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Recruitment of the sea urchin *Heliocidaris erythrogramma* and the distribution and abundance of inducing bacteria in the field

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ABSTRACT: Bacterial biofilms induce settlement in many marine invertebrate organisms. However, there remains a lack of understanding regarding the specific components of biofilms *in situ* that are responsible for the high inducing activity of some biofilms. The correlation of field recruitment patterns with laboratory settlement preferences for particular biofilms is also not well understood. We investigate if recruitment of the sea urchin *Heliocidaris erythrogramma* is related to the field distribution of highly inducing bacterial genera. Newly recruited sea urchins were found in highest numbers on the coralline algae *Amphiroa anceps* and *Corallina officinalis* and in low numbers on rubble and the brown alga *Ecklonia radiata*. Most bacterial genera that induce high levels of larval settlement of *H. erythrogramma* had previously been identified by 16S rRNA sequencing as *Pseudoalteromonas*, *Vibrio* or *Shewanella*. Oligonucleotide probes were developed for each of these genera to enable their quantification in environmental samples via catalysed reporter deposition-fluorescence *in situ* hybridisation (CARD-FISH). The probes were applied to biofilms on several algal species from the adult urchin habitat. All 3 genera were found in biofilms on all species of macroalgae examined. *Pseudoalteromonas* were found in highest numbers on the coralline alga *C. officinalis* and in higher numbers on red algae in comparison to brown algal species. *Shewanella* strains were also found in highest densities on *C. officinalis.* This is the first example demonstrating that bacteria that are able to induce larval settlement in the laboratory are also present in the field in the juvenile recruitment habitat.

KEY WORDS: Larval settlement · FISH · Probe development · Sea urchin · Recruitment · *Heliocidaris erythrogramma*

INTRODUCTION

A wide variety of invertebrate larvae settle and metamorphose in response to bacterial biofilms (reviewed by Wieczorek & Todd 1998), including larvae of sea urchins (Pearce & Scheibling 1991), polychaetes (Unabia & Hadfield 1999), corals (Webster et al. 2004), barnacles (Thompson et al. 1998), bivalves (Weiner et al. 1989), starfish (Johnson & Sutton 1994), molluscs (Bao et al. 2007), tunicates (Wieczorek & Todd 1997), jellyfish (Schmahl 1985) and bryozoans (Keough & Raimondi 1995). Biofilms are also important for the settlement of some algal zoospores (Patel et al. 2003). Importantly, it appears that particular bacterial strains within mixed environmental communities may be responsible for the high inducing ability of some biofilms. Several studies have isolated a variety of bacterial species from marine biofilms on surfaces such as rocks (Patel et al. 2003), algae (Johnson et al. 1991), plastic or glass (Unabia & Hadfield 1999) submerged in

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seawater for various time periods. From these mixed communities, single-species biofilms of some bacteria are able to induce high rates of settlement, while others have little or no effect (e.g. Lau et al. 2002, 2003, Webster et al. 2004).

Ultimately, the goal of such biofilm/larval settlement studies is to understand how the distribution and abundance of inducing species of bacteria relate to recruitment of larvae in the field. The few field studies that have been done (Keough & Raimondi 1995, 1996) proposed that larval settlement varies in response to different types of biofilms, as based on general features of the biofilm (e.g. biofilm age) without characterising their species composition. Patterns of larval settlement in the field have not yet been related to the more detailed taxonomy of bacteria that induce settlement in the laboratory or the composition of biofilms that exist on recruitment surfaces in the field. A recent and compelling study correlated site-specific variations in bacterial community composition with larval choice of barnacles (Hung et al. 2007); however, this study did not identify particular bacterial species or groups in biofilms that attract or inhibit larval attachment. At present, there remains a challenge to demonstrate that the distribution of recruits in the field is actually linked with the distribution of bacteria that are able to induce metamorphosis in the laboratory. Another important question not yet addressed is the specificity of highly inducing bacterial strains to surfaces in the field. For example, there is a growing body of evidence that suggests that bacteria from the surfaces of coralline algae are important for settlement of larvae (Johnson & Sutton 1994, Negri et al. 2001, Huggett et al. 2006), but it is not known whether or not these bacteria are specific to or most commonly associated with corallines or distributed broadly and non-discriminately throughout the habitat.

In the present study, we used catalysed reporter deposition-fluorescence *in situ* hybridisation (CARD-FISH) to measure the distribution and abundance of the 3 bacterial genera *Pseudoalteromonas*, *Vibrio* and *Shewanella* on the surfaces of 5 co-occurring algal species collected from the habitat of adult *Heliocidaris erythrogramma.* These 3 genera contained the majority of strains which induced *H. erythrogramma* settlement both in the laboratory and in the field, in comparison to seawater alone (Huggett et al. 2006). *H. erythrogramma* is an endemic Australian sea urchin found in coastal waters from 1.5 to 35 m depth. *H. erythrogramma* feeds on a wide range of sources including seagrasses, macroalgae, encrusting coralline algae and diatoms (Keesing 2001) and currently supports a commercially important fishery. Despite being common and widespread within Australia, the ecology of *H. erythrogramma* is relatively unknown. We also measured the distribution and abundance of newly recruited *H. erythrogramma* juveniles onto these same algae over a period of ca. 18 mo. Settlement was defined as the ability to induce larvae to metamorphose in the laboratory, while recruitment refers to the presence of small juveniles in the field. The distribution of the 3 bacterial genera on algal surfaces was then related to the field distribution of juvenile urchins.

MATERIALS AND METHODS

Recruitment surveys. Surveys of newly recruited *Heliocidaris erythrogramma* were conducted using SCUBA at Bare Island, Botany Bay (33° 59′ 38″ S, 151° 14′ 00″ E), New South Wales, Australia, approximately. every 4 wk between December 2002 and May 2004. Recruits were placed into 2 subclasses: new recruits (<5 mm test diameter) and older recruits (5.1 to 10 mm test diameter). This method was successful in finding juveniles as small as 1.2 mm test diameter. In the laboratory, newly settled juveniles (48 h after metamorphosis from the non-feeding larval stage) have a test diameter of ca. 1 mm (M. J. Huggett pers. obs.), and anecdotal evidence (Keesing 2001) suggests that individuals with >10 mm test diameter are ca. 6 mo old. A number of algae common in the habitat were surveyed, as well as adult urchins and sediment. Algae included the 2 coralline turfing algae *Corallina officinalis* and *Amphiroa anceps*, the turfing or foliose red and brown algae *Delisea pulchra*, *Homeostrichus olsenii* and *Sargassum linearfolium* and small individuals of the brown alga *Ecklonia radiata.* Whole plants were sampled rather than patches of mixed species, enabling recruitment onto specific algal species to be measured. Five plants of each species were collected and placed in clip seal bags underwater, except for the coralline algae, which were sampled as 5 randomly chosen 100 cm^2 quadrants. Rubble (a mixture of sand, small rocks and dead shells) was also collected at 5 replicate 100 cm^2 areas. Five adult urchins were also collected, and the area within the surrounding 100 cm^2 patch was scraped and placed into clip seal bags underwater. Samples were transported to the University of New South Wales (UNSW), sorted and examined for the presence of recently recruited *H. erythrogramma* juveniles. The wet weight of all algae and sediment samples, and the test diameter of adult urchins, were recorded.

Probe design from isolates and probe synthesis. Previously, bacteria were isolated from the coralline algae *Amphiroa anceps* and *Corallina officinalis* and evaluated for their ability to induce *Heliocidaris erythrogramma* larval settlement (Huggett et al. 2006). Of ca. 250 strains, 60% of highly inducing strains were from the 3 genera *Pseudoalteromonas*, *Shewanella*

and *Vibrio.* Thus, probes were designed to target each of these genera.

Three probes (PSU730, SHEW227 and VIB572a), specific to these 3 genera (Table 1), were designed using the probe design tool in the ARB software package (Ludwig et al. 2004) using the SSUjun02 database, expanded to contain all available closely related sequences. While the probe VIB572a targets validly described genera other than *Vibrio*, namely some members of *Photobacterium* and *Listonella* (see Fig. 1), for ease of description and discussion the target group for this probe is broadly referred to as *Vibrio* hereafter. A fourth oligonucleotide, VIB572b (ACCGCCTGCATGCGCTTT) was designed to target the entire order *Vibrionales*, but bound to 2-mismatch negative controls up to formamide concentrations of 55%, and was not evaluated further. Sequences were subsequently confirmed for specificity using BLAST (Altschul et al. 1990). The specificity of the 3 probes is illustrated in Fig. 1 using an evolutionary distance tree created using the Neighbour Joining tool in ARB. Probes were synthesised and labelled at the 5′ end with Cy3 (Thermo Electron) and evaluated via FISH using *Pseudoalteromonas* strain A213 (DQ005864), *P. tunicata* (DQ005902), *Telluria mixta* (DQ005909), *Vibrio harveyii* (DQ005908), *V. alginolyticus* (DQ005910), *Shewanella* strain A317 (DQ005868), *Shewanella* strain C111 (DQ005889), *Photobacterium phosphoreum* (DQ099331) and *Aeromonas salmonicida* (DQ099332). The optimal formamide concentration was determined as previously described (Schmid et al. 2000). A competitor probe was designed to accompany the SHEW227 probe (Table 1) and was successful in eliminating non-target binding of the SHEW227 probe during subsequent hybridisations.

CARD-FISH. This method was conducted to quantify the distribution of the 3 high inducing bacterial genera *Pseudoalteromonas*, *Shewanella* and *Vibrio* on algae from the adult urchin habitat. Algal samples were collected using SCUBA from Bare Island, Botany Bay, during summer 2004, corresponding with the annual *Heliocidaris erythrogramma* spawning and recruitment season. The 5 algae that were collected included *Corallina officinalis* and *Amphiroa anceps* which induced the highest levels of settlement and which had the highest numbers of recruits in the recruitment surveys. For comparison, the co-occurring red alga *Delisea pulchra* and the brown algae *Sargassum linearfolium* and *Ecklonia radiata* were also examined. Triplicate samples of each alga were collected on each of the 2 sampling dates (January and April 2004).

The CARD-FISH method followed that established for marine samples by Schönhuber et al. (1999) and Pernthaler et al. (2002) and was subsequently adapted for the enumeration of bacteria on the surface of algae (Tujula et al. 2006). Algae were hybridised with HRP-labelled probes (Thermo Electron) targeting either *Pseudoalteromonas*, *Shewanella* or *Vibrio.* Briefly, samples were embedded using a 1% agarose solution, and permeabilisation of cells was performed via incubation at 37°C in a 1% w/v lysozyme solution for 90 min. Samples were then rinsed with Milli-Q water, incubated in a solution of hydrogen peroxide (0.15%) in methanol for 30 min to inactivate endogenous peroxidase and finally rinsed in Milli-Q water and dehydrated with ethanol before storing at −20°C. Subsequently, hybridisation was performed at 46°C for 90 min in a 30% formamide hybridisation buffer, followed by washing at 48°C (Ishii et al. 2004, Tujula et al. 2006). Tyramide signal amplification was achieved following the enhancer method for signal amplification (Pernthaler et al. 2004) using the carboxyfluorescein $Alexa_{546}$-labelled tyramide. Samples were counterstained with SYBR Green II (2× concentration, Molecular Probes) and mounted in 1% DABCO anti-fade solution (Sigma Aldrich). SYBR Green II is a general nucleic acid stain and enabled enumeration of the whole biofilm community. Cells were observed with a Leica TCS-SP confocal laser scanning microscope (CLSM) mounted on a Leica DCM IRB epifluorescence microscope equipped with a 50 W mercury lamp, PL APO 60×/1.4 na oil objective and appropriate filter sets for $Alexa_{546}$ and SYBR Green II (Leica Microsystems). An argon laser with excitation peak at 488 nm with an emission line of 500 to 537 nm was used as the excitation source for the SYBR Green II. Two helium/neon lasers with an excitation peak at 543 nm and emission line at 555 to 620 nm were used for $Alexa_{546}$. Z-sections were prepared following the protocol of Tujula et al. (2006) due to the uneven topography of the algal surfaces.

Table 1. Oligonucleotide probes used in the present study

Probe	Sequence	Specificity	% formamide	Source
EUB338i-iii	GCTGCCTCCCGTAGGAGT	*Bacteria*	0 to 50	Daims et al. (1999)
PSU730	TTGACCCAGGTGGCTGCC	*Pseudoalteromonas*	40	Present study
SHEW227	AGCTAATCCCACCTAGGTWCATC	*Shewanella*	40	Present study
cSHEW227	AGCTAATCCCACCTAGGCWTATC	Use with SHEW227	40	Present study
VIB572a	ACCACCTGCATGCGCTTT	*Vibrio/Photobacterium*	40	Present study

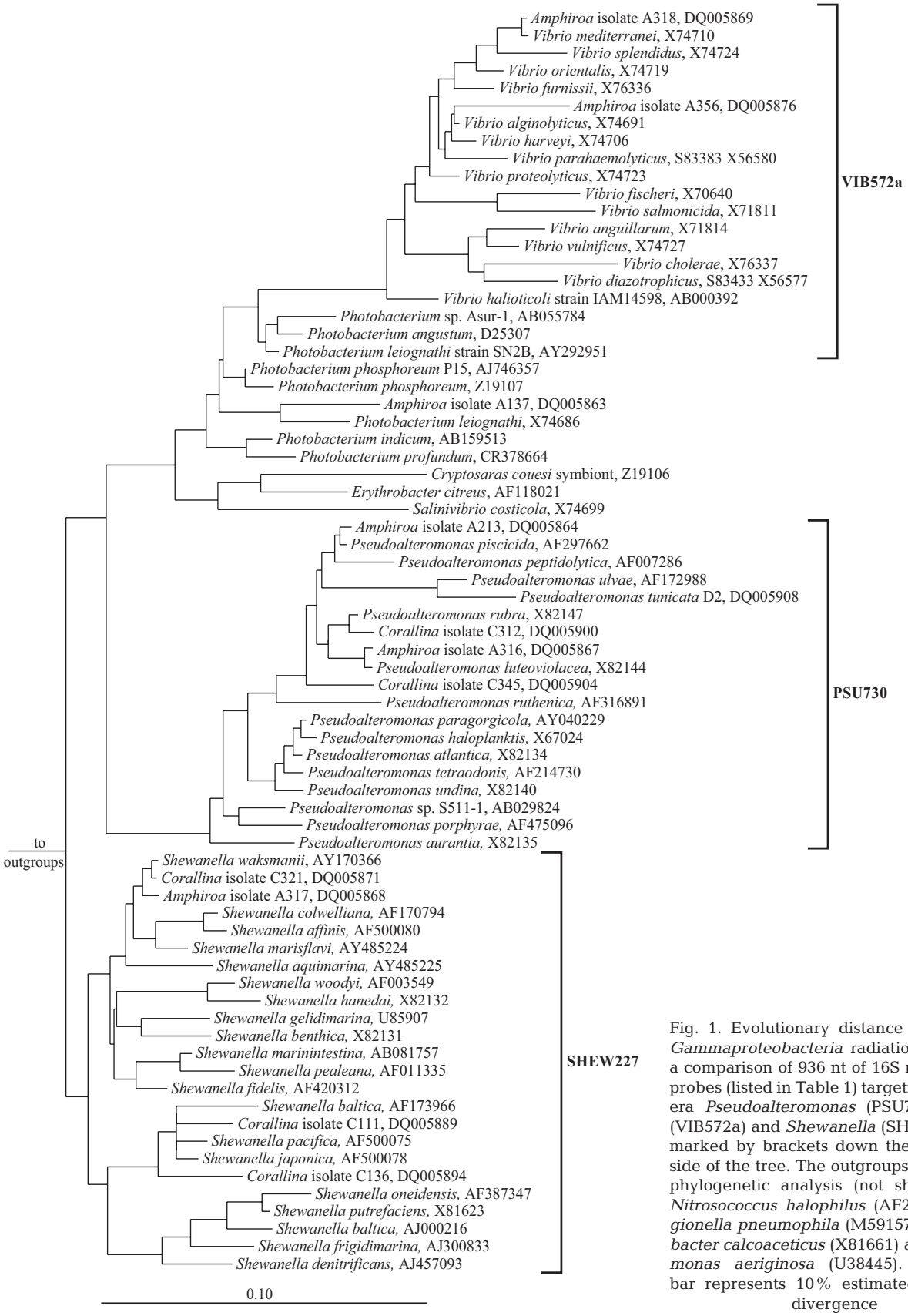

Fig. 1. Evolutionary distance tree of the *Gammaproteobacteria* radiation based on a comparison of 936 nt of 16S rDNA. FISH probes (listed in Table 1) targeting the genera *Pseudoalteromonas* (PSU730), *Vibrio* (VIB572a) and *Shewanella* (SHEW227) are marked by brackets down the right-hand side of the tree. The outgroups used in the phylogenetic analysis (not shown) were *Nitrosococcus halophilus* (AF287298), *Legionella pneumophila* (M59157), *Actinetobacter calcoaceticus* (X81661) and *Pseudomonas aeriginosa* (U38445). The scale bar represents 10% estimated sequence divergence

Quantification of *Pseudoalteromonas*, *Shewanella* and *Vibrio*. Previous studies on algae from the same sites resulted in no non-specific binding as evaluated with non-EUB and a detectability of ca. 90% of all bacteria present as indicated by hybridisation with probe EUB (Tujula et al. 2006). Despite the improved usefulness of CARD-FISH, the background fluorescence of algal cells was still extremely high in some algal species, most conspicuously the coralline algae *Amphiroa anceps* and *Corallina officinalis*. Control experiments containing no probes indicated that some cells, notably large host algal cells and chains of diatoms, emitted high levels of autofluorescence. Unlike the even surface topography of other algae such as *Ulva australis*, the significant contribution of green and red pixels from algal cells in most optical sections (see Fig. 4) prevented the use of automated pixel counting using software such as Image J (http://rsb.info.nih. gov/ij). Instead, manual cell counts were conducted. Each image was viewed using Adobe Photoshop, and the number of cells in the red channel (representing the genus probe) were recorded as a percentage of all biofilm cells (shown in the green channel).

In order to minimise counting time, a pilot study was done to establish the least number of fields of view needed before the mean numbers of bacteria per field of view reached an asymptote in a cumulative sampling curve. Fifteen fields of view were captured using the PSU730 probe labelled with horseradish peroxidase (HRP) ($Alexa_{546}$ and counterstained with SYBR Green II). The running mean and standard error of bacterial numbers were plotted against the numbers of fields of view counted, and it was established that the mean estimates of numbers of bacteria derived from 8 fields of view did not differ from 15 fields of view. To be conservative, 10 fields of view were counted for each replicate sample. The density and percentage of the whole community of each genus of bacteria on plants were then calculated.

Data analysis. Statistical analyses were carried out using SYSTAT 7.0. The number of recruits kg^{-1} algae mo^{-1}, and the mean percentage of bacteria from each genus on each alga were analysed. Assumption of normality and homogeneity of variance were checked graphically, and data were transformed where necessary. One-way analyses of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) post hoc tests was used for all analyses.

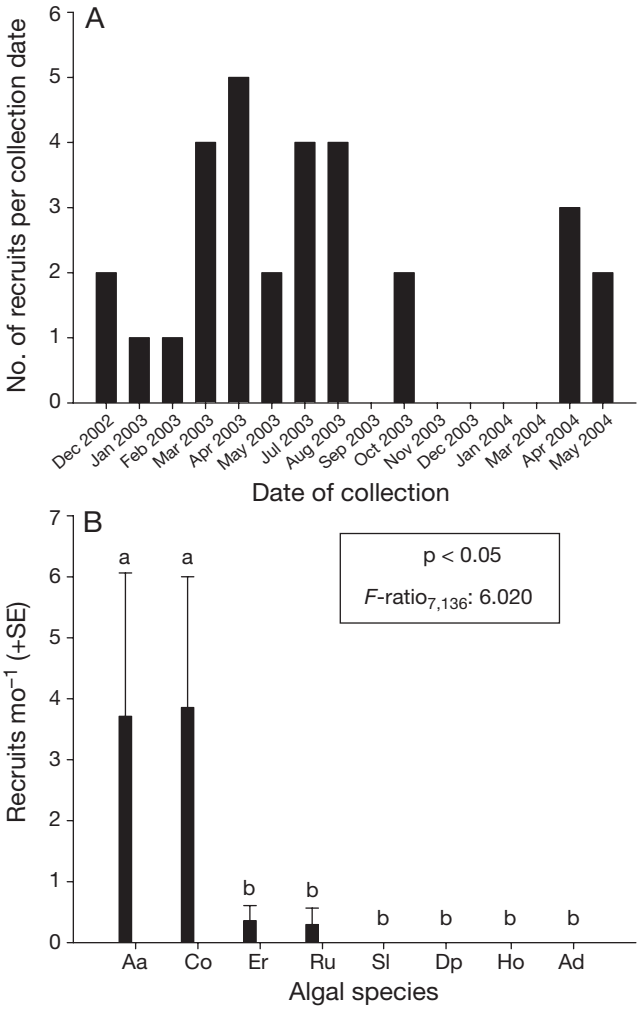

Fig. 2. *Heliocidaris erythrogramma*. Recruitment of sea urchin at Bare Island, Botany Bay, Australia, from December 2002 to May 2004 (A) per month and (B) on various substrata. Numbers are mean recruits (±SE) per kg (wet weight) algae or rubble, or per individual adult urchin per month. Co: *Corallina officinalis*, Aa: *Amphiroa anceps*, Er: *Ecklonia radiata*, Ru: rubble/sediment, Sl: *Sargassum linearfolium*, Dp: *Delisea pulchra*, Ho: *Homeostrichus olsenii*, Ad: adult *H. erythrogramma*. Data were log transformed before ANOVA. Treatments that do not share a letter differ significantly from one another (1-way ANOVA)

RESULTS

Recruitment surveys

Recruitment of juvenile *Heliocidaris erythrogramma* was low, but occurred throughout the year (Fig. 2A). This was somewhat unexpected as adults spawn from November through May (Williams & Anderson 1975, M. J. Huggett pers. obs.), producing a short-lived (3 to 4 d) non-feeding larva. New recruits (<5 mm test diameter) were found each month, from December 2002 through October 2003, with the exception of September. Highest recruitment occurred from March to August 2003. A similar recruitment peak was not observed in 2004. A total of 3 or 4 recruits was usually found, and the most ever detected on a single sampling date was 5.

On the coralline turfing algae *Corallina officinalis* and *Amphiroa anceps* 95% of recruitment occurred. A small number of recruits were also found on the lamina

of the brown alga *Ecklonia radiata* and on rubble. No recruits were found on or near adults or on the foliose red algae *Homeostrichus olsenii*, *Delisea pulchra* or the brown alga *Sargassum linearfolium.* Recruitment was highly variable, but new recruits were found in significantly higher numbers on both *C. officinalis* and *A. anceps* than on any other substrate (Fig. 2B; $F_{7,136}$ = 6.020, $p < 0.05$).

Probe development

Probes targeting the 3 genera *Pseudoalteromonas*, *Vibrio and Shewanella* were designed and tested. Appropriate target strains and controls, listed below, were tested with newly designed probes labelled with Cy-3 and EUBi-iii mix labelled with FITC concurrently. All strains bound the EUB338i-iii at high pixel intensity between formamide concentrations of between 10 and 50%. Outside of this formamide range pixel intensity decreased for EUBi-iii.

For the probe VIB572a, the positive isolates *Vibrio harveyii* (DQ005908) and *V. alginolyticus* (DQ005910) were used with 2 controls — *Photobacterium phosphoreum* (DQ099331), which has a 1-base mismatch, and *Aeromonas salmonicida* (DQ099332), which has a 2-base mismatch — to the target sequence. In testing the VIB572a probe, the positive isolates exhibited the maximum fluorescence up to 40% formamide concentration, while neither *P. phosphoreum* nor *A. salmonicida* demonstrated probe binding at any significant level (Table A1, Fig. A1 in Appendix 1, available online as AME Supplementary Material at: www.int-res.com/articles/suppl/a053p161_app.pdf).

The probe SHEW227, together with its unlabelled competitor, was optimised using 2 positive isolates, correlating to each of the 2 target sequence combinations, due to the ambiguous base (W = A/T) at *Escherichia coli* position 245. Isolate A317 (DQ005868) was used as the '245T' positive, and isolate C111 (DQ005889) was used as the '245A' positive. *Vibrio harveyii* and *Aeromonas salmonicida*, each with a 1-base mismatch to the target sequence, were used as negative controls. Both positive controls strongly bound the SHEW227 probe up to formamide concentrations of 40%, and the negative controls did not bind the probe at any significant level at any formamide concentration (Table A2, Fig. A2 in Appendix 1).

To determine the optimal formamide concentration for PSU730, the positive isolates A213 (DQ005864) and *Pseudoalteromonas tunicata* (DQ005908) were used with the negative control *Telluria mixta* (DQ005909), which has a 1-base mismatch to the target sequence. Isolate A213 and *P. tunicata* most strongly hybridised with probe PSU730 up to 40% formamide concentrations. *T. mixta* did not bind the probe at any significant level at any formamide concentration (Table A3, Fig. A3 in Appendix 1).

For each probe, the optimal formamide concentration was determined as 40%. Probe specificity and target groups are demonstrated in Fig. 1.

Distribution of *Pseudoalteromonas*, *Shewanella* and *Vibrio*

Three genera of bacteria were quantified in biofilms on the surface of 5 species of algae, during January and again in April 2004, using CARD-FISH. CARD-

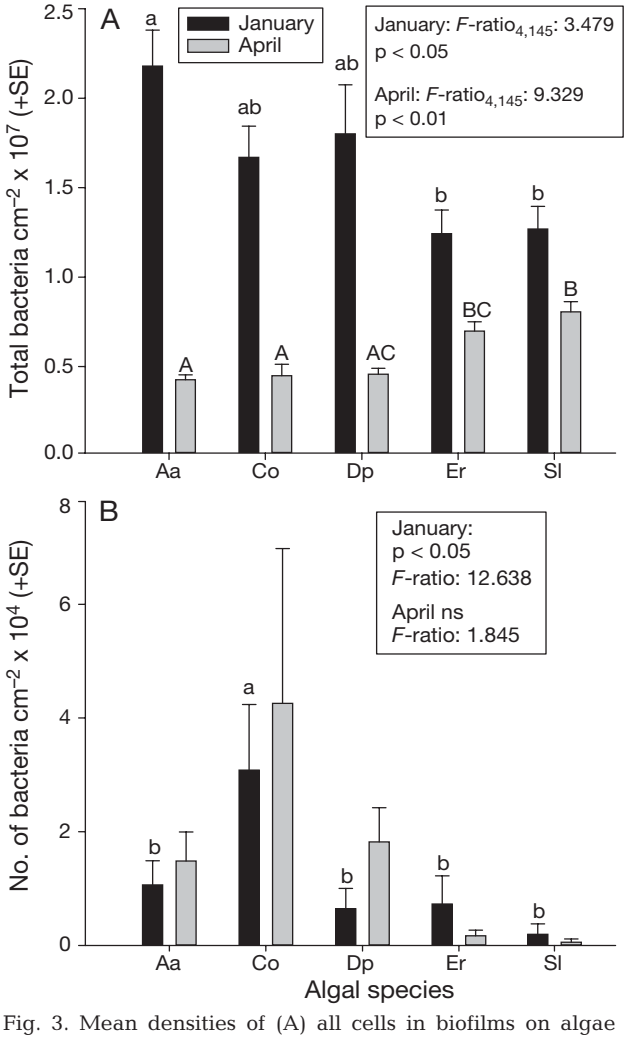

Fig. 3. Mean densities of (A) all cells in biofilms on algae for January and April and (B) total of all 3 genera: *Pseudoalteromonas, Vibrio* and *Shewanella.* Aa: *Amphiroa anceps*, Co: *Corallina officinalis*, Dp: *Delisea pulchra*, Er: *Ecklonia radiata*, Sl: *Sargassum linearfolium.* Lowercase and uppercase letters refer to cell densities from January and April, respectively; bars that do not share the same letter differ significantly from one another (1-way ANOVA). Details of ANOVA are indicated within each panel

FISH probe binding on the surface of *Amphiroa anceps* and *Corallina officinalis* is shown in Fig. 4. This figure also demonstrates the high level of autofluorescence observed in some algae. The density of *Vibrio*, *Pseudoalteromas* and *Shewanella* cells on algae is shown in Fig. 5. Maximum densities for a single genus ranged from 1 to 4×10^4 cells cm^{-2} (Fig. 5) compared to whole biofilm densities of ca. 0.5 to 2.1×10^7 cells cm^{-2} (Fig. 3A). In April, there were higher densities of *Pseudoalteromonas* in biofilms on the surfaces of red versus brown algae (1-way ANOVA; $F_{4,10} = 9.322$; $p < 0.01$; data log transformed), and densities of *Pseudoalteromonas* across all algae were higher than either *Shewanella* or *Vibrio*. *Shewanella* occurred in lower numbers, but across all plants for both months, and *Vibrio* occurred in highest densities on *C. officinalis* in January, and *A. anceps* in April (Fig. 5).

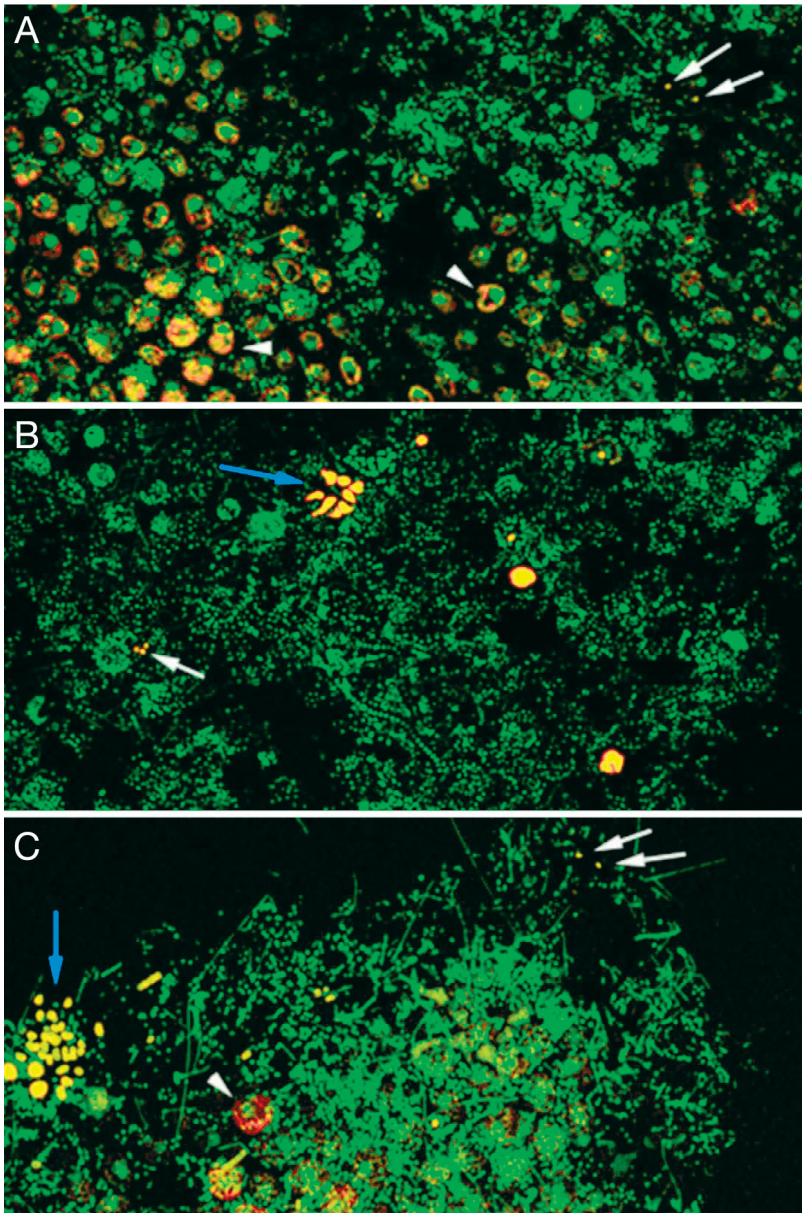

Fig. 4. Biofilms on the surface of (A,B) *Amphiroa anceps* and (C) *Corallina officinalis* stained with SYBR Green II. CARD-FISH probes applied to samples were (A) PSU730, (B) SHEW227 and (C) VIB572a. All probes were labelled with $Alexa_{546}$ tyramide for signal amplification. Green cells are stained only with SYBR Green II and yellow cells have stained with SYBR Green II and also bound to the probe. White arrows indicate cells that bound to probes (yellow). Blue arrows indicate autofluorescent cells, which were not counted as they exhibited fluorescence during control experiments containing no labelled probes. White arrowheads indicate large algal cells, which were also highly autofluorescent and were not counted

We also compared total numbers of bacteria from the 3 genera on different hosts (Fig. 3B). Density of the total number of all 3 genera combined was highest on *Corallina officinalis* in both January and April (3.08×10^4 and 4.26×10^4 cells cm^{-2}, respectively). *Amphiroa anceps* and *Delisea pulchra* had similar total numbers of all genera combined, and both were slightly lower than *C. officinalis* for both months. *Ecklonia radiata* had comparable numbers of all 3 genera to *A. anceps* (1.06×10^4) and *D. pulchra* (6.44×10^3) in January (7.28×10^3 cells cm^{-2}) but lower amounts in April (1.68×10^2 cells cm^{-2}). *Sargassum vestitum* had low overall numbers of all 3 genera for both months.

DISCUSSION

Recruitment surveys of *Heliocidaris erythrogramma* revealed that juvenile urchins occur predominantly on the coralline algae *Amphiroa anceps* and *Corallina officinalis*. CARD-FISH analysis of various macroalgae indicated that the 3 bacterial genera *Pseudoalteromonas*, *Vibrio* and *Shewanella* also occur in the highest total numbers on the coralline algae *C. officinalis* and *A. anceps*. Previously, larvae of *H. erythrogramma* were shown to settle in response to these 2 coralline algal species and also to single-species biofilms isolated from these algae (Huggett et al. 2006). These isolates are from a wide range of taxa, but are dominated by the genera *Pseudoalteromonas*, *Shewanella* and *Vibrio*. The

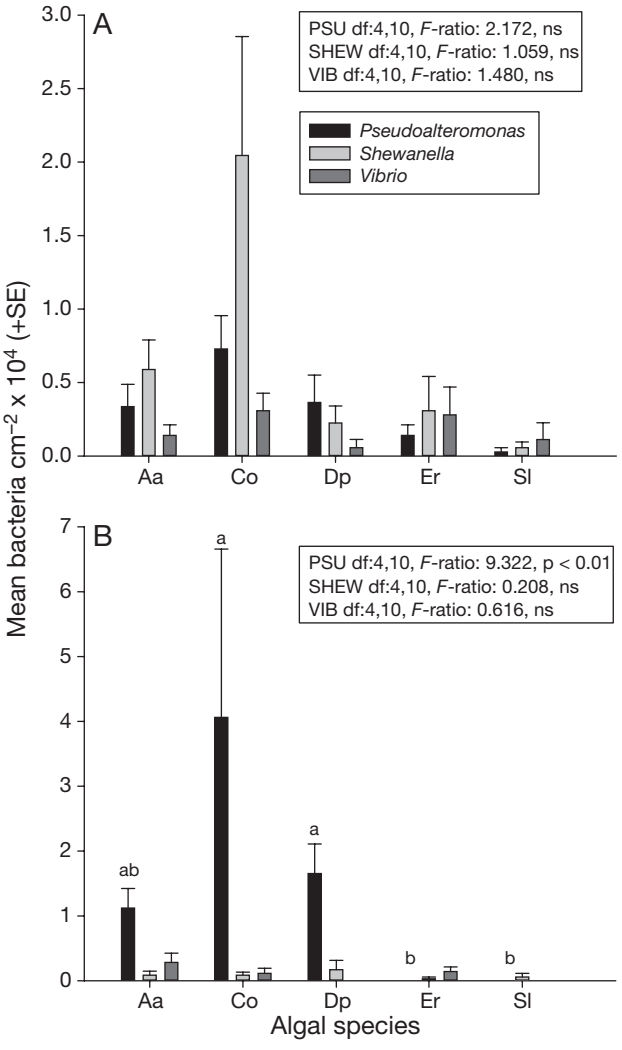

Fig. 5. Mean densities of *Pseudoalteromonas* (PSU), *Shewanella* (SHEW) and *Vibrio* (VIB) cells in biofilms on algae in (A) January and (B) April. Aa: *Amphiroa anceps*, Co: *Corallina officinalis*, Dp: *Delisea pulchra*, Er: *Ecklonia radiata*, Sl: *Sargassum linearfolium*. Bars that do not share the same letter differ significantly from one another (1-way ANOVA). Details of ANOVA are indicated within each panel

results of the present study suggest that settlement and recruitment of *H. erythrogramma* in the field may be quantitatively related to the distribution of inducing strains within bacterial biofilms.

Our CARD-FISH procedure used oligonucleotide probes targeting the 3 bacterial genera *Pseudoalteromonas*, *Vibrio* and *Shewanella*. New *Vibrio* and *Pseudoalteromonas* probes were designed, because when existing oligonucleotides such as G-V823 (Giuliano et al. 1999), VIB643 (Moreno et al. 1999), PSA184 (Eilers et al. 2000) and Psalt815 (Holmes et al. 2001) were tested against our ARB database containing 16S rRNA gene sequences from our in-house culture collection, we found that they did not target several of these isolates within *Vibrio* and *Pseudoalteromonas*. Single-species probes would give a much more accurate estimate of the actual numbers of high inducing species in biofilm communities. However, it is likely that single-species probes would detect only quite low, scattered and variable numbers of bacteria. The drawbacks of single-species probes are primarily the extremely low numbers of a single species that are likely to be present, the difficulty in locating probe sites for areas that are exclusive to one bacterial species only and the labour-intensive microscopy and counting that would be required to gain an accurate measure of bacterial numbers. These factors all contributed towards the decision to use genus-level probes instead of several species-level probes. Genus-level probes provide a useful starting point (and in this case the first reported starting point) for investigating the field distribution of inducing bacteria. This hierarchical probe approach to FISH investigations into microbial community structure has been successfully employed previously (Bond et al. 1995), contributing significantly towards subsequent discoveries using more specific oligonucleotide probes (Crocetti et al. 2000).

These results show an association between *in situ* bacterial distribution and sea urchin recruitment that emulate previously reported laboratory results. Numerous studies have focussed on settlement cues for marine invertebrate larvae (reviewed by Pawlik 1992, Fusetani 1997, Slattery 1997, Hadfield & Paul 2001, Steinberg & de Nys 2002). These cues should not be considered ecologically relevant until they can be correlated to (or tested against) the actual recruitment of juveniles in the field. For settlement cues to be ecologically relevant they must be present in the habitat that larvae recruit in and in quantities that larvae are able to detect and respond to (Williamson et al. 2000). While the evidence has been mounting for the importance of *Gammaproteobacteria* in promoting larval settlement, it is often unclear whether or not these strains are actually present on settlement surfaces in the field, and if so, how they relate to recruitment. Through CARD-FISH, we were able to monitor the distribution and quantification *in situ* of *Pseudoalteromonas*, *Shewanella* and *Vibrio* in biofilms on 5 species of algae during both January and April 2004, correlating with the timing of *Heliocidaris erythrogramma* settlement. During this time, bacterial densities, as well as settlement and recruitment of urchins, were all highest on corallines. The red alga *Delisea pulchra* had slightly lower, but comparable, numbers of all 3 bacterial genera to the coralline alga *Amphiroa anceps*, but does not induce larval settlement in the laboratory, or recruitment in the field. This alga is known to produce a range of biologically active compounds, furanones,

which inhibit larval settlement of other invertebrate larvae (De Nys et al. 1995), and these may also have a deterrent effect on *H. erythrogramma* larvae. Furthermore, *D. pulchra* was observed to have a lethal effect on some *H. erythrogramma* larvae in the laboratory if left together in small Petri dishes in static seawater for 24 h (M. J. Huggett pers. obs.). Despite similarities in the numbers of the 3 bacterial genera we investigated, *D. pulchra* appears to present a different settlement 'signature' to larvae, as no recruits were found on it, and it does not induce settlement in the laboratory (Huggett et al. 2006). Importantly, these results have shown that the distribution and relative densities of the 3 bacteria genera *Pseudoalteromonas*, *Vibrio* and *Shewanella* relate to both settlement in the laboratory and distribution of juveniles in the field.

A critical factor relating to the significance of larval settlement induction by bacteria is the density of cells required to induce a response. For any single genus in biofilms on the algae we investigated, cell density ranged from 4.06×10^4 to 0 cells cm^{-2} (Fig. 5). The highest total density of all 3 genera was 4.26×10^4 cells cm^{-2} on *Corallina officinalis* in April (Fig. 3A.). Recently, Rao et al. (2007) showed that densities as low as 10^2 to 10^3 cells cm^{-2} are active in inhibiting settlement by fouling organisms, and the inhibitory effect of a *Pseudoaltermonas* biofilm on the bryozoan *Bugula neritina* occurs at densities as low as 1×10^4 cells cm^{-2} (Dahms et al. 2004), suggesting that the numbers we found here are certainly within the range required to produce a response by larvae. These seemingly low densities of active strains may be able to be effective by means of concentration of the settlement, inducing or inhibiting molecules due to accumulation in biofilms while continuously being produced.

Despite the increasing accessibility of molecular methods such as clone libraries, quantitative PCR and FISH, characterisation of marine biofilms is still largely unexplored. *Gammaproteobacteria* have been isolated from a wide variety of marine surfaces including algae (Tujula et al. 2006), sponges (Friedrich et al. 1999), tunicates (Holmström et al. 1992) and bivalves (Jaksic et al. 2002) and also from water samples (Franklin et al. 2005). However, they have rarely been quantified in marine biofilms. The few existing reports of quantification of the *Gammaproteobacteria* indicate that their numbers vary widely in marine bacterial communities, ranging from <1% in oceanic waters (Eilers et al. 2000), between 20 and 25% in coral reef biofilms (Webster et al. 2004) and over 87% in bacterial communities from the sea surface microlayer (Franklin et al. 2005). Only one other study has attempted to quantify a single genus within the *Gammaproteobacteria* division in biofilms from marine surfaces. This was recently achieved for *Pseudoalteromonas* through real-time quantitative PCR (Skovhus et al. 2004). This method revealed that *Pseudoalteromonas* cells on the green algae *Ulva australis* and *Ulvaria fusca* varied, but on average comprised 1.55 and 0.06% of the whole community, respectively, and the tunicate *Ciona intestinalis* averaged 0.10% (Skovhus et al. 2004). The percentages of *Pseudoalteromonas* cells in microbial communities from both the 2 algae and the tunicate (Skovhus et al. 2004) are comparable to the percentages (between 0 and 0.12%) that we found in communities on the surfaces of algae from Bare Island.

The present study has revealed that the algal species on which *Heliocidaris erythrogramma* larvae settle in the laboratory are also providing habitat for juvenile recruits in the field. A range of single-species bacterial biofilms, from a wide range of taxa, but dominated by *Pseudoalteromonas*, *Vibrio* and *Shewanella*, also induce high rates of larval settlement. These genera are found in low numbers across a range of algae from the *H. erythrogramma* adult habitat, with more of these cells present on the high inducing coralline algae *Corallina officinalis* and *Amphiroa anceps.* From other studies of bacterial densities, it is feasible that the *in situ* densities of *Pseudoalteromonas*, *Vibrio* and *Shewanella* on algae are high enough to be detected by competent larvae, although this has not been demonstrated for *H. erythrogramma* larvae in this investigation. It is also apparent that although these high inducing genera are representative of less than half a percent of the whole biofilm community, the densities found are high enough that they could be detected as settlement cues for larvae. This is the first example demonstrating that bacteria that are able to induce larval settlement in the laboratory are also present in the field in the juvenile recruitment habitat.

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