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PLANT ANIMAL INTERACTIONS

Larval settlement of the common Australian sea urchin *Heliocidaris erythrogramma* in response to bacteria from the surface of coralline algae

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Abstract Bacterial biofilms are increasingly seen as important for the successful settlement of marine invertebrate larvae. Here we tested the effects of biofilms on settlement of the sea urchin *Heliocidaris erythrogramma*. Larvae settled on many surfaces including various algal species, rocks, sand and shells. Settlement was reduced by autoclaving rocks and algae, and by treatment of algae with antibiotics. These results, and molecular and culture-based analyses, suggested that the bacterial community on plants was important for settlement. To test this, approximately 250 strains of bacteria were isolated from coralline algae, and larvae were exposed to single-strain biofilms. Many induced rates of settlement comparable to coralline algae. The genus *Pseudoalteromonas* dominated these highly inductive strains, with representatives from *Vibrio*, *Shewanella*, *Photobacterium* and *Pseudomonas* also responsible for a high settlement response. The settlement response to different bacteria was species specific, as low inducers were also dominated by species in the genera *Pseudoalteromonas* and *Vibrio*. We also, for the first time, assessed settlement of larvae in response to characterised, monospecific biofilms in the field. Larvae metamorphosed in higher numbers on an inducing biofilm, *Pseudoalteromonas luteoviolacea*, than on either a low-inducing biofilm, *Pseudoalteromonas rubra*, or an unfilmed control. We conclude that the bacterial community on the surface of coralline algae is important as a settlement cue for *H. erythrogramma* larvae. This study is also an example of the emerging integration of molecular microbiology and more traditional marine eukaryote ecology.

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Introduction

Most benthic marine invertebrates have a complex life cycle involving a sessile, benthic adult phase, and a planktonic larval phase. The series of events whereby tiny larvae, weeks or months after fertilisation, are able to locate, settle and metamorphose in a habitat, where juveniles are then capable of successfully establishing themselves, is a key part of the life cycle of such organisms. This process is crucial to population and community dynamics, biogeography, gene flow, and macroevolution of marine invertebrates, and remains one of the central foci of marine research (Thorson 1950; Butman 1987; Caley et al. 1996; Pechenik 1999).

Settlement and recruitment of propagules are complex processes, determined by the interaction of biotic

and abiotic factors that operate at different spatial and temporal scales (Rodriguez et al. 1993). At a large scale, a variety of hydrodynamic factors, e.g. flow (Abelson and Denny 1997), light and gravity (Kobak 2001) play important roles in settlement. Within a habitat, more environmentally specific factors become important (Butman 1987). Environmental cues including physical factors such as surface texture (Callow et al. 2002) and small-scale flow (Boxshall 2000), biological factors such as the presence of adult conspecifics (Zhao and Qian 2002) and chemical factors associated with various aspects of the habitat (Fusetani 1997) are thought to be most influential at small scales. Chemical cues in particular, from both natural and artificial sources, have received considerable attention in the literature (reviewed by Pawlik 1992; Steinberg and de Nys 2002). Compounds arising from plants (Morse et al. 1984; Morse 1992; Williamson et al. 2000), prey (Pawlik 1992), conspecifics (Burke 1984, 1986; Pawlik 1992), and biofilms (Johnson and Sutton 1994) can all act as settlement cues for larvae.

As early as 1935, Zobell and Allen (1935) noted that many marine invertebrate larvae require a bacterial biofilm to enable settlement to occur, and we now know that larvae from a number of phyla including echinoderms (Johnson et al. 1991b), cnidarians (Negri et al. 2001), polychaetes (Unabia and Hadfield 1999), gastropods (Rodriguez et al. 1995) and crustaceans (Neal and Yule 1994), settle in response to biofilms of either single or mixed bacterial communities (reviewed by Hadfield and Paul 2001). Factors such as the age of the biofilm (Keough and Raimondi 1995), origin of the biofilm (Johnson et al. 1991a), and whether or not the biofilm is presented in combination with another surface (Johnson and Sutton 1994; Negri et al. 2001) all mediate the response of settling larvae (reviewed by Wieczorek and Todd 1998; Holmström and Kjelleberg 2000). With respect to particular assemblages or taxa present in inducing biofilms, bacteria associated with coralline algae are important for settlement of a number of invertebrates such as the crown-of-thorns starfish (Johnson et al. 1991b; Johnson and Sutton 1994), corals and other cnidarians (Leitz 1997; Negri et al. 2001), and bacteria from the genus *Pseudoalteromonas* are common as mediators of larval settlement as inducers (Lau and Qian 2001; Negri et al. 2001) or inhibitors (Holmström et al. 1992; Holmström and Kjelleberg 2000).

Beyond these few observations, however, there are few generalities regarding the types of interactions that occur between settling invertebrate larvae and biofilms. One generalisation regarding the effect of biofilms on larval settlement was proposed by Steinberg et al. (2001). They suggested that larvae of generalist marine herbivores, i.e. those that feed on a wide range of host plants and are not associated with any particular plant species, respond to bacterial biofilms, while larvae of specialist marine herbivores, i.e. those that feed on a discrete range of plants, and are often small, mobile and live on their food source, respond to cues associated with their host plant. For specialist herbivores, there are now a number of examples of larvae that settle in response to compounds secreted by their host alga (Boettcher and Targett 1998; Krug and Manzi 1999; Williamson et al. 2000; Swanson et al. 2004). For generalists (Pearce and Scheibling 1991; Gosselin and Jangoux 1996; Lamare and Barker 2001), larvae may be responding to more broadly distributed cues, such as the bacterial biofilms associated generally with surfaces in shallow subtidal habitats.

A second gap that currently exists in biofilm/larval settlement studies is the linking together of laboratory and field studies. There are a number of laboratory studies that have focussed on bacterial cues as triggers for settlement and metamorphosis. Far fewer studies have been conducted in the field in an attempt to understand in situ larval responses (e.g. Keough and Raimondi 1995) and how the distribution and/or amount of various cues might actually influence larval settlement (Underwood and Keough 2000) and none of these have manipulated or characterised biofilm-derived cues except in a very general way (e.g. biofilm age). A clear challenge for the future is to develop reliable techniques, incorporating modern microbiological methods, to monitor and manipulate biogenic cues and rates of larval arrival in experiments under field conditions.

Here, the prediction that larvae of generalist herbivores will respond to biofilm-derived cues, rather than host plant cues, is explored using the generalist marine herbivore *Heliocidaris erythrogramma*. *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 (CCA) and diatoms (Keesing 2001), and currently supports a commercially important fishery. Despite being common and widespread within Australia (Keesing 2001), the ecology of *H. erythrogramma* is relatively unknown. Factors affecting the settlement and recruitment of this urchin have not previously been studied. Here we ask:

1. Does *H. erythrogramma* respond to host plants, or to associated biofilms?
2. If *H. erythrogramma* does respond to biofilms, are there specific bacterial strains that induce settlement, or are there many strains that cause the same response?

3. Do larvae of *H. erythrogramma* respond to characterised biofilms in the field?

Materials and methods

Culturing of larvae

Heliocidaris erythrogramma larvae were reared in filtered seawater (FSW) in aerated beakers at 19°C. After approximately 3 days larvae were competent (Williams and Anderson 1975) and a haphazard selection was used in assays.

Study organisms and study sites

Settlement assays were done using local substrata collected from Shark Bay (33°51′09″S, 151°16′00″E) and Bare Island (33°59′38″S, 151°14′00″E). The green alga *Ulvella lens* was also tested and was grown on plastic plates at Melbourne University, and maintained at the University of New South Wales (UNSW) at 19°C. *U. lens* induces settlement of the generalist herbivore *Haliotis rubra* (Daume et al. 2000) but was not found at local sites. Recruitment surveys and field experiments were done at Bare Island.

Protocol for settlement assays

Here we define "settlement" as the attachment of a larva to the substrate after metamorphosis. Pieces of algae (50 mg wet weight) were used for assays, with the exception of *U. lens* that was grown on plastic plates and cut into 1-cm^2 pieces and CCA, presented as a minimum of 95% cover on small rocks ~ 1 cm^2. Small rocks and shells as well as sand and shell grit were also used. Each replicate piece of alga, rock or square of plate was selected from a new plant, rock or plate. Assays had ten replicates of each treatment including FSW controls. Algal pieces and five competent larvae were added to sterile 5-ml Petri dishes containing FSW, except for the first algal assay, where only one larva per dish was added, and the initial assay (see Culturing of larvae) testing for gregarious settlement. After 48 h the number of settled individuals per dish was counted using a dissecting microscope and recorded.

Gregarious settlement

To determine if competent larvae respond gregariously to conspecifics, an assay was conducted with densities of 1, 5, 10 and 50 larvae per dish. Positive controls of CCA were included for each density.

Settlement in response to algae and biofilmed (but otherwise non-living) surfaces

Dictyota dichotoma, *Ecklonia radiata*, *Dilophus marginatus*, *Sargassum vestitum*, *Sargassum linearfolium*, *Delisea pulchra*, two species of geniculate coralline algae, *Corallina officinalis* and *Amphiroa anceps*, mixed unidentified CCA, *Gracilaria* sp., *Homeostrichus* sp., *Codium fragile*, *Caulerpa filiformis*, *Enteromorpha* sp., *Ulva australis*, and *Ulva lens* were used to assess the settlement response of larvae to algae from the adult habitat. To assess larval response to biofilmed (but otherwise non-living) surfaces, sand, shell, shell grit and rocks were collected from Shark Bay, in the vicinity of adult *H. erythrogramma*. *C. officinalis* was used as a positive control.

Settlement in response to sterile rocks and *A. anceps*

Larvae settled broadly in response to various surfaces, suggesting a common, broadly distributed cue. To determine if this cue was associated with bacterial biofilms, 20 rocks were collected from Bare Island. Ten of these were scrubbed and autoclaved to remove the surface biofilm. Twenty pieces of *A. anceps* were also collected and ten pieces were autoclaved (but not scrubbed, so as to present the plants intact). The remainder were used as controls.

Controls for effect of antibiotics

The results from Settlement in response to sterile rocks and *Amphiroa anceps* suggested that biofilms could be important as settlement cues. We then conducted a series of experiments aimed at separating the effect of the algae or non-living surfaces (on settlement) from that of their biofilms. Prior to commencing these assays, the effect of antibiotics was assessed.

To test the effects of antibiotics against larvae, a batch was divided into two cultures immediately following fertilisation. One was reared in FSW containing antibiotics (20 mg/l streptomycin sulphate, 10 mg/l penicillin G, 2 mg/l neomycin sulphate and 10 mg/l kanamycin sulphate) and the other in FSW only. An assay was conducted with five larvae from each treatment with either *C. officinalis* or FSW only.

To test the effects of antibiotics on settlement to a known chemical cue, *H. erythrogramma* larvae were exposed to histamine, which induces settlement in some species of sea urchins (Swanson et al. 2004). Treatments were histamine with antibiotics (same concentrations as above) and histamine alone. FSW and *A. anceps* were used as controls.

To test the effects of antibiotics on a cue produced by a plant, the red alga *D. pulchra* was used. *D. pulchra* produces a chemical cue (histamine) that rapidly induces settlement of the sea urchin, *Holopneustes purpurascens* (Swanson et al. 2004). Pieces of *D. pulchra* were submitted to one of the following five treatments, and exposed to larvae.

Antibiotic treatment

Algae were soaked in a 10% Betadine solution in FSW for 5 min, rinsed, then soaked in FSW containing streptomycin sulphate (20 mg/l), penicillin G (10 mg/l), neomycin sulphate (2 mg/l) and kanamycin (10 mg/l) for 48 h (modified from Xue-wu and Gordon 1987; Johnson and Sutton 1994). Algae were rinsed before addition to assay dishes.

Antibiotic treatment with agar wipe

Identical to Antibiotic treatment with the addition of wiping algae over the surface of sterile agar both before soaking in Betadine and after soaking in the antibiotic solution. This treatment was included in order to facilitate the physical removal of the biofilm from the surface of the algae.

Control treatment for the agar wipe process

The plant was wiped over the surface of sterile agar and gently rinsed in FSW.

Control treatment for soaking

Algal pieces were soaked in FSW for 48 h.

Unmanipulated control

A piece of algae.

Bacterial community characterisation

To determine the effect of the antibiotic treatments in removing bacteria from the algal surfaces, the number and diversity of bacteria on each of the treatments was first assessed using culturing methods. *C. officinalis* and *A. anceps* were treated with the same five treatments listed in Controls for effect of antibiotics. Triplicate pieces of each were added to 1 ml FSW, vortexed then serially diluted to 10^{-4}. One hundred microlitres of each dilution was spread evenly onto Marine Agar 2216 plates. Plates were observed daily for 2 weeks, and the number and diversity of colonies per plate counted (Prescott et al. 1995).

To further assess the affect of antibiotic treatments on the microbial community diversity of plants treated with antibiotics, a DNA fingerprinting technique, denaturing gradient gel electrophoresis (DGGE), was done. For each antibiotic treatment, five replicate samples of *C. officinalis* and *A. anceps* were prepared via DNA extraction and polymerase chain reaction (PCR), and run on a BioRad Dcode system following the methods of Dahllöf et al. (2000). DGGE gels were run using the rpoB gene, rather than 16S rDNA as it is known to produce a single band per bacterial species, while 16S rDNA primers can yield up to four bands per species, biasing community analyses (Dahllöf et al. 2000). Three representative samples of each treatment were selected for DGGE analysis.

Antibiotic assays

The above experiments established that antibiotics did not affect a known plant cue and had no apparent direct effect on larval settlement. We then used these techniques to assess whether *H. erythrogramma* larvae responded to a cue emitted by the plant itself, or to the biofilm on the algal surface. The experiment was done using *H. erythrogramma* larvae and the two coralline algal species *A. anceps* and *C. officinalis*, which were subjected to the treatments described in Controls for effect of antibiotics.

Settlement in response to single-strain bacterial films

Each bacterial morphotype cultured as described in this section was re-streaked until purity and stored in 30% glycerol at −80°C. Approximately 125 strains were isolated from *C. officinalis* and a further 125 from *A. anceps*. From these, single-strain biofilms were created by placing five sterile coverslips in 10 ml marine broth inoculated with a single strain. Cultures were grown overnight, allowing biofilms to form on the coverslips. These were then gently rinsed and used in a series of settlement assays (method adapted from Negri et al. 2001).

To determine if any particular phylogenetic group of bacteria were responsible for approximately 70 strains, 35 that induced high settlement, and a further 35 that induced low settlement, were sequenced. Isolates were grown overnight in Marine Broth 2216, spun, and the pellet retained. The DNA extraction procedure was identical to that for the whole community analysis. The 16S rDNA primers used were F27 (5′-GAGTT TGATCCTGGCTCAG−3′) and R1492 (5′-ACGGTT ACCTTGTTACGACTT-3′). One hundred nanograms DNA was added to a 17 µl PCR mixture with

2 µl Sigma REDtaq buffer, 2.5 mM each DNTP, 5 pmol of each primer, 3.2 µg BSA and sterile milliQ water. The PCR conditions were 94°C for 3 min, then 25 cycles each of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. A final extension step of 72°C for 5 min was then performed. Purified PCR product (~ 100 ng) was sequenced unidirectionally using BigDye terminator cycle sequencing reaction mix (Applied Biosystems), with either F27, R1492 or 530F (5′-GT GCCAGCMGCCGCGG-3′), then analysed on an ABI 310 DNA sequencing system. Sequences were analysed using the BLAST search algorithm (Altschul et al. 1990) available through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Larval traps—monitoring recruitment to high- and low-inducing biofilms

Recruitment of *H. erythrogramma* to biofilms was monitored using larval traps from November 2003 until March 2004 at Bare Island, Botany Bay. Traps were made of PVC piping with a rectangular window in the top and lined with Astroturf. Traps were filmed with either the high inducer, *Pseudoalteromonas luteoviolacea* (strain A316, see below) or the low inducer, *Pseudoalteromonas rubra* (strain C312, see below), or used as unfilmed controls. Each week, ten replicates of each treatment were randomly assigned to holders ($n = 30$) secured to the rocky reef at 3 m depth, left in the field, then collected, sealed underwater and replaced with freshly filmed traps. At UNSW traps were examined for recruits.

Traps were sampled to verify the persistence of the biofilm that was incubated inside them. Triplicate swabs were taken from inside three of each treatment at the end of incubation and from three more of each after 48 h in the field. Swabs were wiped over the surface of marine agar plates, and the number and diversity of colonies that grew were recorded. To verify that the purple pigmented colonies were strain A312 (*P. luteoviolacea*), and that the red pigmented colonies were pigmented strain C312 (*P. rubra*), four colonies of each colour were selected from plates and sequenced as described above.

There was no natural recruitment of *H. erythrogramma* to traps. In order to overcome this, larvae were cultured then released in the field. Ten replicates of each trap treatment were prepared, filled with FSW and approximately 150 larvae added to each. A piece of 100-µm mesh was fitted across the window and secured in place with rubber bands, forcing larvae to remain inside the trap. Traps were left in the field for 48 h, collected and examined at UNSW. A second experiment tested the ability of larvae to detect, locate and recruit to biofilms in the field. Ten replicates of each treatment were deployed and approximately 250 larvae were gently syringed into the water adjacent to (< 10 cm) each trap. After 24 h traps were collected and examined at UNSW.

Data analysis

All settlement assays were analysed using SYSTAT (Wilkinson 1997). Data for ANOVAs were checked for normality and heterogeneity of variance using frequency histograms of residuals and plots of residuals versus means, respectively. Arcsin square root transformations were performed where appropriate. Assays that used only one larva per dish were analysed using Pearsons χ^2. Single larva data for the gregarious settlement assay were excluded from the analysis. Post-hoc tests (Tukey's multiple range) were performed where appropriate. Differences in bacterial community composition on seaweeds were analysed using non-metric multidimensional scaling (MDS) and analysis of similarities (ANOSIM) (Clarke and Warwick 1994). Following MDS a one-way ANOSIM was conducted with seaweed as the factor.

Results

Gregarious settlement

There was no evidence of gregarious settlement by *H. erythrogramma* larvae. When larvae were alone in assay dishes, six out of ten settled in response to CCA. This was comparable to higher density treatments (47 ± 7, 45 ± 5 and 46 ± 10%; $n = 5$, 10 and 50 larvae, respectively). Larval settlement also did not increase with increasing density when in the presence of CCA in the dishes (one-factor ANOVA, $F_{2,27} = 0.226$, $P = 0.779$, data arcsin transformed). In sterile seawater alone, no settlement occurred at any density.

Settlement in response to algae and biofilmed (but otherwise non-living) surfaces

Larvae settled in response to most algae tested, although settlement rates varied. Larvae responded most strongly to coralline algae. Three assays were conducted, and the result of one representative assay is given in Fig. 1a ($\chi^2 = 1$, $P < 0.05$, $df = 5$). Over the three assays settlement in response to the coralline red algae *A. anceps*, *C. officinalis* and CCA, the brown alga *S. linearifolium*, and the green alga *C. fragile* was

consistently high. The brown alga *E. radiata*, the green algae *U. australis* and the red alga *D. pulchra* only induced low rates of settlement. No settlement was observed in FSW alone.

Larvae settled in response to all biofilmed surfaces (Fig. 1b, $F_{6,63} = 15.649$; $P < 0.05$). However, the positive control, *C. officinalis*, induced a much higher rate of settlement (94 ± 3%) than rocks (58 ± 9%), sand (50 ± 11%), shell grit (46 ± 10%) and shell (46 ± 14%). All surfaces induced higher rates of settlement than seawater collected from the adult habitat (0 ± 0%) and FSW (10 ± 8%).

Settlement in response to sterile and filmed rocks and algae

"Sterilisation" via autoclaving and, for rocks, scrubbing and autoclaving, reduced settlement on both rocks and

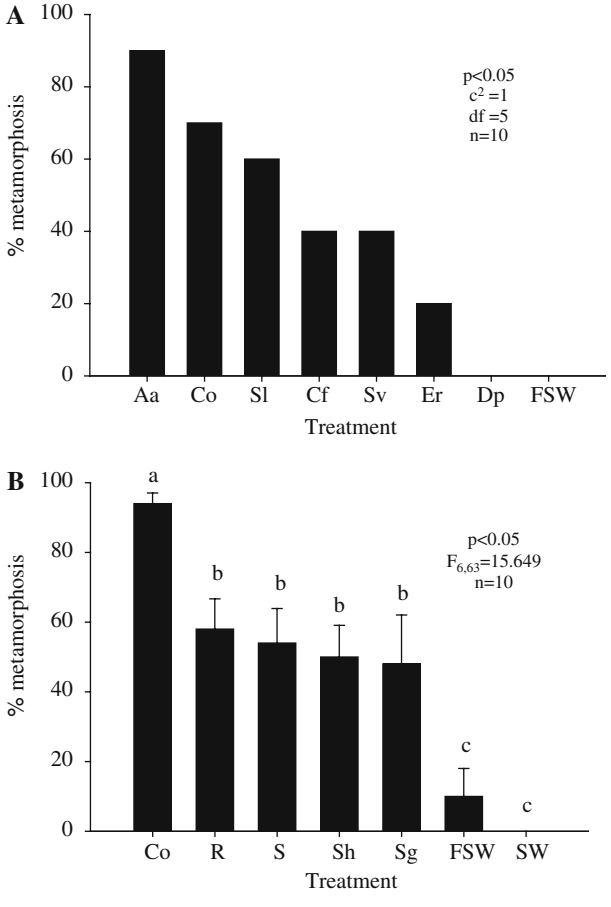

Fig. 1 *Heliocidaris erythrogramma* larval settlement after 48 h in response to **a** local algal species, and **b** biofilmed (but otherwise non-living) surfaces. Data for **a** are proportion of larvae settled, $n = 10$, and **b** mean percent settlement ± SE, $n = 10$. *Aa Amphiroa anceps*, *Co Corallina officinalis*, *Sl Sargassum linearfolium*, *Cf Codium fragile*, *Sv Sargassum vestitum*, *Er Ecklonia radiata*, *Dp Delisea pulchra*, *R* rock, *S* sand, *Sh* shell, *Sg* shell grit, *FSW* filtered seawater, *SW* seawater collected from the adult habitat

A. anceps (Fig. 2, $F_{4,45} = 17.996$; $P < 0.01$). Settlement in response to "sterile" substrata did not differ from that for FSW.

Effect of antibiotics on larvae

Settlement was not affected by the addition of antibiotics to assay dishes (Fig. 3a) as settlement remained high (between 88 ± 64 and 86 ± 5%) for all larvae presented with *C. officinalis* despite being exposed to antibiotics (Fig. 3a, $F_{3,36} = 135.74$; $P < 0.01$).

Effect of antibiotics

H. erythrogramma larvae settled at the same rate in response to histamine, even when antibiotics were included in dishes (Fig. 3b).

Settlement of *Holopneustes purpurascens* was not affected by the addition of antibiotics to *D. pulchra* (Fig. 4, $F_{4,45} = 1.103$; $P = 0.366$). This assay indicates that a known chemical cue arising from a plant was not affected by the antibiotic treatments applied to it. We thus proceeded to extend this treatment to *A. anceps* and *C. officinalis*, the coralline algae that induced settlement of *H. erythrogramma.*

Bacterial abundance and diversity

For both *A. anceps* and *C. officinalis*, the abundance of culturable bacteria was not reduced in the presence of antibiotics, but bacterial diversity was, thus altering the microbial community on the surface of plants (Table 1). The abundance of bacteria actually

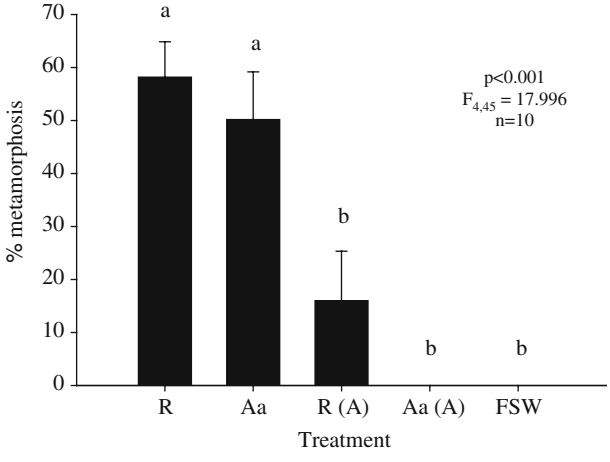

Fig. 2 *H. erythrogramma* larval settlement in response to "sterile" rocks and algae. Data are mean percent settlement ± SE, $n = 10$. *(A)* Rock or alga was autoclaved; for other abbreviations, see Fig. 1

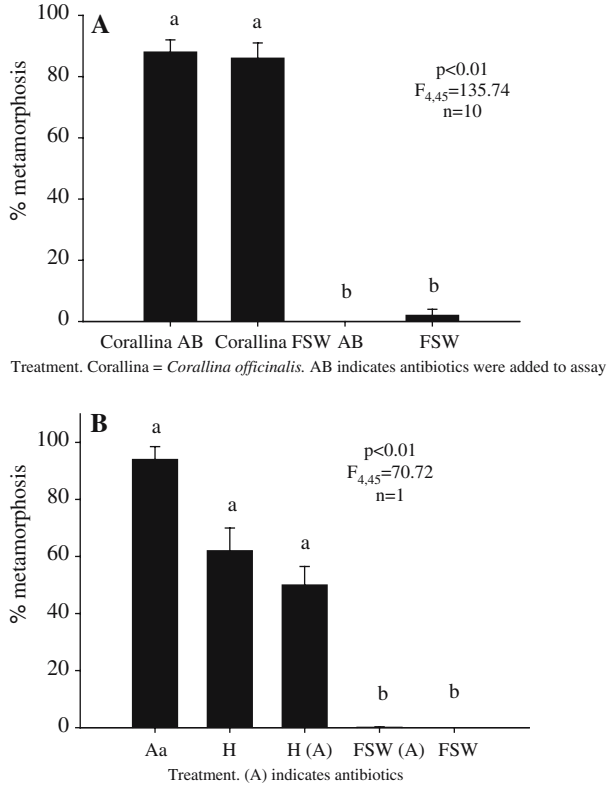

Fig. 3a, b Effect of antibiotics on larvae and on a known chemical settlement cue. **a** *H. erythrogramma* larval settlement after rearing in antibiotics. **b** *H. erythrogramma* larval settlement to histamine (*H*) in the presence (*A*) and absence of antibiotics. Aa was the positive control. For other abbreviations, see Fig. 1

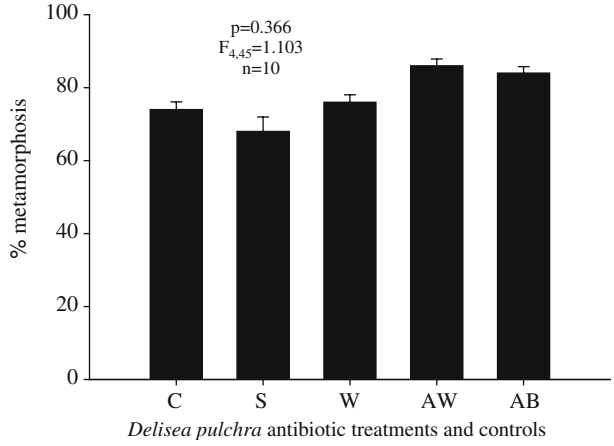

Fig. 4 *Holopneustes purpurascens* settlement in response to *Delisea pulchra* treated with antibiotics. Data are mean percent settlement ± SE, $n = 10$. *C* Unmanipulated control, *S* soaking control, *W* wiping control, *AW* antibiotic treatment plus wipe, *AB* antibiotic treatment

increased on *A. anceps* treated with antibiotics compared to controls. This increase was reduced to natural levels by the agar wipe (Table 1, $F_{4,10} = 7.278$; $P < 0.01$). A similar trend was observed for *C. officinalis* (Table 1, $F_{4,10} = 9.938$, $P = 0.35$). For *A. anceps* treated with antibiotics, the diversity of bacteria was much less than for control plants (Table 1, $F_{4,10} = 15.159$, $P < 0.01$). A similar trend was observed for *C. officinalis* (Table 1, $F_{4,10} = 3.480$; $P = 0.064$).

Molecular analysis also showed that bacterial communities associated with *C. officinalis* and *A. anceps* changed in the presence of antibiotics (*A. ancpes*, $R = 0.558$, significance level 0.1%; *C. officinalis,* $R = 0.343$, significance level 0.3%). Patterns were very similar for both algal species and representative *A. anceps*; figures are shown (Fig. 5). For *A. anceps*, bacterial community composition was >78% similarity within control and unmanipulated plants, whereas

Table 1 Cultured bacterial abundance and diversity on *Amphiroa anceps*[a] and *Corallina officinalis*[b] after treatment with antibiotics. Data are mean numbers per 0.1 g wet weight algae ± SE, $n = 3$. *Letters in parentheses* indicate results of one-way ANOVAs. Data that share a letter do not differ statistically. *FSW* Filtered seawater

Seaweed	Treatment	Diversity (number of colonies per 0.1 g algae ± SE)	Abundance (number of bacteria × 10^5 per 0.1 g algae ± SE)
A. anceps	Soaking control	35.67 ± 3.56 (a)	57.33 ± 0.01 (a)
A. anceps	Unmanipulated control	21.33 ± 3.89 (b)	1.17 ±0.01 (a)
A. anceps	Agar wipe control	13.33 ± 6.18 (bc)	1.19 ± 0.33 (a)
A. anceps	Antibiotics	10.67 ± 0.71 (c)	357.78 ± 0.01 (b)
A. anceps	Antibiotics + agar wipe	5 ± 1.78 (c)	177 ± 0.59 (ab)
C. officinalis	Soaking control	18.67 ± 7.97	2,082.22 ± 1731.26 (a)
C. officinalis	Unmanipulated control	16.33 ± 1.33	39.18 ± 29.88 (abc)
C. officinalis	Agar wipe control	9.33 ± 2.40	3.71 ± 2.12 (bc)
C. officinalis	Antibiotics	3.33 ± 0.33	888.89 ± 528.09 (ab)
C. officinalis	Antibiotics + agar wipe	6.33 ± 0.88	51.56 ± 9.75 (ab)
FSW	–	1.33 ± 0.88	0.01 ± 0.01

[a] *A. anceps* abundance, $F_{4,10} = 7.278$, $P < 0.01$; diversity, $F_{4,10} = 15.159$, $P < 0.01$

[b] Data for *C. officinalis* were log transformed. *C. officinalis* abundance, $F_{4,10} = 9.938$, $P < 0.05$; diversity, $F_{4,10} = 3.480$, $P = 0.064$

those plants treated with antibiotics had < 65% similarity to controls, and most had < 60% similarity (Fig. 5b). For *C. officinalis* all plants treated with antibiotics shared < 70% similarity to controls and unmanipulated plants, with half of these sharing < 50% similarity to controls. Bacterial community diversity within control and unmanipulated plants generally shared at least 75% similarity.

Effect of antibiotics on settlement of *H. erythrogramma*

Settlement in response to both *A. anceps* and *C. officinalis* was significantly reduced in the presence of antibiotics ($F_{4,45} = 13.047$, $P < 0.001$ and $F_{4,45} = 17.063$, $P < 0.01$, Fig. 6). Unmanipulated plants and controls all induced a high rate of settlement.

Settlement in response to single-strain biofilms

Settlement of *H. erythrogramma* on bacterial strains was varied (see Fig. 7). Many strains induced a high settlement response, similar to the coralline algal control. FSW consistently produced negligible settlement and many bacterial strains induced low or no settlement.

Bacteria inducing a high rate of settlement were dominated by the genus *Pseudoalteromonas* (Table 2). *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Photobacterium* and *Alphaproteobacteria* were present in both the high- and low-inductive groups. Other strains that induced high rates of larval settlement included *Alteromonas*, *Aestuariibacter*, *Cytophaga*, *Micrococcus*, *Thalassomonas* and *Pseudomonas*. *Roseobacter*,

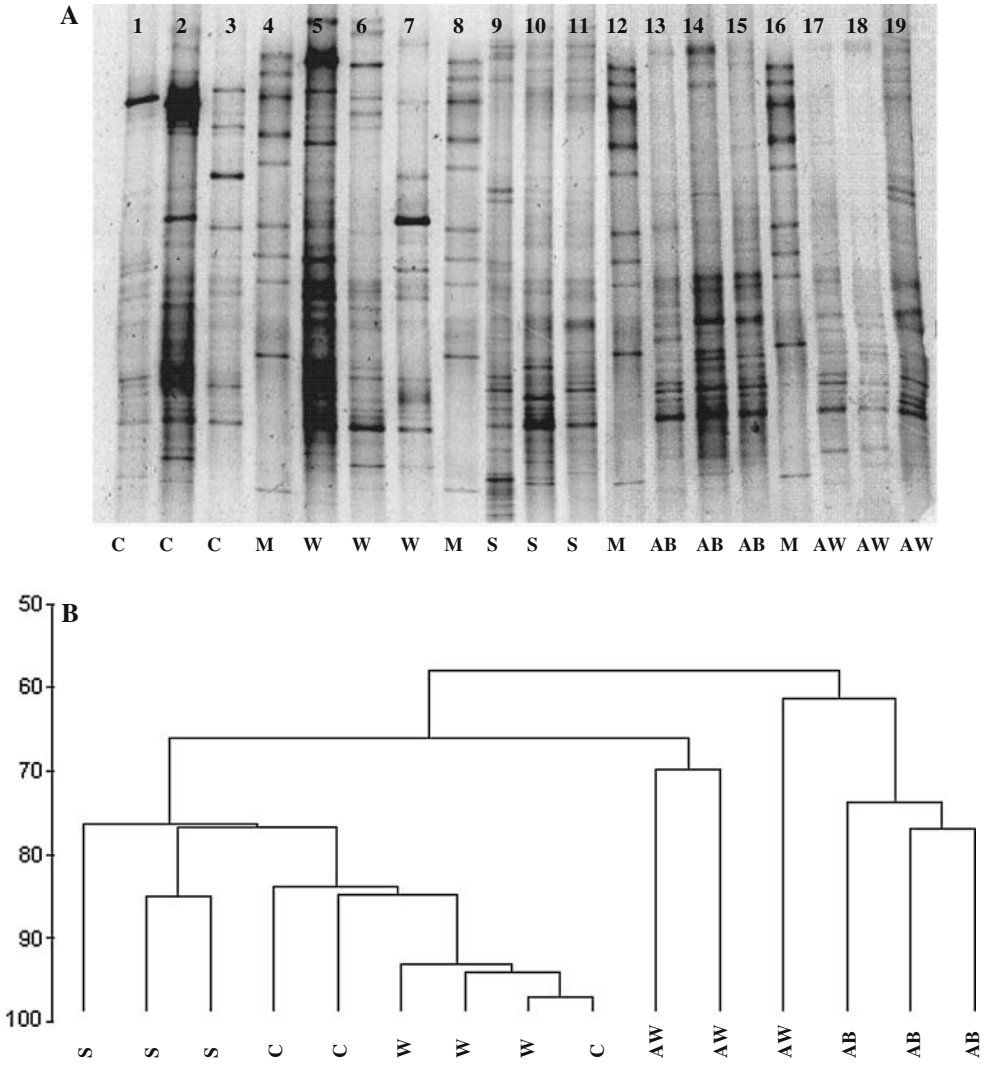

Fig. 5a, b Bacterial community composition of *A. anceps* treated with antibiotics. **a** *rpoB* denaturing gradient gel electrophoresis gel, showing banding patterns across three replicates within each treatment. **b** Cluster diagram representing the presence or absence of bands in **a**. For abbreviations, see Fig. 4. *M* marker lane

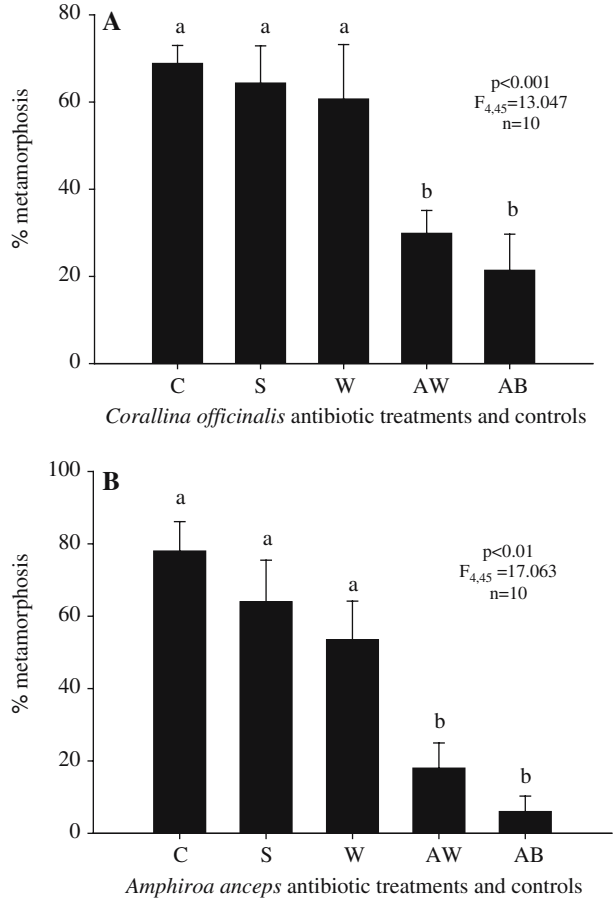

Fig. 6 *H. erythrogramma* larval settlement in response to **a** *A. anceps* and **b** *C. officinalis*, treated with antibiotics. Data are mean percent settlement ± SE. For abbreviations, see Fig. 4

Paracoccus, *Ferrimonas*, *Algoriphagus* and *Ruegaria* were present only among the low inducers.

Counts of bacteria in traps deployed in the field

For both the high and low inducer, biofilms in traps remained dominated by the strain they were inoculated with after 48 h in the field. For traps filmed with *P. luteoviolacea*, a mean of 97.97 ± 1.3% cover was found, and for traps filmed with *P. rubra* a mean of 98.08 ± 1.2% cover was found. Control traps appear to have been slightly contaminated with both *P. luteoviolacea* and *P. rubra*: after forty-eight hours 14.7 ± 6.9% of cells were *P. luteoviolacea* and 0.16 ± 0.16% of cells found in traps were *P. rubra.* This contamination did not result in higher numbers of recruits into traps. For *P. luteoviolacea* and *P. rubra* the three haphazardly selected pigmented colonies matched 100% with the strain they were inoculated with, for > 1,350 bp, validating the culture counts.

Larval traps—monitoring recruitment to high- and low-inducing biofilms

No *H. erythrogramma* larvae were found in any traps, despite traps being in the field for several months during the adult spawning period.

Larval release to traps filmed with high and low inducers

Significantly more larvae metamorphosed in response to high inducers in enclosed traps in the field than to either the unfilmed or filmed control (Fig. 8, $F_{2,27} = 20.69$, $P < 0.001$). Furthermore, larvae released near traps recruited to traps filmed with the high inducer more than to traps filmed with the low inducer or the unfilmed control ($F_{2,27} = 3.354$, $P = 0.054$). This result was not significant as recruitment was very low, with a mean of only 7.1 ± 1.8 urchins recruiting to traps filmed with the high inducer.

Discussion

There is a growing body of evidence that suggests that generalist herbivores have broad patterns of settlement. Urchin examples include *Evichinus chloroticus*, which settles in response to rocks, shells, coralline algae and biofilms (Lamare and Barker 2001), *Paracentrotus lividus* which settles in response to algae, and detritus particles (Gosselin and Jangoux 1996) and *Strongylocentrotus droebachiensis* which settles in response to various red and brown macroalgae, filmed cobbles and biofilms (Pearce and Scheibling 1991). Abalone also settle in response to a suite of stimuli including macroalgae (Daume et al. 2000), diatoms (Gordon et al. 2004), mucus trails (Gallardo and Buen 2003) and coralline algae (Daume et al. 1999). *H. erythrogramma* also exhibits high settlement in response to a range of macroalgae and biofilmed surfaces. The response of these larvae of generalist herbivores to a range of stimuli indicates that either: (1) larvae are responding to a variety of different types of cues, or (2) larvae are responding to one cue that is shared by all of these stimuli. Conversely, specialist marine herbivores with narrow host ranges often settle specifically in response to host-derived cues. For example *Alderia modesta*, metamorphoses exclusively in response to the obligate adult food, the green alga *Vaucheria longicaulis* (Krug 2001). Other ascoglossans including several *Aplysia* species (Switzer-Dunlap and Hadfield 1977; Switzer-Dunlap 1978) and *Placida dendritica* (Towbridge 1992) also settle in response to algae that

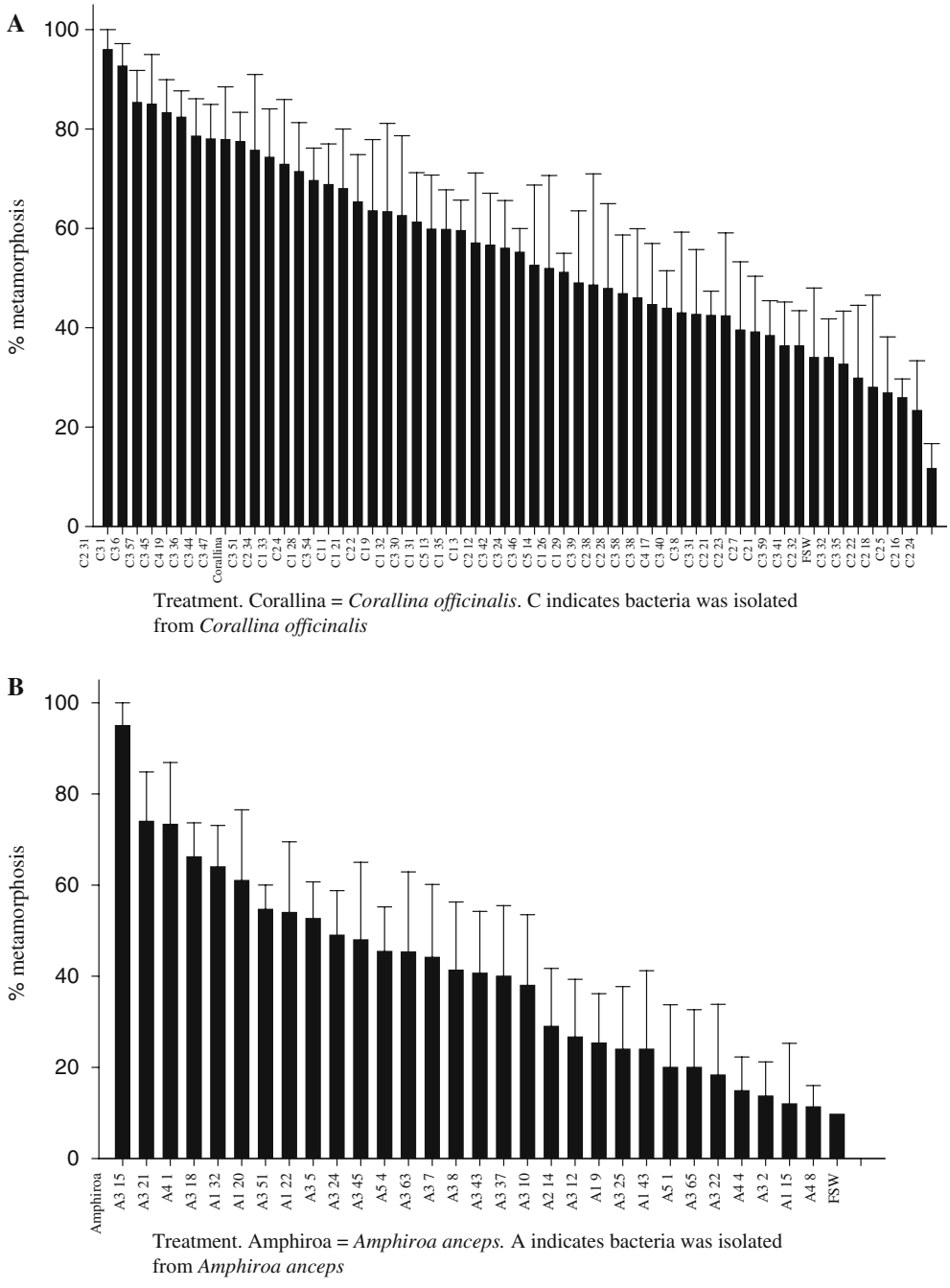

Fig. 7 *H. erythrogramma* larval settlement in response to bacteria isolated from the surface of **a** *C. officinalis* and **b** *A. anceps*. Data are mean percent settlement ± SE, $n = 5$. *C* Strains from *C. officinalis*, *A* strains from *A. anceps*, *Corallina C. officinalis*, *Amphiroa A. anceps*

constitute the preferred adult diet. For larvae such as these, the restricted diet of adults appears to be reflected in the restricted response of larvae to stimuli for settlement. Larvae will only settle when they locate the required adult habitat.

Our results indicate that bacterial biofilms, associated with a range of algae and non-living surfaces, are important for *H. erythrogramma* larval settlement. Interestingly, phylogenetically similar bacterial species do not necessarily have the same effect on eukaryote larval settlement. Some bacteria within certain genera induce settlement of *H. erythrogramma*, while others from the same genera had no effect. Perhaps some bacteria within these groups are capable of producing biologically active metabolites, while others are not. Larvae of the polychaete *Hydroides elegans* also show both high and low settlement to various bacterial isolates (Unabia and Hadfield 1999) and strains

Table 2 16S rDNA sequence analysis of isolates. *H* High inducer, *L* low inducer

Isolate[a]	Accession number	BLAST closest match	Percentage of similarity	Accession number of closest match	Inducer
A14	DQ005853	*Aestuariibacter halophilus*	94.9	AJ391191	H
C115	DQ005890	*Alphaproteobacteria*	94.5	AF365994	H
A323	DQ005873	*Alteromonas* sp.	96.7	AJ391191	H
C239	DQ005899	*Cytophaga* sp.	95.9	ABO73567	H
C327	DQ005903	Marine gammaproteobacterium	96.8	AF366050	H
C36	DQ005885	*Micrococcus* sp.	96.9	AY258119	H
A137	DQ005863	*Photobacterium* sp.	97.3	AY582934	H
C16	DQ005883	*Photobacterium* sp.	93.1	AY147861	H
C419	DQ005906	*Photobacterium* sp.	97	AJ842344	H
A360	DQ005878	*Pseudoalteromonas bacterolytica*	94.2	AF173962	H
A41	DQ005858	*Pseudoalteromonas denitrificans*	97.7	X82138	H
C59	DQ005888	*Pseudoalteromonas denitrificans*	97.9	X82138	H
A316	DQ005867	*Pseudoalteromonas luteoviolacea*	99.4	X82144	H
A13	DQ005852	*Pseudoalteromonas* sp.	98.9	AY217773	H
A213	DQ005864	*Pseudoalteromonas* sp.	99.6	AJ391204	H
A22	DQ005854	*Pseudoalteromonas* sp.	98.7	AJ874351	H
A315	DQ005866	*Pseudoalteromonas* sp.	99.1	U80834	H
A321	DQ005871	*Pseudoalteromonas* sp.	96.7	AJ874351	H
A359	DQ005877	*Pseudoalteromonas* sp.	94.7	AY626830	H
C345	DQ005904	*Pseudoalteromonas* sp.	97.2	AJ874351	H
C127	DQ005892	*Pseudomonas* sp.	97.3	AFS00211	H
C235	DQ005898	*Pseudomonas* sp.	97.2	AFS00211	H
C123	DQ005891	*Pseudomonas stutzeri*	97.2	AY125329	H
A367	DQ005879	*Shewanella* sp.	97.0	AF145921	H
C111	DQ005889	*Shewanella* sp.	97.3	AF145921	H
C136	DQ005894	*Shewanella* sp.	94.8	AF145921	H
A317	DQ005868	*Shewanella waksmanii*	99	AY170366	H
C357	DQ005905	*Shewanella waksmanii*	95.0	AY170366	H
A510	DQ005882	*Thalassomonas viridans*	95.6	AJ294748	H
A318	DQ005869	*Vibrio mediterranei*	98.4	X74710	H
A53	DQ005859	*Vibrio pomeroyi*	99.7	AJ491290	H
A358	DQ005880	*Vibrio ponticus*	93.5	AJ630103	H
C130	DQ005893	*Algoriphagus winogradskyi*	96.6	AJ575263	L
C319	DQ005901	*Alphaproteobacteria*	97.1	AF186699	L
C512	DQ005907	*Ferrimonas balaerica*	93.6	X93021	L
C47	DQ005886	*Gammaproteobacteria*	94.9	AY207503	L
C218	DQ005896	Marine bacteria	96.0	AY626827	L
A510	DQ005881	*Paracoccus* sp.	94.8	AB008115	L
C222	DQ005897	*Photobacterium eurosenbergii*	96.9	AJ842344	L
A55	DQ005860	*Pseudoalteromonas denitrificans*	97.1	X82138	L
C312	DQ005900	*Pseudoalteromonas rubra*	96.9	X82147	L
A25	DQ005851	*Pseudoalteromonas* sp.	96.8	AY626830	L
A349	DQ005875	*Pseudoalteromonas* sp.	96.3	AY258115	L
A58	DQ005861	*Pseudoalteromonas* sp.	98.2	AY626830	L
A333	DQ005874	*Reugeria* sp.	96.2	AJ391197	L
C23	DQ005884	*Roseobacter* sp.	96.5	DQ008594	L
C216	DQ005895	*Roseobacter* sp.	95.6	AJ534238	L
A311	DQ005865	*Shewanella waksmanii*	99.3	AY170366	L
C321	DQ005902	*Shewanella waksmanii*	97.3	AY170366	L
A115	DQ005862	*Silicibacter lacuscaerulensis*	98.9	U77644	L
A356	DQ005876	*Vibrio harveyi*	97.3	AY967728	L
C53	DQ005887	*Vibrio pomeroyi*	96.3	AJ491290	L
A32	DQ005856	*Vibrio* sp.	99.7	AB180386	L
A322	DQ005872	*Vibrio splendidus*	96.9	AY620972	L
A319	DQ005870	*Vibrio splendidus*	95.2	AJ874361	L
A31	DQ005855	*Vibrio* sp.	99.3	AJ845021	L

[a] Codes for isolates are from Fig. 7 and remaining settlement assays

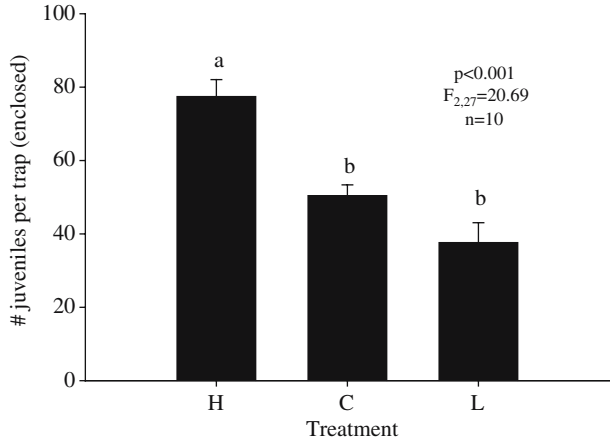

Fig. 8 *H. erythrogramma* settlement in traps in response to the high-inducing biofilm *Pseudoalteromonas luteoviolacea* (*H*), the unfilmed control (*C*), and the low-inducing biofilm *Pseudoalteromonas rubra* (*L*). Larvae were enclosed in traps. Data are mean percent settlement ± SE, $n = 10$

inducing both responses have now been identified as belonging to the genera *Pseudoalteromonas*, *Vibrio*, and *Alteromonas* (Lau et al. 2002). These studies present evidence for a number of different bacteria inducing the same settlement response from a eukaryotic larva, suggesting a redundancy in the function of bacteria on the surface of coralline algae (this study) and on an artificial settlement surface (Unabia and Hadfield 1999; Lau et al. 2002). In both cases, there was not simply a single bacterial species that indicated a suitable settlement surface, but rather a diverse range each performing the same function. While a wide taxonomic range of bacteria induce settlement of these larvae, strains of *P. luteoviolacea* has now been identified as inducing a high settlement response for two species of invertebrates: *H. elegans* (Huang and Hadfield 2003) and *H. erythrogramma* (this study).

H. erythrogramma larvae, when enclosed in traps in the field, metamorphosed in significantly higher numbers on *P. luteoviolacea* than on either the low-inducing biofilm *P. rubra* or the unfilmed control. Raimondi and Morse (2000) were also able to demonstrate that the coral *Agaricia humilis* settles at various depths inside traps in the field in response to a chemical cue. To our knowledge, ours is the first field experiment to show that larvae are able to respond to a characterised bacterial biofilm in the field. Browne and Zimmer (2001) demonstrated that when larval traps emitting tracers of a waterborne peptide were deployed in an estuary, significantly more *Balanus amphitrite* larvae were able to successfully recruit in response to the presence of the peptide. For *H. erythrogramma* larvae, when larvae were released into the water column near traps, only a very small number of individuals were able to locate the inducing biofilm. One explanation is that local hydrodynamic conditions may have transported larvae away from traps. Alternatively larvae in traps may have been subject to heavy predation. Demonstrations of the effects of settlement inducers in the complex ecological and hydrodynamic environment of natural habitats remain a significant challenge for studies of settlement cues.

Bacteria that induced high rates of settlement of *H. erythrogramma* larvae were dominated by *Gammaproteobacteria* contributing to a growing body of work suggesting that *Gammaproteobacteria* are important as inducers of larval settlement. *Pseudomonas* A3 induces larval settlement of the coral larvae *Acropora millepora* and *A. willisae* (Negri et al. 2001), the scyphozoan larvae *Cassiopea andromeda* settle when exposed to *Vibrio* sp. (Hofmann and Brand 1987), and bacteria from the genus *Alteromonas* induce settlement of larvae of the hydrozoan *Hydractinia echinata* (reviewed by Leitz 1997). The fouling polychaete *H. elegans* also settles to a range of bacterial isolates dominated by *Gammaproteobacteria* (Lau et al. 2002), exopolymers produced by a marine *Pseudoalteromonas* strain enhance attachment of the ascidian *Ciona intestinalis* (Szewzyk et al. 1991) and *Alteromonas colwelliana* promotes settlement by larvae of the oyster *Crassostrea gigas* (Weiner et al. 1989). *Gammaproteobacteria*, particularly bacteria from the *Pseudoalteromonas*, are frequently isolated from eukaryotic hosts such as marine algae (reviewed by Holmström and Kjelleberg 1999) and are known to produce biologically active metabolites that mediate interactions such as antifungal (Barbieri et al. 2001), antifouling (Holmström et al. 1992; Holmström and Kjelleberg 2000) and antibacterial activity (Hentschel et al. 2001). Given their broad association with eukaryotic hosts in the marine environment, it is not surprising that these bacteria are important as mediators of settlement.

Most studies that have sought to investigate the importance of surface-associated bacteria to settlement of marine invertebrate larvae have focused only on the members of the bacterial community that are able to be cultured. Ours, and other recent studies, employ a more holistic approach through use of various molecular techniques (e.g. Webster et al. 2004; Lau et al. 2005). DGGE, a DNA fingerprinting method that compares microbial community diversity among samples, revealed that community composition of biofilms on plants is important for larval settlement of *H. erythrogramma.* Similarly, two species of barnacles, *B. amphitrite* and *Balanus trigonus*, also display differential settlement in response to changes in biofilm commu-

nity composition (Lau et al. 2005). Conversely, Webster et al. (2004) were unable to detect differences between coral reef biofilms that did and did not induce coral larval settlement. They concluded that differential coral settlement was due to changes of single bacterial species within biofilms which could not be detected using the techniques employed. Future development and application of these and other molecular techniques should substantially increase our understanding of important components of marine biofilms for larval settlement.

Settlement assays with larvae and surfaces treated with antibiotics can be challenging because of potential artefacts (Johnson and Sutton 1994). To control for the possible effect of antibiotics on algae, we tested the settlement response of larvae of the sea urchin *H. purpurascens*, which settles in response to histamine isolated from the host alga *D. pulchra* (Swanson et al. 2004), in the presence and absence of antibiotics. Both our study and Swanson et al. (2004) found that the application of antibiotics did not reduce the settlement response to *D. pulchra* by *H. purpurascens* larvae, suggesting that antibiotics do not affect the efficacy of this algal chemical cue. The application of antibiotics to two coralline algae saw a corresponding drop in *H. erythrogramma* larval settlement. This drop was not seen in the equivalent assays using *H. purpurascens* larvae, suggesting that different sources are responsible for the settlement cues of these co-occurring urchin species, and that the cue for settlement by *H. erythrogramma* larvae is not an algal chemical cue. We also tested the response of larvae that were reared in FSW containing antibiotics and larvae that were exposed to antibiotics during assays in comparison to controls. These experiments indicated that antibiotics did not have any visible effects on the larval settlement behaviour of *H. erythrogramma.*

Many larvae settle in response to coralline algae (reviewed by Roberts 2001). For *Acanthaster planci* larvae, no settlement occurs on either boiled or autoclaved CCA (Johnson et al. 1991b). However, neither mixed- nor single-strain biofilms of bacteria isolated from CCA induce settlement, initially suggesting that induction is not bacterially induced (Johnson et al. 1991b). Further experiments indicated that when bacteria were re-infected onto CCA previously treated with antibiotics, settlement was once again strongly induced (Johnson and Sutton 1994). Another interesting case is for larvae of the corals *A. willisae* and *A. millepora* (Negri et al. 2001). Both species settle in response to CCA; however this response is not reduced either through treatment of CCA with antibiotics, or by autoclaving. A single bacterium, isolated from CCA, was able to induce settlement, but only in the presence of *Porites* sp. (Negri et al. 2001). These studies suggest that bacteria isolated from CCA are important for larval settlement, but that compounds from a source of calcium carbonate, such as CCA, are also needed to provide a cue. This was not the case for *H. erythrogramma* larvae though, as single-strain biofilms grown on glass slides were able to induce rates of settlement as high as on coralline algae (Fig. 7).

While the DGGE analyses conducted here include changes in cyanobacteria, one aspect of biofilms that has not been specifically investigated in this study is the photosynthetic component. Changes to ephemeral algal cover by grazers can impact the composition of intertidal community assemblages (Anderson and Underwood 1997) and the availability of both bacteria and diatoms impacts the growth and success of intertidal grazing molluscs (Thompson et al. 2000). Furthermore, for a number of invertebrate larval species, both the bacterial and diatom components of biofilms are important for settlement (Harder et al. 2002; Lau et al. 2003; Dahms et al. 2004; Patil and Anil 2005), and the importance of diatoms for settlement of abalone larvae has been well documented (reviewed by Roberts 2001). While we have demonstrated the importance of bacteria in settlement of *H. erythrogramma* larvae, it remains possible that diatoms and unicellular algal species within mixed biofilm communities also influence larval settlement of this species.

Here we have addressed the hypothesis that larvae of generalist herbivores settle in response to broadly distributed settlement cues, such as bacterial biofilms. A wide range of algae and biofilmed (but otherwise non-living) surfaces all induced settlement of *H. erythrogramma* larvae, with coralline algae inducing settlement at the highest rates. Settlement was reduced when coralline algae were autoclaved and when they were treated with antibiotics. Antibiotic treatments caused a drop in the diversity of culturable bacteria and a shift in the microbial community diversity. A diverse range of bacteria isolated from coralline algae also induced settlement of larvae, both in the laboratory and in the field. The distribution of new recruits in the field confirms the settlement results obtained in the laboratory—recruitment predominantly occurs on coralline algae with small numbers of recruits also found on co-occurring algae and rubble (M. J. Huggett et al., in preparation). These results support the hypothesis that larvae are responding to biofilm cues, and that these biofilms are likely to be distributed across many algal species and biofilmed (but otherwise non-living) substrata. However, we have also addressed this hypothesis for larvae of the generalist herbivore *H.*

rubra (Huggett et al. 2005). In contrast to the results found here for *H. erythrogramma*, *H. rubra* larval settlement does not appear to be primarily driven by biofilm-based cues, despite displaying broad spectrum settlement to a range of macroalgae. The difference in the types of cues that are important for larval settlement of *H. rubra* and *H. erythrogramma* indicate that despite similarities in adult habitat use, generalist invertebrate herbivores do not all respond to the same type of cues, and in particular, that while bacterial biofilm cues are important for some generalist larvae, including *H. erythrogramma*, they are not necessarily a source of settlement cues for all generalists.

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