the larval bioassay.

Expt 3. Stillwater attachment response of barnacle larvae to biofilms developed in 2 different habitats. Unlike the previous experiment, biofilms were developed on polystyrene Petri dishes and polystyrene plates. The bacterial community composition was analyzed using FISH. The larval attachment response to biofilms was examined using the double-dish bioassay method.

Development of biofilms: Natural biofilms were developed on polystyrene Petri dishes (FALCON, No. 1006) and polystyrene plates (7 cm × 2.5 cm, used for FISH analysis) according to Qian et al. (2003). Dishes and plates were placed in nylon mesh bags (mesh pore size = 110 µm) in order to prevent the attachment of invertebrate larvae. The bags were submerged for 10 d at mid-intertidal height (~1 m above the mean low water level) at 2 sites (OA and HKUST) in summer. Prior to bioassays, dishes were dip rinsed 10 times in FSW to remove loosely attached bacteria.

Bacterial community composition in biofilms analyzed by FISH: Biofilm plates were fixed on site using formaldehyde solution (3.7% vol/vol in FSW) and stored in a 1:1 mix of ethanol and 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) at –20°C until further processing. Before hybridization, fixed biofilm plates were washed with 1× PBS (phosphate-buffered saline) for 3 min, dehydrated in ethanol (50, 80 and 96%, 3 min each) and dried at room temperature. Hybridization experiments were conducted using the fluorescently labeled oligonucleotide probes listed in Table 1. A negative control probe (NonEUB338) was used to check for nonspecific hybridization. Hybridization buffer was prepared with 0.9 M NaCl, 20 mM Tris-HCl (pH 8), 0.01% SDS (sodium dodecyl sulphate) and a variable amount of formamide depending on the choice of the probe (Table 1). For hybridization with the probes BET42a, GAM42a, and HGC69a, equimolar concen-

Table 1. Oligonucleotide probes and formamide concentrations used in fluorescence *in situ* hybridization

Probe	Sequence	Specificity	Formamide (%)	Source
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	20	Amann et al. (1990)
ALF968	GGTAAGGTTCTGCGCGTT	*Alphaproteobacteria*	20	Manz et al. (1992)
BET42a	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	35	Manz et al. (1992)
GAM42a	GCCTTCCCACATCGTTT	*Gammaproteobacteria*	35	Manz et al. (1992)
CF319a	TGGTCCGTGTCTCAGTAC	*Cytophaga–Flavobacterium*	35	Manz et al. (1996)
HGC69a	TATAGTTACCACCGCCGT	*Actinobacteria*	25	Roller et al. (1994)
LGC354a, -b and -c	YSGAAGATTCCCTACTGC	Part of low-G+C gram-positive division	35	Meier et al. (1999)
NonEUB338	ACTCCTACGGGAGGCAGC	Bacteria (negative control probe)	20	Amann et al. (1990)

trations of the unlabeled competitor oligonucleotides BET42c, GAM42c, and HGC69c, respectively, were added to the hybridization mixtures to ensure hybridization specificity (Manz et al. 1992). Each biofilm plate was then placed in a humid chamber (50 ml FALCON No. 352070 polystyrene conical tube, with paper towel saturated with hybridization buffer) and incubated for 90 min at 46°C. Biofilm plates were subsequently washed for 15 min in 50 ml wash buffer prewarmed to 46°C. The wash buffer was prepared with 20 mM Tris-HCl (pH 8), 5 mM EDTA, 0.01% SDS and the concentration of NaCl corresponding to the formamide concentration used during hybridization (Table 1). All hybridization and washing steps were performed in the dark.

For microscopic analysis, biofilm plates were air dried and mounted with a drop (~25 µl) of antifading mountant supplemented with 1.5 µg ml^{-1} of DAPI solution (VETASHIELD, Vector laboratories, Inc.). Bacterial cells were counted at a magnification of 1000× under an epifluorescent microscope, equipped with a 100 W mercury burner and specific filter sets for DAPI (wide-band UV excitation, exciter filter BP330-385) and Cy3 (wide-band interference Green excitation, exciter filter BP520-550). Each microscopic field was first viewed with the Cy3 filter set before switching to the DAPI filter set, to avoid bleaching of Cy3 during the DAPI examination (Glöckner et al. 1999). Cells positive with FISH (under Cy3 filter), corresponding to the target bacterial group, appeared red, and cells positive with DAPI (under UV light), corresponding to all bacteria, appeared blue. For each probe and sample, 10 to 20 randomly chosen fields, corresponding to at least 1000 DAPI-stained cells, were counted.

***Balanus amphitrite* larval attachment bioassay:** The attachment response of cyprids to biofilms was investigated in a still-water choice bioassay (or double-dish bioassay) according to Harder et al. (2001). Briefly, the cyprids were placed within 2 polystyrene dishes that were joined and sealed using parafilm, named attachment vessels. Three treatments were included (1 dish from OA, paired with 1 dish from HKUST; 1 dish from OA, paired with 1 clean dish; 1 dish from HKUST, paired with 1 clean dish). The control vessels consisted of 2 clean dishes. For each combination, there were 6 replicate vessels, each receiving 80 to 100 cyprids (the attachment was independent of cypris density within this range) in FSW. Vessels were placed vertically in a tray and incubated for 24 h at 28°C under a 15 h light:9 h dark photoperiod. The dishes were separated after incubation, and the number of both attached and metamorphosed individuals on each dish was counted under a dissecting microscope.

Statistical analysis. All percentage and count data were subject to angular and log transformation, respectively, before statistical analysis (Zar 1999). In Expt 1, differences in mean recruitment among sites were analyzed using 1-way ANOVA and Tukey's multiple comparisons test. In Expt 2, differences in mean numbers of larval attachment among biofilm treatments (including control) in the laboratory bioassay were analyzed using Friedman's test, followed by nonparametric Tukey-type multiple comparisons tests (Zar 1999). For the field bioassay, effects of row, column, site (fixed factor) and replicate frames (random factor) on larval attachment were analyzed using a replicated Latin-square ANOVA. If the effects of row, column and site on larval attachment were not consistent among replicate frames, the numbers of attached larvae in each replicate frame were analyzed using 1-way Latin-square ANOVA. If a significant main effect was detected, Tukey's multiple comparisons test was used to determine the difference between the control and the sites. For the comparison of bacterial community composition in biofilms originating from different sites, peak patterns in T-RFLP chromatographs and band patterns in DGGE gels were transformed to binary characters (1 or 0 corresponding to the presence or absence of a given peak/band) using the Genetic Profiler and GelCompar II program packages, respectively. A similarity matrix was constructed based on the total number of T-RFs/bands observed in biofilm samples from all sites and the presence or absence of these T-RFs/bands at each site. For both T-RFLP and DGGE,

similarity matrices were used to construct non-metric multidimensional scaling (nMDS) plots to visually evaluate variations in the bacterial community composition among sites (Clarke & Warwick 1994). One-way analysis of similarities (ANOSIM) was used to analyze the difference in bacterial community composition of biofilms among sites. One-way ANOVA and Tukey's multiple comparisons test were used to analyze the differences in the abundance of bacterial cells in biofilms. In Expt 3, attachment response of cyprids to OA and HKUST biofilms was compared to the null hypothesis of 50:50 distribution of attached cyprids on either side of the vessel using replicated *G*-tests for goodness of fit. The *G*-value was calculated as a measure of heterogeneity among replicate chambers within the experiment. Homologous data sets were pooled, and corresponding *G*-values were transformed by Williams' correction using replicated *G*-tests for goodness of fit (Zar 1999). For the bacterial community composition in OA and HKUST biofilms, 1-way ANOVA and Tukey's multiple comparisons test were used to analyze the abundance of different groups of bacteria in biofilms.

RESULTS

Expt 1. Recruitment of barnacles on artificial substratum

In both winter and summer, significantly higher recruitment of *Balanus* sp. was found at OA than at the other sites (Fig. 2).

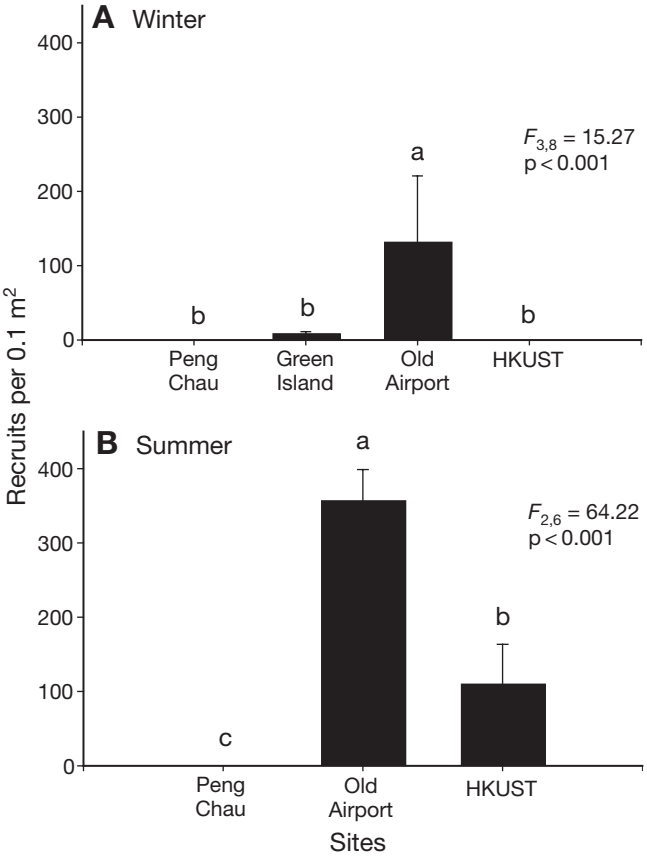

Fig. 2. *Balanus* sp. Recruitment of barnacles on artificial substrata at different sites in (A) winter and (B) summer. Data are expressed as means (+1 SD) of 3 replicates. Data that are significantly different at $\alpha = 0.05$ in Tukey's test are indicated by different letters above the bars. HKUST: Hong Kong University of Science and Technology. Green Island not sampled in summer

Expt 2. Attachment response of barnacle larvae to biofilms originating from different habitats

Enumeration of bacterial abundance in biofilms

In winter, both repeats showed that the bacterial abundance was significantly higher in GI and OA biofilms than in those at other sites (Fig. 3A). Similarly, in summer, the bacterial abundance was significantly higher in OA biofilms than in those at other sites, regardless of experimental repeats (Fig. 3B). There was no significant difference in the bacterial abundance between PC and HKUST biofilms, regardless of experimental repeats and seasons (Fig. 3A,B).

Bacterial community composition in biofilms

In T-RFLP profiles of biofilms (Fig. 4), bacterial community composition was semi-quantitatively represented by detected T-RFs, each of them could be associated with an operational taxonomic unit (OTU), whereas the position of OTUs could be used to distinguish bacterial communities qualitatively. According to the total number of OTUs, OA biofilms had a higher number of bacterial types (~42) compared to PC (~36), GI (~34) and HKUST (~30) in winter. Whereas, there was no obvious difference in the number of OTUs in summer samples (i.e. 32, 30 and 28 in PC, HKUST and OA biofilms, respectively). In both winter and summer samples, nMDS plots separated biofilm samples according to sites (Fig. 5) and indicated that the positions of some OTUs were different among samples. The ANOSIM showed a significant difference in bacterial community composition of biofilms among sites (winter—Repeat 1: Global R = 0.975, $p < 0.001$; Repeat 2: Global R = 0.509, $p < 0.001$; summer—Repeat 1: Global R = 0.897, $p < 0.01$; Repeat 2: Global R = 0.79, $p < 0.01$). In addition to T-RFLP fingerprinting, DGGE was used to compare the bacterial community composition in biofilms originating from different sites in summer (Fig. 6). Visual in-

spections of DGGE band patterns and ANOSIM results showed that bacterial community composition in biofilms developed at 3 different sites were different in terms of band position and number, and nMDS plots also separated biofilm samples according to sites (Fig. 6).

Larval attachment response to biofilms (laboratory bioassay)

In both winter and summer, there was a significant difference in larval attachment among biofilms originating from different sites (Fig. 7). For example, a higher number of larvae attached on biofilms at OA compared to those at other sites, regardless of season (winter and summer) (Fig. 7A,B). There was no clear trend in larval attachment pattern among biofilms from the other 3 sites (including control) in winter (Fig. 7A). On the other hand, there was no difference in larval attachment among biofilms from PC, HKUST and the control in summer (Fig. 7B).

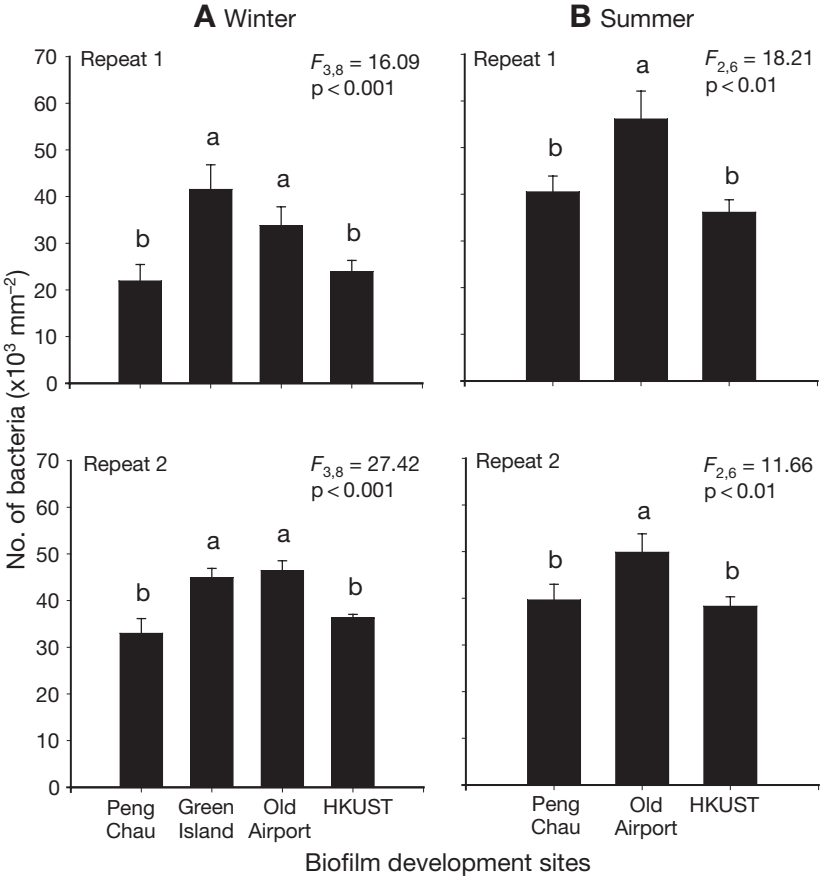

Fig. 3. Mean abundance of bacteria in biofilms originating from different sites in (A) winter and (B) summer. Data are expressed as means (+1 SD) of 3 replicates based on counts of 10 randomly chosen fields of view at 1000× magnification. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letters above the bars

Larval attachment response to biofilms (field bioassay)

The number of attached larvae differed significantly among biofilms originating from different sites and the control, and those differences were consistent among the replicate frames (Latin-square ANOVA, $p > 0.05$). The differences in mean larval attachment among biofilms from different sites were examined in each frame separately (Fig. 8). The number of larvae attached on biofilms at OA was significantly higher than that at the other sites. Interestingly, HKUST biofilms attracted slightly higher numbers of larvae than did PC biofilms and the control (Fig. 8), although these 2 biofilms had a similar abundance of bacteria (Fig. 3B). These differences in larval attachment among biofilms from different sites cannot be accounted for by the arrangement of plates in the frame, because neither the row nor column effect was significant (Latin-square ANOVA, $p > 0.05$).

During the bioassay period, we also examined the bacterial community composition in biofilms (using T-RFLP) before and after exposure to the field for larval attachment. Overall, the results showed that exposure of biofilms for <18 h in the field did not markedly change the native bacterial community composition in the biofilm (data not shown), which is in agreement with our previous experiments (Hung et al. 2005). For example, PC biofilms had 32 ± 5 OTUs before the bioassay, and after 18 h of exposure in the field both the number and position of the OTUs remain highly similar.

Expt 3. Still-water attachment response of barnacle larvae to biofilms developed in 2 different habitats

Comparison of bacterial community composition in OA and HKUST biofilms (FISH)

Probe EUB338 was used to determine the abundance of bacteria that could be detected by FISH (i.e. relative abundance of hybridizing bacteria). The bacterial abundance relative to total cell counts ranged from 86.3 to 88.2% and 89.4 to 90.4% in OA and HKUST biofilms, respectively. Signals due to non-specific probe binding or autofluorescence were determined by the negative control probe NonEUB338, with bacterial abundance relative to total cell counts being 8.2 and

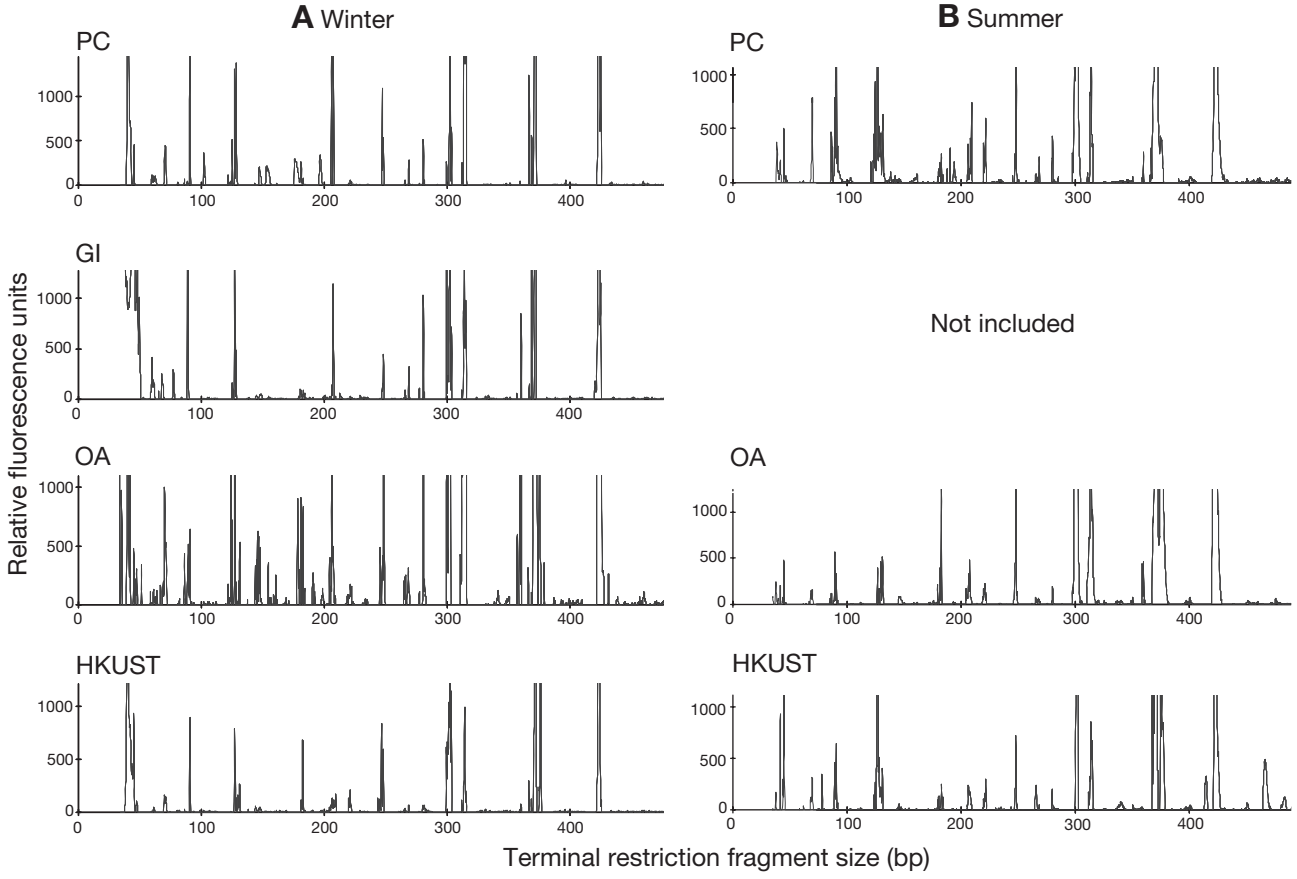

Fig. 4. T-RFLP profiles of biofilms originating from different sites in (A) winter and (B) summer. Since all 3 replicate samples were clustered in 1 group (see Fig. 5), the T-RFLP profile of 1 replicate sample (selected randomly) is shown. See Fig. 1 for site abbreviations

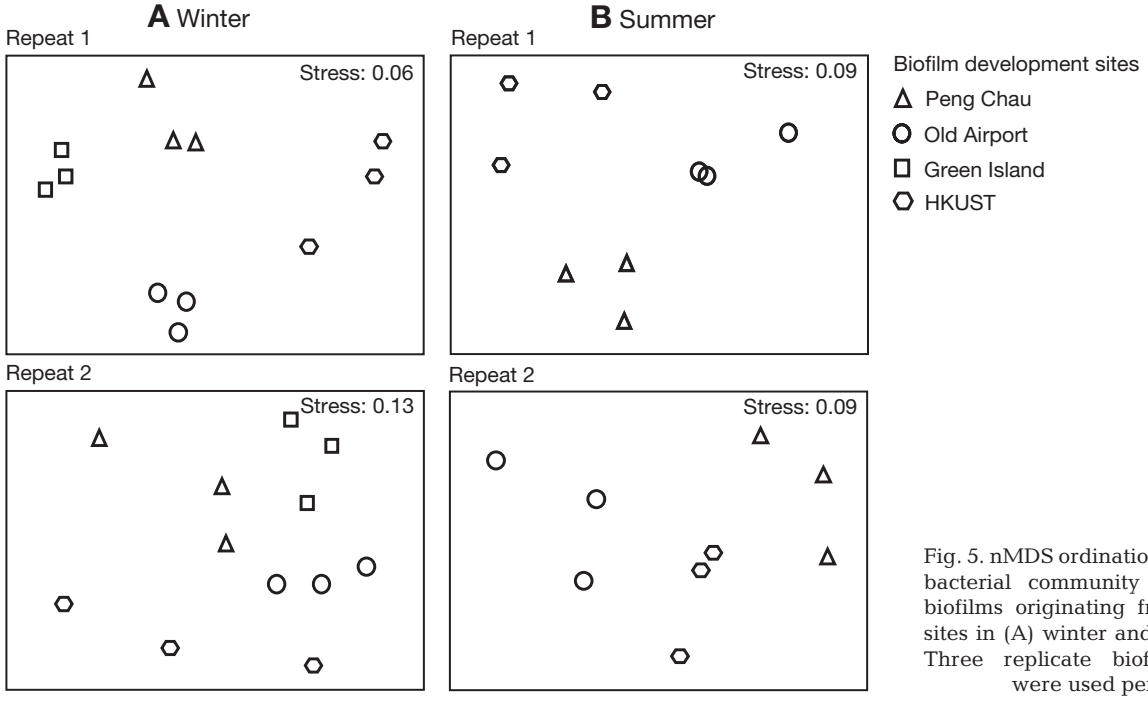

Fig. 5. nMDS ordinations comparing bacterial community structure in biofilms originating from different sites in (A) winter and (B) summer. Three replicate biofilm samples were used per site

7.0% in OA and HKUST biofilms, respectively. In OA biofilms, the abundance of *Gammaproteobacteria* was significantly higher than that of other bacterial groups (Fig. 9A), with bacterial abundance relative to total cell counts being 50.8 ± 10.3%. In HKUST biofilms, both *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant groups (Fig. 9B), with bacterial abundance relative to total cells counts being 42.6 ± 11.4 and 40.8 ± 10.8%, respectively. According to the nMDS plot, biofilms clearly separated into 2 groups according to sites (stress value: 0.01).

Larval attachment response to biofilms (laboratory bioassay)

In the paired dishes between OA biofilms and unfilmed dishes, cyprids attached preferentially to OA biofilmed surfaces and strongly avoided unfilmed dishes (G = 374.65, $p < 0.001$; Fig. 10). In the paired dishes between HKUST biofilms and unfilmed dishes, cyprids attached preferentially to HKUST biofilmed surfaces and strongly avoided unfilmed dishes (G = 337.71, $p < 0.001$). On the other hand, in the paired dishes between OA biofilms and HKUST biofilms, cyprids attached preferentially to OA biofilmed surfaces (G = 59.06, $p < 0.001$). In the paired control dishes, cyprids showed no preference and attached with equal frequency to both unfilmed dishes (G = 2.36, $p > 0.1$).

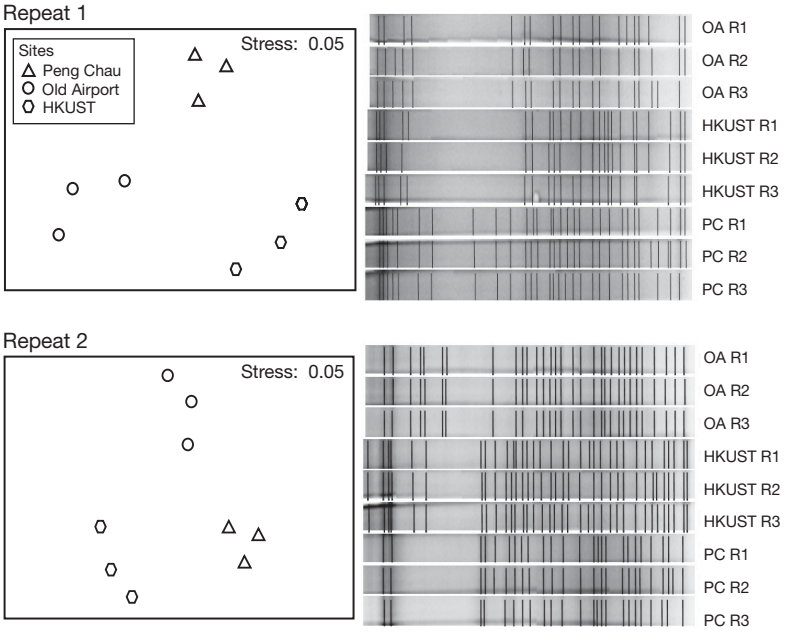

Fig. 6. nMDS ordinations (left panels) and DGGE profiles (right panels) comparing bacterial community structure in biofilms originating from different sites. Three replicate (R1 to R3) biofilm samples were used per site. See Fig. 1 for site abbreviations

DISCUSSION

This is the first study to investigate the attachment choice of barnacle larvae to biofilms originating from contrasting environments in the intertidal region. It was hypothesized that larvae can distinguish biofilms developed in habitats that support higher recruitment. Both laboratory and field multiple-choice bioassays demonstrate that larvae prefer to attach on biofilms originating from habitats where juvenile growth and survival are the best, thereby supporting the hypothesis proposed. The molecular fingerprinting techniques used have allowed a comparison of habitat-specific changes in bacterial community composition in biofilms and larval choice.

Bacterial community composition in biofilms

Biofilms used in this study had only few micro-algae (~1 to 20 cells mm^{-2} $site^{-1}$), probably due to the tidal height at which biofilms were developed and the mesh that was used to prevent the recruitment of invertebrates during biofilm development. Bacteria were most abundant in biofilms; therefore, their abundance and community composition were examined using epifluorescent microscopy and molecular fingerprinting techniques, respectively. In both winter and summer, OA and GI biofilms had a slightly higher number of bacteria (~40 to 50 × 10^3 cells mm^{-2}) than PC and HKUST biofilms. As we expected, differences in bacterial community composition in biofilms were observed among sites, regardless of fingerprinting techniques (see Figs. 4, 5, 6 & 9). Species richness did not differ greatly among sites (determined according to the number of T-RFs in TRFLP and of bands in DGGE), but species diversity (relative position of T-RFs and bands) differed dramatically. Although identity of either T-RFs in the TRFLP profile or bands in the DGGE was not revealed in this study, *in situ* analysis using a comprehensive suite of rRNA-targeted probes visualized individual cells within the *Alphaproteobacteria* and *Gammaproteobacteria*, as well as within the *Cytophaga–Flavobacterium* group, as major parts of the bacterial community in biofilms. In OA biofilms, *Gammaproteobacteria* were present predominantly (>50%), whereas both *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant groups in HKUST biofilms (see Fig. 9). Our obser-

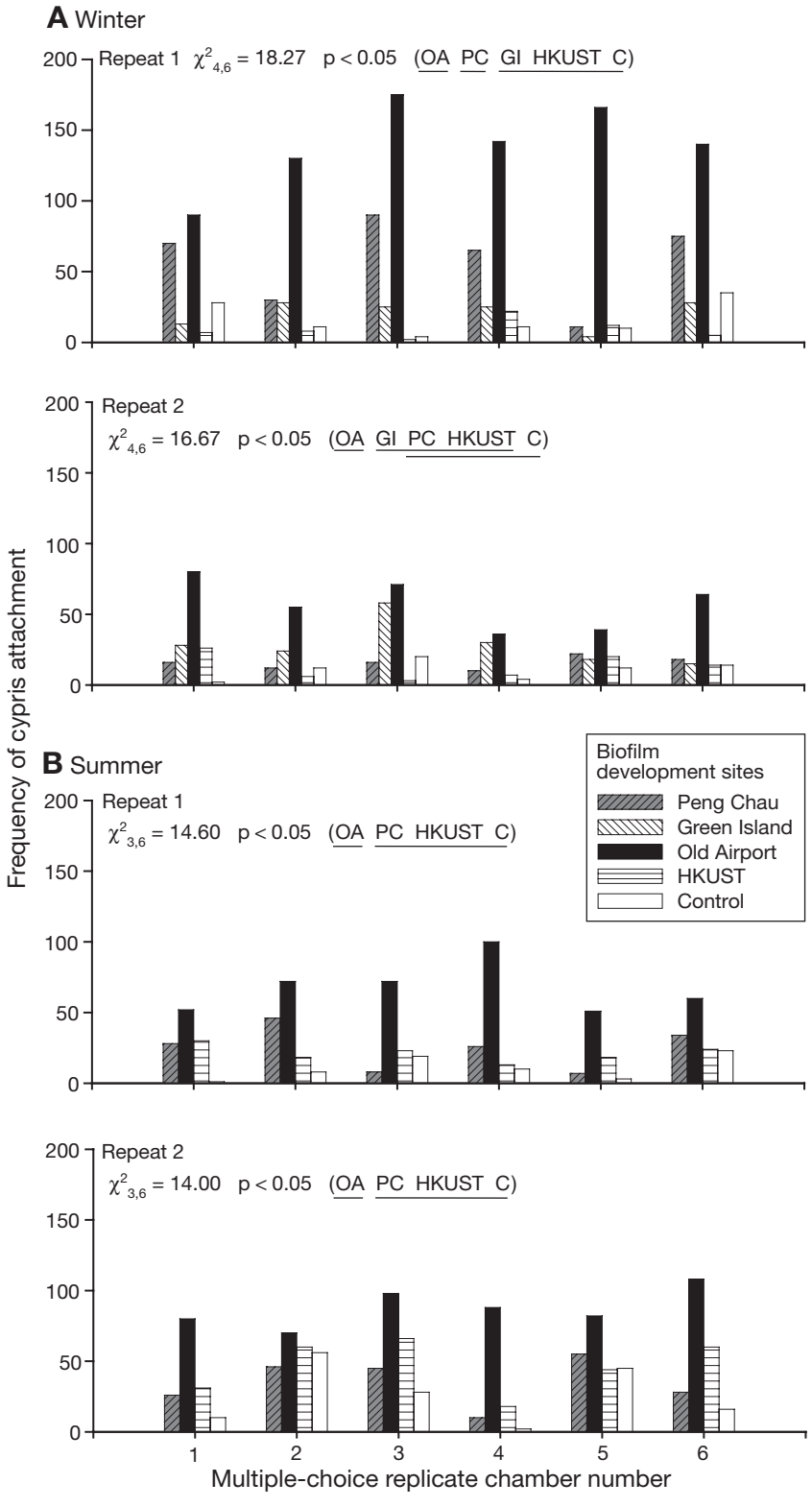

Fig. 7. *Balanus amphitrite*. Multiple-choice bioassay: attachment response of *B. amphitrite* cyprids to biofilms originating from different sites in (A) winter and (B) summer in a multiple-choice bioassay chamber. In Tukey-type multiple comparisons test, values are arranged from left to right in descending order, and those not significantly different ($\alpha = 0.05$) are connected by line. C: control (initially clean dish). See Fig. 1 for site abbreviations

vations of bacterial community dynamics with respect to different environmental conditions corresponded well with the results of our previous study (Chiu et al. 2005). The observed differences in bacterial community in biofilms among sampled sites are likely associated with the prevailing contrasting environment. For example, OA is subject to sewage pollution, PC is strongly influenced by input of riverine water from the Pearl River, and HKUST has strong influence from the South China Sea.

Larval choice to biofilms originating from contrasting environments

According to our multiple-choice attachment bioassay results, larvae discriminately attached and metamorphosed on biofilms that originated from contrasting environmental conditions. For instance, larvae attached in significantly higher numbers on biofilms from OA, a highly productive site, than on biofilms from GI, PC and HKUST. Although a significantly higher number of larvae attached on HKUST biofilms than on PC biofilms in the field bioassays, there was no difference in larval attachment between these sites in the laboratory bioassays conducted during summer. Differential attachment response of barnacle larvae to biofilms originating from different tidal heights (Miron et al. 1999, Olivier et al. 2000, Qian et al. 2003, Thiyagarajan et al. 2006) has been reported in previous investigations, but this is the first experimental demonstration of preferential attachment of larvae to biofilms originating from different environmental conditions in the intertidal region. Examination of bacterial abundance in biofilms revealed that the observed preferential attachment response could not be accounted for by bacterial abundance, which is in agreement to previous studies (e.g. Lau et al. 2005 and references therein). Analysis of bacterial community composition in biofilms suggests that a particular bacterial group and/or bacterial species may be involved. Other authors have also emphasized the role of biofilm composition on larval attachment (reviewed by Hadfield & Paul 2001), but the exact mechanism for detec-

tion of variations in biofilms by larvae is unknown. In any case, the present study provides strong evidence to support the argument of Keough & Raimondi (1995) that spatial variation in larval recruitment may be a function of variability in biofilms.

Relationship between larval choice and bacterial community composition in biofilms

The importance of bacteria in biofilms for the induction or inhibition of invertebrate larval attachment has been hypothesized for many years. Bacterial films on hard substrata can serve as larval inductive and/or inhibitive cues depending on their composition, which can be inferred from several laboratory and field studies. For example, biofilms composed of *Vibrio* spp. (Aveline et al. 1993), *Deleya marina* (Maki et al. 1988, O'Connor & Richardson 1998), *Roseobacter* sp. (Lau et al. 2003) and biofilms originating from low-shore habitat (e.g. Qian et al. 2003) were reported to inhibit the attachment of barnacle *Balanus amphitrite* larvae. On the other hand, biofilms originating from adult habitats (e.g. Olivier et al. 2000), and mono-species films of several bacterial species that were isolated from conspecific adults (Khandeparker et al. 2003) significantly induced the attachment of more barnacle *B. amphitrite* larvae relative to the control. Although only few studies have examined the role of natural biofilms on larval attachment under field conditions, existing data suggest that larvae of several marine invertebrates can discriminate between biofilm compositions that origi-

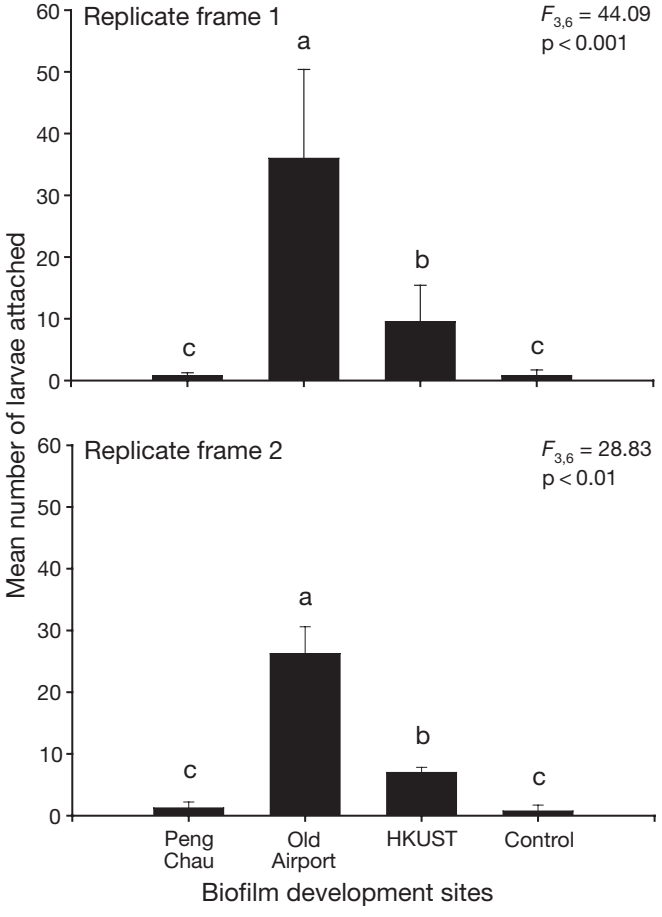

Fig. 8. *Balanus amphitrite.* Field bioassay: attachment response of *B. amphitrite* cyprids to biofilms originating from different sites. Data are expressed as means (+1 SD) of 4 replicates. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letters above the bars. Control: initially clean dish

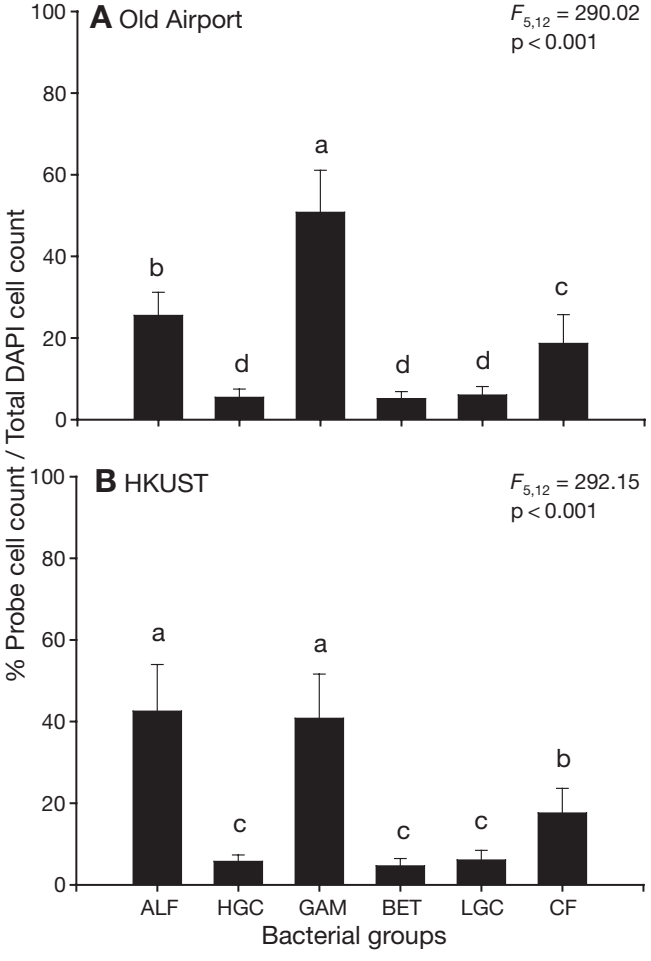

Fig. 9. Relative percentages of probe-positive cells with respect to counterstained positive cells for all samples and probes in (A) Old Airport and (B) HKUST biofilms. Data are expressed as means (+1 SD) of 3 replicates based on counts of 10 to 20 randomly chosen fields of view at 1000× magnification. Data that are significantly different at α = 0.05 in Tukey's test are indicated by different letters above the bars (ALF: *Alphaproteobacteria*; HGC: *Actinobacteria*; GAM: *Gammaproteobacteria*; BET: *Betaproteobacteria*; LGC: part of low-G+C gram-positive division; CF: *Cytophaga–Flavobacterium*)

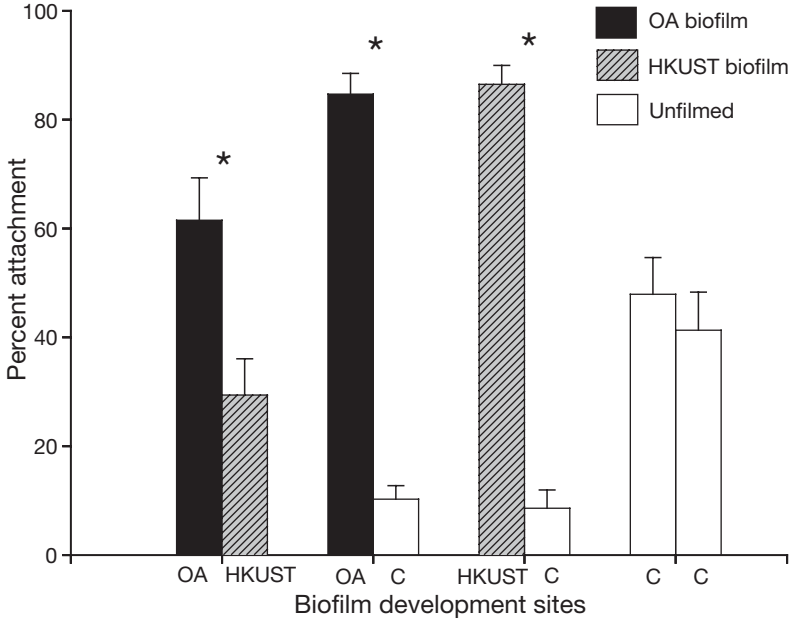

Fig. 10. *Balanus amphitrite*. Double-dish bioassay: attachment response of *B. amphitrite* cyprids to biofilmed or unfilmed surfaces, 2 biofilmed surfaces from different sites in the paired dishes. The control treatment had unfilmed surfaces on both sides. Data are expressed as means (+1 SD) of 6 replicates. Asterisk indicates a significant difference in log-likelihood ratio analysis. C: control (initially clean dish). See Fig. 1 for site abbreviations

nate from different subtidal habitats (Todd & Keough 1994), tidal heights (Miron et al. 1999, Olivier et al. 2000, Thiyagarajan et al. 2006), biofilms of different ages (Keough & Raimondi 1995) and biofilms developed during different seasons (Wieczorek et al. 1996). All this evidence, including the present study, supports the argument that the bacterial community composition in biofilms plays a predominant role in the modulation of larval behavior at the time of attachment. Although different molecular techniques employed to characterize bacterial community composition in biofilms have improved our understanding of the relationship between biofilm composition and larval attachment, the source of larval attachment cues in biofilms remains unknown.

Can larvae distinguish biofilms originating from habitats that support high recruitment?

The answer is yes. Barnacle larvae preferentially attached on biofilms originating from a habitat that supported higher recruitment, juvenile growth and survival (i.e. OA biofilms). For example, according to juvenile growth and survival, the sites were ranked as follows: OA > PC > HKUST (Thiyagarajan et al. 2003). The results of this study also ranked those sites in the same order based on larval preference to biofilms. Such preference for OA biofilms was not only demonstrated in laboratory bioassays but also in the field. As we expected, larvae did not prefer PC biofilms (see Fig. 4), in which juveniles grew slowly and suffered higher mortality (Thiyagarajn et al. 2005). We observed a similar trend in both winter and summer, despite substantial seasonal variation in biofilm composition. Numerous studies have demonstrated that larvae of marine invertebrates may choose to settle in favorable habitats, and this process may have established the patterns observed in the field (reviewed by Underwood & Keough 2001). The potential importance of biofilm as one of the associated cues for larval attachment (see 'Introduction') has been documented in several studies, but only a few studies have addressed the role of biofilms during the habitat selection process by larvae (see Wieczorek & Todd 1998). Early field studies correlated site-specific variation in biofilm and larval choice (e.g. Keough & Raimondi 1996); however, biofilm composition was not determined in those studies. Our laboratory bioassay results, complemented by field experiments, accentuated, not only the significance of bacterial community composition in biofilms on larval habitat selection, but also showed that larvae may use biofilm cues to locate a suitable habitat at the time of attachment.

Though barnacle larvae were capable of attaching on the artificial plates (acrylic and polystyrene) used in this study, potential interactions between substratum (e.g. natural rocks) and biofilm are known to affect the attachment choices of marine larvae (e.g. Neal & Yule 1994, Faimali et al. 2004). Our results are, however, valuable in demonstrating the role of spatial variation in the bacterial community composition in biofilms at the time of larval attachment. Thus, results of this study suggest that small-scale variations in biofilms may contribute significantly to the variation in recruitment at small spatial scales. But the relative importance of larval supply, attachment cues such as biofilms and post-settlement performance on recruitment successes of invertebrates remain to be tested. All these factors are likely to be important in establishing recruitment patterns.

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