Investigating a possible role for the bacterial signal molecules N-acylhomoserine lactones in *Balanus improvisus* cyprid settlement

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Abstract

Increased settlement on bacterial biofilms has been demonstrated for a number of marine invertebrate larvae, but the nature of the cue(s) responsible is not well understood. We tested the hypothesis that the bay barnacle *Balanus improvisus* utilises the bacterial signal molecules N-acylhomoserine lactones (AHLs) as a cue for the selection of sites for permanent attachment. Single species biofilms of the AHL-producing bacteria *Vibrio anguillarum*, *Aeromonas hydrophila* and *Sulfitobacter* sp. BR1 were attractive to settling cypris larvae of *B. improvisus*. However, when AHL production was inactivated, either by mutation of the AHL synthetic genes or by expression of an AHL-degrading gene (aiiA), the ability of the bacteria to attract cyprids was abolished. In addition, cyprids actively explored biofilms of *E. coli* expressing the recombinant AHL synthase genes *luxI* from *Vibrio fischeri* (3-oxo-C6-HSL), *rhlI* from *Pseudomonas aeruginosa* (C4-HSL/C6-HSL), *vanI* from *V. anguillarum* (3-oxo-C10-HSL), and *sulI* from *Sulfitobacter* sp. BR1 (C4-HSL, 3-hydroxy-C6-HSL, C8-HSL and 3-hydroxy-C10-HSL), but not *E. coli* that did not produce AHLs. Finally, synthetic AHLs (C8-HSL, 3-oxo-C10-HSL and C12-HSL) at concentrations similar to those found within natural biofilms (5 µM) resulted in increased cyprid settlement. Thus, *B. improvisus* cypris exploration of and settlement on biofilms appears to be mediated by AHL signalling bacteria in the laboratory. This adds to our understanding of how quorum sensing inhibition may be used as for biofouling control. Nonetheless, the significance of our results for larvae settling naturally in the field, and the mechanisms that underlay the observed responses to AHLs, are as yet unknown.

Key words: *Balanus improvisus*, settlement response, quorum sensing, N-acylhomoserine lactone, biofilm

Introduction

Many reports have described enhanced settlement of algal spores and invertebrate larvae on bacterial biofilms, (e.g. ascidians, barnacles, bryozoans, corals, echinoderms, polychaetes, molluscs, and sponges; reviewed by Wieczorek and Todd, 1998; Hadfield and Paul, 2001; Hadfield 2011). The microbially-derived agents that mediate this induction are not only important for selection of surfaces, but can also trigger metamorphological events in certain species (Wieczorek and Todd, 1998; Hadfield and Paul, 2001; Hadfield 2011). Reports of both surface-attached and water-bourne attractants derived from microbial films have been described (Leitz and Wagner, 1993; Wieczorek and Todd, 1988; Harder et al., 2002), but until recently, very few have identified the cue responsible (reviewed in Hadfield, 2011). There is evidence to suggest that for larvae of some marine invertebrates, the receptor that detects the presence of a biofilm is a lectin. This includes the spirorbid polychaete *Janua brasiliensis* (Maki and Mitchell, 1985), the ascidians *Herdmania curvata* (Woods et al., 2004) and *Boltenia villosa* (Roberts et al., 2007), and the barnacle *Balanus amphitrite* (Khandeparker et al., 2003). In addition Grasso et al. (2008) found high levels of transcripts for a protein that includes a C-type lectin domain in the anterior tip of larvae of the coral *Acropora millepora*. In the polychaete, *Hydroides elegans*, inhibiting the activity of a p38 mitogen-activated protein kinase inhibited the biofilm-induced larval settlement (Wang and Qian, 2010), and a similar protein has been shown to regulate settlement of the barnacle *Balanus amphitrite* (He et al, 2012).

An alternative settlement cue has been described for the zoospores of the problematic biofouling macro-algae *Ulva*: N-acylhomoserine lactone signal molecules (AHLs) (Joint et al., 2002). Production of AHLs by biofilms affect swimming behaviour of the zoospores through a process of chemokinesis, which brings about decreased swimming speed (Wheeler

et al., 2006) and increased settlement within areas of high AHL production, such as dense biofilm micro-colonies (Tait et al., 2005). These AHL signal molecules are used by bacteria to co-ordinate their behaviour on a population level: a process known as 'quorum sensing' (QS). QS links the concentration of signal molecule to the expression of multiple genes, including those involved in secondary metabolism, virulence and biofilm development in a variety of bacteria (Swift et al., 2001). Along with Proteobacteria, AHL-production has been reported in Cyanobacteria and Bacteroidetes (Sharif et al., 2008; Huang et al., 2008), indicating AHL-mediated signalling is particularly widespread amongst marine bacteria. Specialist niches, such as biofilms, promote the growth of dense microbial populations in which AHL signalling can be detected (Huang et al., 2009), and concentrations of AHLs of ~ 600 pmol cm^{-2} can be detected within natural rocky shore biofilms (Tait et al. 2009).

Since the initial discovery of the involvement of AHLs in *Ulva* zoospore settlement, N-butanoyl-L-homoserine lactone (C4-HSL) has been shown to up regulate sporulation in the red algae *Acrochaetium* sp. (Weinberger et al., 2007) and a possible role for QS has also been suggested in the settlement of invertebrate larvae: using the QS blockers 5-hydroxy-3[1(R)-1-hydroxypropyl]-4-methylfuran-2(5H)-one, (5R)-3,4-dihydroxy-5-[(1S)-1,2-dihydroxyethyl]furan-2(5H)-one and triclosan Dobretsov et al. (2007) inhibited the establishment of a bacterial biofilm, and thereby decreased the settlement of larvae of the polychaete *H. elegans* and the bryozoan *Bugula neritina*. However, although synthetic AHLs (> 100 μM) induced crawling behaviour in *H. elegans* (a prerequisite to larval settlement) none of the AHLs tested induced larval settlement to the same extent as natural biofilms (Huang et al., 2007). Dobretsov et al. (2009) also refers to similar but unpublished results for the barnacle *Balanus amphitrite*.

The response of *B. amphitrite* to bacterial biofilms has been the most widely studied, but several other barnacle species are known to settle preferentially on bacterial biofilms, including *Balanus improvisus* (O'Connor and Richardson, 1996), *Balanus trigonus* (Thiyagarajan et al., 2006), *Semibalanus balanoides* (Thompson et al., 1998) and *Elminius modestus* (Neal and Yule, 1994). Although the response of barnacles to a glycoprotein termed settlement-inducing complex (SIPC), isolated from adult shells has been well documented (Matsumura et al., 1998; Dreanno et al., 2007), the nature of the cue derived from biofilms is not well understood. It is possible that marine biofilms produce a compound similar to SIPC, or that they are likely to be responding to multiple cues (Hadfield, 2011) such as a component of biofilm EPS (Khandeparker et al., 2003) or alternative, currently undetermined biofilm properties. Interestingly, for B. amphitrite, it is known that settling cypris larvae can distinguish between biofilms of varying community composition, preferring to settle on biofilms characteristic of their adult habitat (Lau et al., 2005).

The aim of the present study was to assess the impact of AHL signals on settlement of cypris larvae of the bay barnacle *B. improvisus*. This invasive species is thought to have originated in North America, but now has a world-wide distribution as a result of dispersal as a biofouling agent on the hulls of ships. Similar to the more widely studied *B. amphitrite* (Harder et al., 2001; Qian et al., 2003; Hadfield, 2011), *B. improvisus* has been shown to settle preferentially on bacterial biofilms (O'Connor and Richardson, 1996). There are, however, key differences: *B. amphitrite* has a preference for hydrophilic surfaces, but the presence of older biofilms enhance larval attachment, irrespective of the type of substrate (Hung et al., 2008). In contrast, *B. improvisus* has shown a clear preference for hydrophobic substrates (Dahlström et al., 2004) and smooth substrata (Berntsson et al., 2000), and the presence of a biofilm can alter the response of *B. improvisus* cyprids to particular surfaces,

decreasing detachment to hydrophobic polystyrene but increasing attachment to hydrophilic glass (O'Connor and Richardson, 1996). This indicates that the nature of the biofilm and perhaps also the *B. improvisus* cyprid-settlement cue may be altered by properties of the underlying substratum.

To investigate the role of AHL signal molecules on the settlement of cyprid larvae of *B. improvisus*, we adapted methodologies used to investigate the role of AHLs in Ulva zoospore settlement (Joint et al., 2002; Tait et al., 2005). Live single species biofilms of the marine bacteria *Vibrio anguillarum*, *Aeromonas hydrophila* and *Sulfitobacter* sp. BR1 were used to provide a natural supply of AHL signal, and the response of *B. improvisus* cyprids compared with AHL-deficient variants of the three strains. Attempts were also made to assess cyprid responses to biofilms of *E. coli* expressing recombinant AHL synthases, as well as to synthetic AHLs.

Materials and Methods

Bacterial strains

All bacterial strains and plasmids are described in Table 1. The influence of AHL signal molecules on cyprid settlement were assessed using three AHL-producing strains and their signal-deficient mutants *V. anguillarum* and *A. hydrophila* each contain a mutation to the AHL synthases: *vanM* in *V. anguillarum* (Tait et al., 2005) and *ahyI* in *A. hydrophila* (Lynch et al., 2002). In addition, we also used a strain of *V. anguillarum* that expresses an inducible copy of *aiiA*, a lactonase enzyme which has been shown to degrade AHLs (Tait et al., 2005). An AHL-deficient variant of *Sulfitobacter* sp. BR1 was constructed first by transforming with the luxR::luxI' Gfp-based AHL reporter plasmid pRK-C12 (Reidel et al., 2001) to produce a strain that self-reported AHL production (BR1 pRK-C12). Transposon mutagenesis of BR1

pRK-C12 with the EZ-Tn5™ <R6Kγori /KAN-2>Tnp Transposome kit (Epicentre Biotechnology) was used to randomly mutate the genome of BR1. The transformants were plated onto marine agar containing both gentamicin and kanamycin and the colonies were then screened for the lack of Gfp production. The absence of AHL production was confirmed in dark colonies. As EZ-Tn5™ contains its own origin of replication the insertion site was located by extracting the DNA (DNeasy extraction kit, Qiagen), partially digesting the DNA with EcoRV and self-ligating to form mini-plasmids. *E. coli* pir+ was transformed with the ligated DNA fragments and kanamycin resistant colonies selected. An insertion in a gene with homology to luxI genes was located and designated *sulI*. This gene was amplified from BR1 using the primers sulIF (AGTTGCGATCATGGCAGAACC) and sulIR (TACAAGGATATCGACCAGCA), cloned into pGEM to generate pKT11 and transformed into chemically competent JM109. Using thin layer chromatrography (TLC) plates overlaid with the AHL biosensor *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF372) (Fuqua and Winans, 1996), AHL production by wildtype BR1 and *E. coli* pKT11 was clearly visible, but there was no AHL production in BR1 with the mini-Tn5 insertion in the *sulI* gene (Figure 1). Culture supernatants of the BR1 WT, the *sulI* mutant and *E. coli* pKT11 were extracted with dichloromethane and evaporated to dryness. The extracts were applied to RP18 F_{245} TLC plates (20 x 20 cm; VWR International) and a mobile phase of 60% (v/v) methanol used to separate the extracts. TLC plates were overlaid with the biosensor NTL4 (pCF218; pCF372) (Fuqua & Winans, 1996) following the methodology of Mohammed et al., (2007). Following incubation at 30 °C overnight, the TLC plates were examined for the presence of blue spots, indicative of AHL production. The same AHLs produced by the BR1 WT were also produced by the *E. coli* expressing the recombinant *sulI*. No AHLs were detected in the presence of the BR1 *sulI* mutant, confirming the disruption to the AHL synthases in this bacterium.

The miniTn7 system developed by Lambertsen et al. (2004) was used to make Gfp-tagged varients of *V. anguillarum* WT and the *vanM* mutant. A four parental mating between *V. anguillarum* NB10 or DM28 (recipients), *E. coli* pRK6000 (conjugation helper), *E. coli* pMiniTn7(Gm)P_{rrnB1}gfp.-a (donor) and *E. coli* pUX-BF13 (transposition helper) was carried out, and transconjugants selected on TSB supplemented with 50 μg ml^{-1} gentamycin. Site-specific insertion of Tn7 downstream of the *glmS* gene was verified by PCR (Lambertsen et al., 2004).

Escherichia coli JM109 biofilms expressing *vanI* from *V. anguillarum* (producing 3-oxo-C10-HSL), *luxI* from *Vibrio fischeri* (3-oxo-C6-HSL), *rhlI* from *Pseudomonas aeruginosa* (C4-HSL/C6-HSL) and *sulI* from *Sulfitobacter* sp. BR1 (C4-HSL, 3-hydroxy-C6-HSL, C8-HSL and 3-hydroxy-C10 (Figure 1) were compared to biofilms containing the vector plasmids without the luxI homologues (Table 1).

Sulfitobacter sp. BR1 was routinely grown in Difco Marine Broth. *V. anguillarum* strains were grown in Tryptic Soy Broth (TSB), and *A. hydrophila* strains and *E. coli* strains in Luria Broth. Temperatures for incubation were 37 °C for *E. coli* and 25 °C for *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* sp. BR1.

Preparation of biofilms

Biofilms were prepared as previously described (Tait et al., 2005). Briefly, cultures were grown overnight in rich media, the cells harvested by centrifugation, washed and resuspended in sterile, filtered seawater (0.2 μm, salinity 15 ‰) to an OD of 1.0. Varying volumes of cell suspension (50 – 100 μl) were used to inoculate biofilm culture vessels which contained 10 ml sterile, filtered seawater (0.2 μm, salinity 15 ‰) and sterile microscope cover glasses, and

the vessel incubated for 24 h at room temperature. By adjusting the volume of the inocula, similar densities of signal-producing and non-producing biofilms were achieved.

Preparation of *Balanus improvisus* cyprids and settlement assays

Balanus improvisus cyprids were reared in a laboratory culture system at the Sven Lovén Centre for Marine Sciences in Tjärnö, Sweden as described by Berntsson et al. (2000). Settlement assays were performed by placing cover glass biofilms, synthetic AHLs plus clean cover glasses, or clean cover glasses only (controls) into each well of 6-well culture plates (Corning Costar Cell Culture Plates) containing 10 ml sterile, filtered seawater (0.2 μm, salinity 15 ‰). Between 10 and 12 cyprids were added to a minimum of 12 replicates, and incubated at 18 °C with a light/dark cycle of 9:15 h for a period of 7 days. The vessels were monitored daily using a dissecting microscope (x10 magnification), and the numbers of (1) permanently settled cyprid larvae (following expulsion of cement), (2) exploratory cyprids (non-permanent settlement or active crawling on vessel surface) and (3) dead cyprids was recorded daily. Experiments with *V. anguillarum* were repeated with three separate batches of cyprids and experiments with *Sulfitobacter* sp., *E. coli* or synthetic AHLs repeated with two separate batches of cyprids. Due to varying quantities of cyprids within the different batches, experiments with *A. hydrophila* experiments were conducted only once. As the *E. coli* died during the long incubations in seawater, biofilms were only monitored for 2 days. Each cyprid batch was derived from different multiple barnacle parents.

As biofilm density influences AHL production, care was taken to ensure biofilms of signal-producing and signal-deficient strains were of similar densities. The proportion of the surface area covered by bacteria was determined with microscope image analysis, using an Image ProPlus imaging system attached to a Reichert Jung Polyvar microscope and a

Optronics Magna Fire SP camera. Biofilm material was stained with crystal violet 1% aqueous solution and counts were made of 20 random fields of view from each of four replicates. Measurements revealed similar percent coverage for signal-producing and signal-deficient mutants of all three bacteria. The percent coverage for *V. anguillarum* WT biofilms was 26.04% ± 1.54, for *V. anguillarum vanM* mutant biofilms, 25.43% ± 1.14 and for *V. anguillarum* expressing the recombinant AiiA lactonase, 25.33% ± 1.59. *A. hydrophila* WT biofilm densities were 21.8% ± 1.3 and the *ahyI*- mutant, 23.77% ± 1.18. For *Sulfitobacter* sp. BR1, biofilm densities were 42.42% ± 2.21 for the WT and 37.29% ± 3.67 for the signal-deficient mutant.

Quantification of introduced bacteria during cyprid settlement assays

To calculate the numbers of bacteria introduced to the biofilm along with the cyprids during the long experiments, Gfp-variants of *V. anguillarum* and the *vanM* mutant were used. Similarly, to detect if any of the introduced bacteria were making AHLs, a *V. anguillarum vanM* mutant carrying a gfp-based AHL biosensor luxR-P_{luxI}-RBSII::gfpmut3*-T_0 was used (Tait et al., 2005). This strain does not produce any AHLs, but expresses Gfp when an exogenous source of AHL is detected. This was compared to the number of Gfp-producing bacteria within biofilms of the *V. anguillarum* wildtype strains containing the same construct. Biofilms were counterstained with 1 mg ml^{-1} DAPI and viewed using a Reichert-Jung Polyvar microscope. A blue light filter (excitation, 450–495 nm; emission, 510 nm; dichroic, 510 nm) was used for Gfp fluorescence and an ultraviolet filter (excitation, 330–380 nm; emission 420 nm; dichroic 420 nm) for DAPI. Image Pro+ 5 (Media Cybernetics) was used to estimate the percentage of cells expressing Gfp. Counts were made of 10 random fields of view from each of four replicates.

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230 **Settlement assays using synthetic AHLs**

231 To quantify cyprid response to synthetic AHLs, 0.5, 5 and 50 μM C6-HSL (N-hexanoyl-L-
232 homoserine lactone), C8-HSL (N-octanoyl-L-homoserine lactone), C12, (N-dodecanoyl-L-
233 homoserine lactone) and OC10-HSL (N-(3-oxodecanoyl)-L-homoserine lactone) (Sigma-
234 Aldrich), were embedded in a 1% agarose/distilled water matrix (Tait et al., 2005). A
235 consistent thin coating of agarose/AHL was applied to cover glasses using a mould. This
236 agarose film was used in cyprid settlement assays. For each AHL concentration, 12 replicates
237 were used and agarose films without AHLs were included as controls.

238 Given the rapid diffusion of AHLs from surfaces, which can occur within minutes for
239 very short chain AHLs (Tait et al., 2005), and the long incubation times of these experiments,
240 AHLs were also added directly to seawater. AHL concentrations of 0.5, 5 and 50 μM were
241 maintained through-out the incubation. This first required measurement of the rate of
242 degradation of AHLs in natural seawater. AHL degradation varies with temperature, acyl side
243 chain length and also the presence of substitutions on the acyl chain (Tait et al., 2005; Hmelo
244 &Van Mooy, 2009), and is much higher in natural, unsterilised seawater than in artificial
245 seawater (Hmelo &Van Mooy, 2009). To measure the rate of degradation during incubation,
246 natural seawater containing AHLs was incubated for 3 hours and residual AHLs extracted
247 with ethyl acetate and evaporated to dryness. Extracts were then resuspended in acetonitrile,
248 added to white/clear bottomed microtitre plate wells (Corning, UK) and 200 μl of the lux-
249 based *E. coli* pSB401 AHL biosensor added. The microplates were incubated at 37 °C and the
250 luminescence and absorbance (600 nm) monitored for a period of 8 h using a Berthold
251 Mithras plate reader. Measurements of the areas under each curve were made, and a standard
252 curve of relative light units (RLU)/OD_{600} as a function of AHL concentration constructed for

each of the 4 AHLs. For each sample, five values were obtained and the mean determined. The percent degradation of each AHL in the seawater was calculated as 1.54 ± 0.23% h^{-1} for C6-HSL, 1.02 ± 0.49% h^{-1} for C8-HSL, 4.57 ± 1.21 % h^{-1} for OC10-HSL and 0.68 ± 0.24% h^{-1} for C12-HSL with reference to the calibration curve. Using these values, AHLs were replenished in the cyprid settlement assays every 8 hours to maintain the desired concentration. For each AHL concentration, 12 replicates were used and agarose films without AHLs were included as controls.

Statistical Analysis

Data are reported as a means with 95% confidence intervals. The software package PRIMER 6 (Clarke & Gorley 2006) with PERMANOVA+ (Anderson et al. 2008) was used for all statistical analysis. Multivariate permuational analysis of variance (PERMANOVA) based on Euclidean distance was used for analyses of the cyprid exploratory behaviour (see above) and settlement responses on *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* sp. BR1 biofilms. Daily measurements of cyprid behaviour were used as response variables and the different treatments and their replicates used as samples. The multivariate nature of this analysis readily accounts for the non-independence of the daily measurements. For experiments using batch 1 and 3 cyprids, at least 18 replicates were analysed for every experiment. For batch 2 cyprids, at least 30 replicates were used. Significant terms were investigated further using pairwise comparisons with 999 permutations (Anderson et al 2008). Tests for *V. anguillarum* biofilms were carried out with the 2 different signal-deficient mutants as separate treatments and also as a single, combined treatment with no differences between the conclusions made. Differences in the response of cyprid batch 2 to each of the 3 bacteria studied, and also differences in the behaviour of the 3 separate cyprid batches in vessels containing *V.*

anguillarum signalling and non-signalling biofilms were investigated by creating combined factors of 'Bacterium × Biofilm Type' and 'Batch × Biofilm Type' respectively. To clearly visualise differences within treatments, replicates were averaged and shown as MDS plots.

For experiments using *E. coli* and synthetic AHLs, where analyses typically used data collected on day 2 or day 7, ANOVA was also used to test for differences in cyprid exploratory behaviour between no biofilm controls and control *E. coli* biofilms, *E. coli* controls and *E. coli* strains expressing AHLs, and also in vessels with and without synthetic AHLs.

Results

Increased settlement of *Balanus improvisus* cyprids in the presence of AHL-producing biofilms

Substantially higher numbers of cyprids settled in treatments containing signal-producing bacteria than in non-signalling biofilm and no-biofilm controls (Figure 2). These differences were statistically highly significant for all bacteria tested and for each of three batches of cyprids (PERMANOVA, Table 2, Figure 2). Pairwise comparisons indicated that the AHL-producing wildtype biofilm caused significantly more settlement than the signalling-deficient mutant biofilms and the no-biofilm controls (Table 2). Settlement on the signalling-deficient biofilms was not statistically different from that on the no-biofilm controls ($p > 0.12$, Table 2), except in one case (larvae from Batch 1 on *Sulfitobacter* sp. BR1 biofilms settled significantly less on no-biofilm controls than on the AHL-deficient biofilms; Figure 2, Table 2). Although more cyprids were recorded crawling on the AHL producing biofilms (with the exception of *V. anguillarum*, batch 3; Figure 2, day 2 data), most settlement occurred on the

sides of the culture dishes. This behaviour is typical for this species under static laboratory conditions (Berntsson *et al*., 2001).

Overall levels of larval settlement varied between different batches of larvae (data for *Sulfitobacter* sp and *V. anguillarum*; Figure 2). The possibility that larvae from different batches (genotypes) may have also responded differently to the different biofilm treatments was tested using data for settlement on *V. anguillarum* (the only bacteria species that was tested using three different larval batches). A significant Batch x Biofilm interaction was detected (Pseudo-F = 1.88; p = 0.036, Table 3). Further investigation of this interaction using multidimensional scaling (MDS) showed clear separation of settlement of the AHL signal-producing (WT) biofilms from that in the non-signalling controls (*vanM* mutant and *V. anguillarum* expressing the recombinant AiiA lactonase; Figure 3A), and that responses in the non-signalling controls grouped much more closely together (Figure 3A). Similar broad separation between AHL-producing WT strains and relatively tight grouping of non-signalling biofilms was also seen for all three bacteria species when compared using batch 1 cyprids (the only batch for which all three species and biofilm types were compared; Figure 3B, Table 2).

After 7 days incubation, the WT and the *vanM* mutant biofilms still contained similar bacterial coverage (WT biofilms: 24.67% ± 2.24; *vanM* mutant biofilms: 26.19% ± 2.19). However, addition of cyprids to the biofilm unavoidably introduced additional bacteria to the culture vessels and this was assessed using Gfp variants of *V. anguillarum* WT and the *vanM* mutant. In control, axenic biofilms, the numbers of *V. anguillarum* still expressing Gfp was 97.6% for the WT and 98.1% for the *vanM* mutant after 7 days. Within the biofilms exposed to cyprids, 91.5 ± 0.98% bacteria within the WT vessels and 89.13 ± 1.23% bacteria within the *vanM* mutant biofilm were producing Gfp after the 7 day incubation period. Very few

cells expressing Gfp were detected within the *V. anguillarum vanM* mutant carrying a gfp-based AHL biosensor (2.14 ±1.24%). This shows that despite the relatively high number of introduced bacteria, very few of these were actively releasing AHLs. In contrast, biofilms of the *V. anguillarum* WT containing the same construct contained 93.65 ±5.12% Gfp-producing bacteria.

Experiments using AHL synthase-producing *E. coli* and synthetic AHLs also show an increase to cyprid exploratory behaviour and settlement

After 2 days, significantly higher numbers of cyprids were actively exploring the AHL synthase-producing *E. coli* biofilms than the control biofilms (Figure 4). In contrast there were no significant differences in cyprid exploration between the *E. coli* control plasmids and the no-biofilm controls (ANOVA $p = 0.683$). This experiment was repeated with 2 batches of cyprids, with similar results each time.

Assays using the synthetic AHLs C6-HSL, C8-HSL, OC10-HSL and C12-HSL, in agarose films showed that only C8-HSL and C12-HSL elicited an increase in the number of cyprids actively crawling on the surface of the vessel after 2 days incubation (Figure 5A; ANOVA $p = 0.037$ and $p = 0.001$, for C8-HSL and C12-HSL, respectively). After 7 days incubation, there was no difference in cyprid responses between vessels containing AHLs and the AHL-free controls (results not shown). When AHLs were added directly to the seawater there was increased settlement within vessels containing 50 μM of all 4 AHLs compared to controls (Figure 5B). Using concentrations of AHLs close to those found in natural biofilms (5 μM), C8-HSL, OC10-HSL and C12-HSL, but not C6-HSL increased cyprid settlement. The response towards OC10-HSL was marginally less significant than the response towards

C8-HSL and C12-HSL (ANOVA $p = 0.023$ for OC10-HSL and $p = 0.001$ for both C8-HSL and C12-HSL).

Discussion

Our results clearly demonstrate that AHL-producing biofilms influence settlement of cypris larvae of the barnacle, *B. improvisus*: AHL-producing variants of the marine bacteria *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* sp. BR1 all significantly increased settlement of *B. improvisus* cyprids in comparison to non-AHL producing biofilms and controls (Figures 2 and 3); cyprids actively investigated biofilms of *E. coli* expressing recombinant AHL synthase genes significantly more than biofilms of *E. coli* not producing AHLs (Figure 4); and synthetic AHLs at environmentally relevant concentrations increased the numbers of settling cyprids (Figure 5B). In the majority of cases, there were no differences between settlement within vessels containing no biofilms and biofilms of the signal-deficient mutants. Taken together this evidence suggests that cyprid settlement in response to biofilms is either mediated directly by an AHL signal or is mediated indirectly, for example, the AHL signal may control the production of an unknown biofilm-derived settlement cue.

Mutation to an AHL synthase is likely to impact other phenotypes, other than AHL production in the bacteria used in this study: quorum sensing is thought to constitute a global regulatory system for many bacteria. For example, transcriptomic studies of *P. aeruginosa* revealed over 500 genes regulated by LasRI and RhlI dispersed throughout the chromosome (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). It is, therefore, not surprising to find a link between quorum sensing and regulation of biofilm formation and development in many bacteria, including *V. anguillarum* and *A. hydrophila*. Biofilms of the AHL-deficient mutants in both these bacteria are less differentiated with no microcolonies (Tait et al., 2005;

371 Lynch et al., 2002). Given the differences in structure for *V. anguillarum* and *A. hydrophila*
372 biofilms, it is possible that the cyprid responses we observed were responses to changes in
373 biofilm architecture rather than the presence or absence of an AHL signal. Conversely, under
374 the conditions used to produce the *Sulfitobacter* sp. BR1 biofilms, there are no visible
375 differences between the wildtype and signal-deficient mutant (data not shown). Nonetheless,
376 our treatments may have caused unintended (and uncharacterised) changes to biofilm
377 phenotypes that influenced in cyprid settlement. For example, EPS production has been
378 linked to AHL production in certain bacteria (Sakuragi and Kolter, 2007) and it has been
379 shown that for some invertebrate larvae, the settlement cue involves recognition of biofilm
380 EPS by lectin receptors (Maki and Mitchell, 1985; Khandeparker et al., 2003; Woods et al.,
381 2004; Roberts et al., 2007).

382 The possibility that additional unidentified features of the AHL-deficient variants of
383 *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* BR1 affected cyprid settlement were
384 investigated using assays with *E. coli* expressing recombinant AHL synthases. As would be
385 expected, the long incubation period of the experiments resulted in the death of the *E. coli*
386 biofilms, and consequently after day 7 there was no difference in the numbers of cyprids
387 settling within vessels containing signalling or non-signalling *E. coli* strains (data not shown).
388 Exploratory behaviour precedes permanent attachment for *B. improvisus* cyprids (Berntsson
389 et al., 2000) and therefore the finding that significantly more cyprids were actively exploring
390 the *E. coli* biofilms that expressed the recombinant AHL synthases (after 2 d) than the control
391 biofilms corroborates the results from our settlement experiments using AHL-producing and
392 AHL-deficient strains.

393 Finally, we assessed the biofilm-independent effects of AHLs on cyprid settlement
394 with a range of synthetic AHLs. C8- and C12-HSL produced significantly more searching by

cyprids after 2 days incubation than other AHLs (Figure 5B). After this time, there were no differences between the numbers of cyprids settling in chambers with or without the presence of AHLs. These findings may be partially explained by the instability of AHLs in seawater (Tait et al., 2005; Hmelo & van Mooy, 2009). AHLs consist of five-membered homoserine lactone rings with varied amide linked acyl side-chains. These acyl side chains can range from 4 to 18 carbons in length, and may be saturated or unsaturated, with or without a substituent (usually an oxo or hydroxy) on the C3 carbon of the N-linked acyl side chain (Chhabra et al., 2005). The alkaline pH of seawater (typically pH 8.1) causes rapid hydrolysis of the lactone ring, and this increases with increasing temperature (Tait et al., 2005) Shorter acyl chain length AHLs and those with substitutions on the acyl chain are also more susceptible. In addition, AHLs diffuse rapidly from surfaces (Tait et al., 2005): for short chain AHLs such as C6-HSL almost complete diffusion from the agarose matrix could be expected within < 1 hour. Thus, AHLs have an extremely short half-life in seawater and would only be expected to be biologically active within micro-niches such as biofilms. Given the long exposure times required for cyprid settlement within these laboratory experiments (days), it is unlikely any synthetic AHLs, whether in seawater or within the agarose matrix, would still be biologically active. This may also explain why previous studies using synthetic AHLs within larval settlement assays (Huang et al., 2007; Dobretsov et al., 2007) have yielded ambiguous results. By calculating the rate of degradation of each AHL within the experimental vessels and replenishing regularly through-out the course of the experiment we ensured AHLs remained close to the target concentration and mimicked the natural release of AHLs from live biofilms. This methodology yielded significant results for seawater containing synthetic AHLs at biologically relevant concentrations (Figure 5B). The response to a synthetic AHL suggests that cyprids can respond to the AHL signal directly. Note that this does not exclude

419 the possibility that cyprids also used other biofilm-derived cues during our experiments with
420 bacteria.

421 The long incubation period before cyprid settlement in our experiments (7 days)
422 produced several potential problems, not least the introduction of 'foreign' bacteria along
423 with the cyprids. By using *V. anguillarum* labelled with Gfp, we found the extent of
424 colonisation by non-Gfp bacteria after 7 days was as high as 10% of the biofilm. The
425 identities of the introduced bacteria are not known. Neither is it known if there was a
426 difference between those colonising the signal-deficient or signal-producing biofilms, nor if
427 there were differences in 'foreign' colonisation between the three marine bacteria used. All
428 these factors may have influenced cyprid settlement in our assays. Our attempts to determine
429 the level of AHL signal produced by these marine bacteria using a *V. anguillarum vanM*
430 mutant carrying a Gfp-based AHL reporter did, however, indicate that few of these were
431 actively producing AHL signal: very low numbers of the *V. anguillarum* reporter bacteria
432 were detecting an AHL signal produced by neighbouring, introduced bacteria (2.14 ±1.24%).
433 Consequently, while the biofilms of the signal-deficient strains may not have been entirely
434 AHL-free through the course of the experiment, the concentration of AHLs in these
435 treatments in comparison to the signal-producing strains was extremely low.

436 We found statistically significant differences in cyprid settlement behaviour from
437 different larval batches (Table 3). Variability in larval response is well known (Raimondi and
438 Keough, 1990). Rearing conditions (Holm, 1990), larval age (Holm et al., 2000) and type of
439 microalgae used to feed the developing larvae (Clare et al., 1994) have all been shown to
440 influence the attachment and metamorphosis of *B. amphitrite*. Consequently, offspring of the
441 same parents raised at different times can respond differently to the same surface (Holm,
442 1990). Therefore, care was taken to ensure larvae used within these studies were reared using

identical conditions in each case. Nonetheless, the number (and genetic identity) of parents that contributed to the larvae within each cyprid batch is unknown. The clear differences between larval responses we observed (Table3) indicate the potential for larval selection and adaptation to different biofilms.

Although the number of cyprids exploring the biofilms of signal-producing bacteria was higher than those exploring the non-signalling biofilms and no-biofilm controls (with the exception of *V. anguillarum*, batch 3; Figure 2, Day 2 data), many cyprids chose to settle on the sides of the vessel and not directly on the biofilms. This settlement behaviour is typical of *B. improvisus* within laboratory experiments (Berntsson, 2001). It is known that *B. improvisus* actively explores a large area before settling: the likelihood of final settlement at a particular site is directly related to searching behaviour which occurs over the entire surface of the dish prior to settlement (Havenhand, unpublished data). While the mechanism behind *B. improvisus* cyprid settlement may still be unclear, the critical point here is that without the presence of the AHL-producing biofilms, settlement was reduced (Figure 2).

The series of experiments described here indicates AHL signalling biofilms may be used by *B. improvisus* as a settlement cue under laboratory conditions and certainly highlights the need for further research, particularly using conditions more closely mimicking field conditions. Hydrodynamics and surface properties are known to have a significant impact on *B. improvisus* settlement (Jonsson et al., 2004; Berntsson et al., 2000), and will also influence the rate of diffusion of AHLs from surfaces. This is essential to clarify the importance of AHLs and AHL-signalling biofilms for larval settlement in the field. It is also not clear if the cyprids are chemotactically attracted to the AHL signal, or if the cyprid response is chemokinetic behaviour as shown to be the case with *Ulva* (Wheeler et al., 2006). Yet, it is becoming increasingly apparent that AHLs have biologically important properties beyond

467 their role in cell-to-cell communication within species of bacteria. In the marine environment,
468 there is now evidence that algae (Joint et al., 2002; Weinberger et al., 2007), polychaetes and
469 bryozoans (Huang et al., 2007; Dobretsov et al., 2007) respond to the presence of a bacterial-
470 derived signal. The effect of AHLs on other plant (Mathesius et al., 2003; Ortiz-Castro et al.,
471 2008; von Rad et al., 2008; Bai et al., 2010), animal (Smith et al., 2002; Telford et al. 1998;
472 Pritchard et al. 2005) and fungal cells (Hogan et al. 2004) has also been well documented.
473 These findings show that AHL signals molecules can modify the behaviour of a wide-range
474 of evolutionarily diverse organisms. Studies of the underlying mechanism in each of these
475 organisms are needed to reveal the origin and scale of this interaction. Here we have shown
476 the potential importance of AHLs for settlement success in a key marine invertebrate species.
477 Enhanced understanding of the role of AHL signalling within marine biofouling
478 communities (Tait et al., 2005; Huang et al., 2007; Dobretsov et al., 2007; Huang et al., 2008;
479 Huang et al., 2009) increases the importance of research into technologies that specifically
480 disrupt AHL-mediated QS for biofouling control, as well as for disease control within
481 aquaculture (Natrah et al., 2011). Screens for AHL inhibitory compounds from compounds
482 obtained from the marine environment have already shown promising results (Dobretsov et
483 al., 2011). Further investigations of the role of AHLs in mediating settlement responses,
484 chemical defence, and inter-specific communication of barnacles and other marine
485 invertebrates are warranted.
486
487

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Data accessibility

Data from all experiments (assays with biofilms of *V.anguillarum*, *A. hydrophila*, *Sulfitobacter* sp. BR1 and *E. coli* and assays with synthetic AHLs) have been stored under the Dryad Digital Data repository (http://datadryad.org/) : doi:10.5061/dryad.c3b75.

Legends to figures

Figure 1

Thin layer chromatography (TLC) showing AHL production by *Sulfitobacter* BR1 WT, similar profiles for *E. coli* expressing the BR1 AHL synthase *sulI*, and no detectable AHLs by the BR1 *sulI* mutant. TLC plates were overlaid with the biosensor NTL4 (pCF218; pCF372) (Fuqua & Winans, 1996) and the presence of spots are indicative of AHL production. AHL synthetic standards were used as markers: 0.5 mM *N*-butanoyl-L-homoserine lactone (C4), 50 µM *N*-hydroxyhexanoyl-L-homoserine lactone (HC6), 0.5 µM *N*-octanoyl-L-homoserine lactone (C8) and 0.5 mM *N*-hydroxydecanoyl-L-homoserine lactone (HC10).

Figure 2

Comparison of cyprid exploration (Day 2) and settlement (Days 3 – 7) in vessels containing biofilms of wildtype *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* sp. BR1 and their signal-deficient mutants. Also indicated on each graph is the cyprid batch used in each case. Black bars indicate wildtype bacteria, the light grey bars are the AHL synthase mutant and the white bars are control surfaces containing clean cover glasses with no biofilm. For *V. anguillarum* assays, *V. anguillarum* expressing the aiiA gene (an AHL lactonase) was also included (dark grey bars). Bars are 95% confidence intervals.

Figure 3

Non-metric multidimensional scaling (MDS) ordination of a Euclidean Distance resemblance matrices calculated using cyprid settlement data from days 3 to 7 (data points are average of replicates within treatments). (A) settlement of 3 separate batches of cyprids on *V. anguillarum* WT (▲) and biofilms of the 2 signal-deficient variants of *V. anguillarum*: the

740 *vanM* mutant (○) and *V. anguillarum* expressing recombinant aiiA (□). Numbers represent
741 cyprid batch number. (B) cyprid batch 2 settlement on signal producing and signal-deficient
742 biofilms of *V. anguillarum* (▲), *A. hydrophila* (■) and *Sulfitobacter* sp.BR1 (●) Letters are
743 wildtype (WT) and mutant (M).
744
745 **Figure 4**
746 Percentage numbers of cyprids actively exploring surfaces of vessels containing biofilms of
747 *E. coli* containing AHL synthases. Strains JM109 containing control plasmids are light grey
748 bars and those containing plasmids with the recombinant *sulI* from *Sulfitobacter* sp. BR1, luxI
749 from V. fischeri, rhlI from Ps. aeruginosa and vanI from *V. anguillarum* are black bars. The
750 white bar indicates control surfaces containing clean cover glasses with no biofilm. Error bars
751 are 95% confidence intervals and asterisk show those values that are significantly different to
752 the controls (* one-way ANOVA $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$).
753
754 **Figure 5**
755 Interaction of cyprids with vessels containing AHLs dissolved in (A) agarose films or (B)
756 seawater. For experiments using agarose films, 5 μM was used and data are percentage
757 number of cyprids actively exploring the vessel surface after 2 days incubation. For
758 experiments using AHLs dissolved in seawater, three concentrations of AHLs were used (0.5,
759 5 and 50 μM), and data is percentage number of cyprids permanently settled after 7 days.
760 Agarose films with no AHLs or seawater containing no AHLs were included as controls.
761 Error bars are 95% confidence intervals and asterisks show those values that are significantly
762 different to the controls (* one-way ANOVA $p \leq 0.05$ and *** = $p \leq 0.001$).
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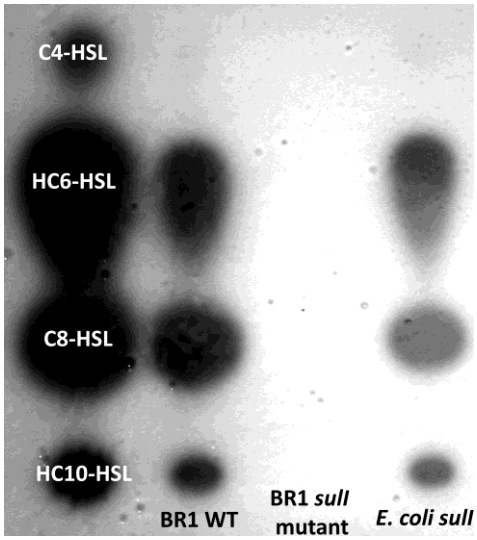

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766 Figure 1

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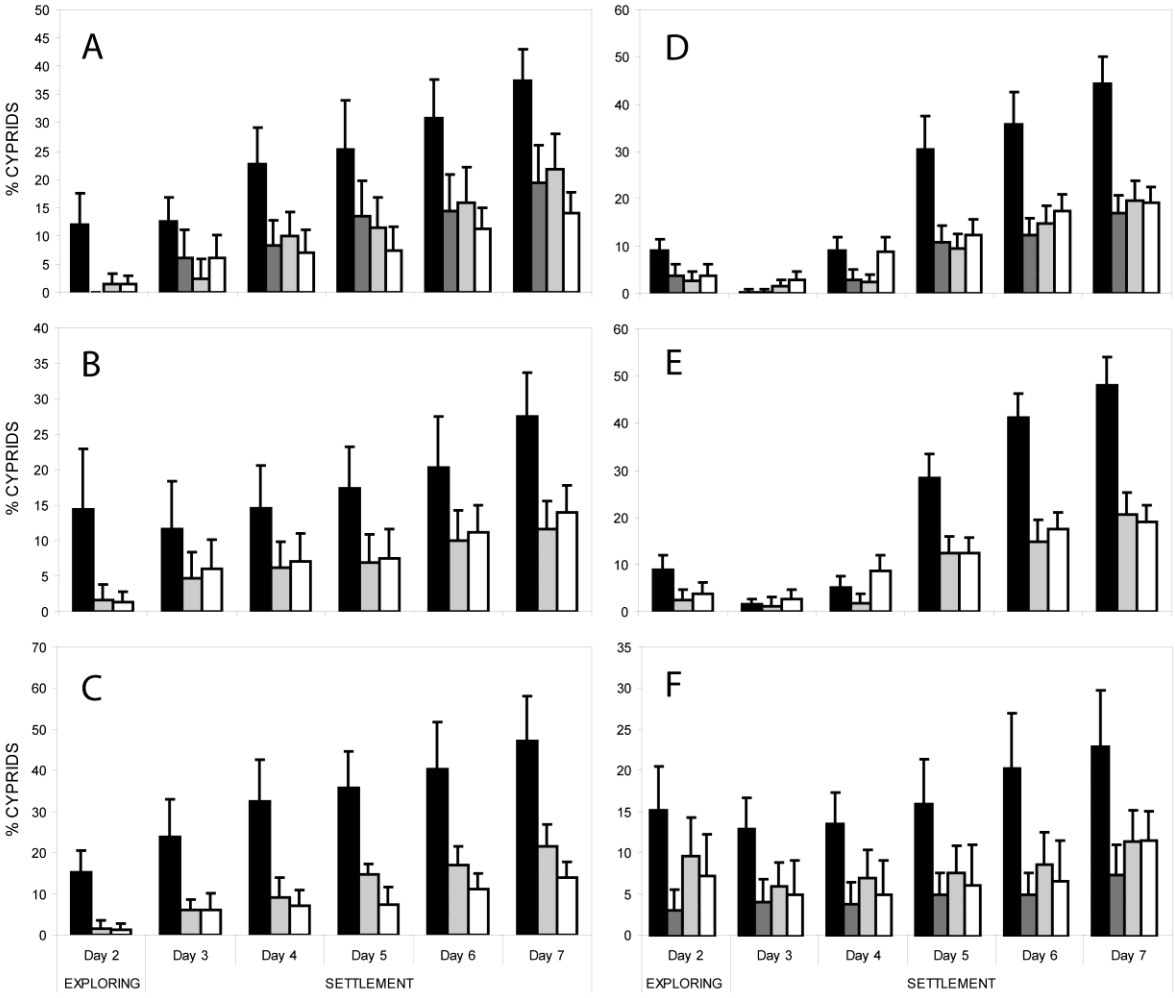

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769 Figure 2

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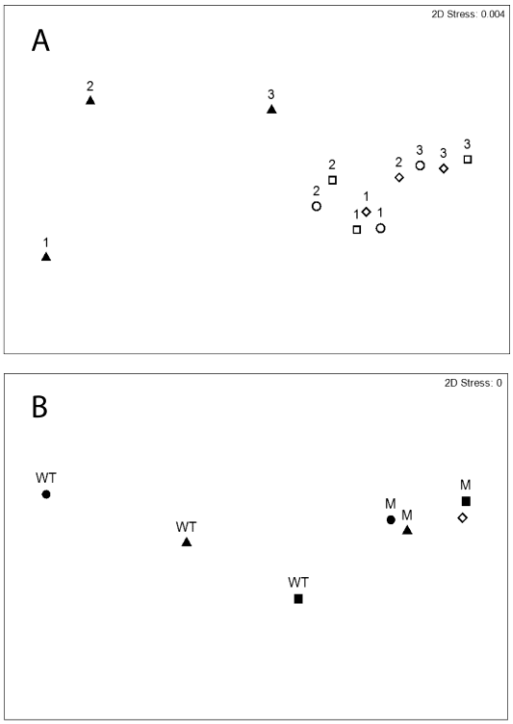

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773 Figure 3

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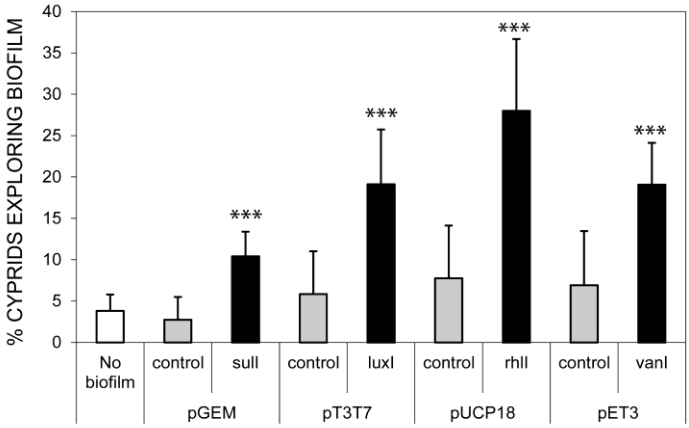

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777 Figure 4

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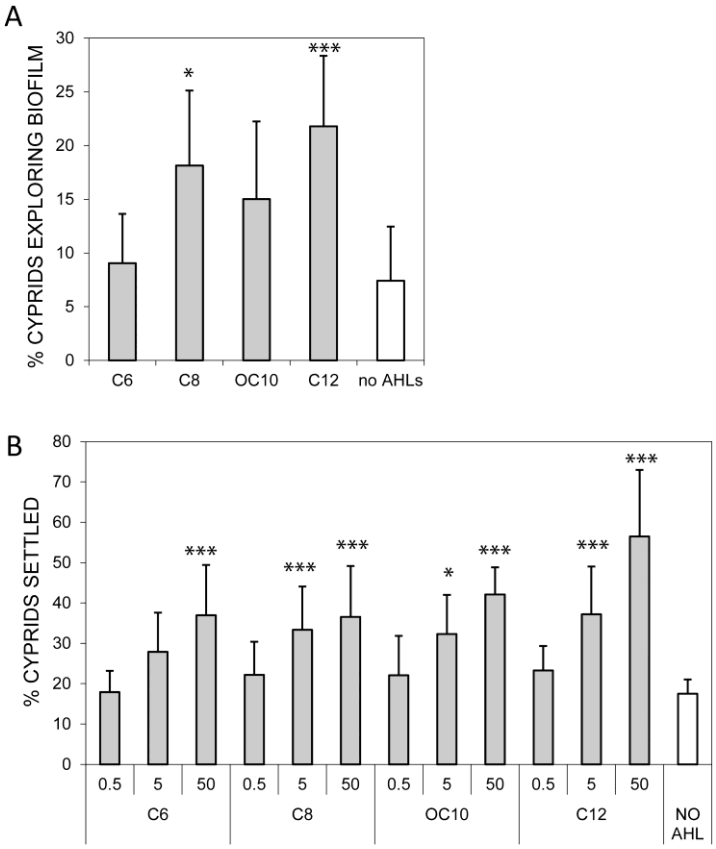

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781 Figure 5

782 **Table 1.** Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description	Reference
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gypA96 relA1 thi* Δ (*lac-proAB*)	Schaefer *et al*. (1996)
A. hydrophila		
AH-IN	Spontaneous mutation of *A. hydrophila* AH-1 lacking S-layer and O-antigen	Swift *et al*. (1999)
AhyI$^-$	AHL-deficient variant:AH-IN with an in frame deletion of *ahyI*	Lynch *et al*. (2002)
V. anguillarum		
NB10	Wild type, serotype 01, clinical isolate from the Gulf of Bothnia	Norqvist *et. al.* (1989)
DM28	AHL-deficient variant: In-frame deletion of *vanM*	Milton *et. al*. (2001)
NB10 Gfp	Gfp-labelled WT: contains mini-Tn7 $P_{A1/04/03}$gfp (Gent^R)	This study
DM28 Gfp	Gfp-labelled AHL-deficient variant: DM28 containing mini-Tn7 $P_{A1/04/03}$gfp (Gent^R)	This study
NB10/pDM44	Wildtype carrying Autoinducer Inactivation protein (AiiA): contains a $P_{A1/04/03}$::aiiA gene fusion (Cm^R)	Tait *et al*. (2005)
NB10/pDM42	Wildtype carrying a Gfp-based AHL reporting construct: contains luxR-PluxI-RBSII::gfpmut3*-T0; (Cm^R)	Tait *et al*. (2005)
DM27/pDM42	AHL-deficient variant containing a Gfp-based AHL reporting contruct: DM28 containing luxR-PluxI-RBSII::gfpmut3*-T0 (Cm^R)	Tait *et al*. (2005)
Sulfitobacter sp.		
BR1	Wild type, isolated from rocky shore	Tait *et al*. (2005)
SulI$^-$	Mini-Tn5 insertion into *sulI* (Kan^R)	This study
Agrobacterium tumefaciens NTL4 (pCF218) (pCF372)	AHL reporter: produces a blue colour in the presence of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) in response to a wide range of AHLs	Fuqua and Winnas (1996)
Plasmids		
pUX-BF13	*mob*$^+$*ori*-R6K; helper plasmid; providing Tn7 transposition functions in trans (Amp^R)	Bao *et al*. (1991)
pRK600	*ori*-ColE1 RK2-mob$^+$ RK2$^-$*tra*$^+$ helper plasmid in matings (Cm^R)	Kessler *et al*. (1992)
pMiniTn7(Gm)P_{rrnB1}–gfp-a	P_{rrnB1}-gfp cloned into NotI site of pBK-miniTn7-ΩGm	Lambertsen et al. (2004)
pSB401	AHL reporter plasmid; *luxR''::luxCDABE* (Amp^R)	Winson *et al.* (1998)
pRK-C12	AHL reporter plasmid; pBBR1MCS-5 carrying P_{lasB}– *gfp*(ASV) P_{lac}- *lasR*	Reidel *et al*. (2001)
pUCP18	pUC18 containing 1.8-kb fragment for maintenance in *Pseudomonas* sp. (Amp^R)	Shweizer (1991)
pMW47.1	2-kb *Pst*I *Pseudomonas aeruginosa* PAO1 DNA insert (*rhlRI*) in pUCP18	Latifi *et al*. (1996)
pT7T3	General cloning vector derived from pUC18 (Amp^R)	Pharmacia
pT7T3luxI	pT7T3 expressing *luxI* from *Vibrio fischeri* 7744	Tait *et al*. (2005)
pET3a	Overexpression vector (Amp^R), T7 promoter, pBR ori	Novagen
PETVanI2	pET3a expressing *vanI* from *Vibrio anguillarum* NB10	Tait *et al*. (2005)
pGEM	General cloning vector derived from pUC18 (Amp^R)	Promega
pKT11	pGEM expressing *sulI* from *Sulfitobacter* sp. BR1 (Amp^R)	This study

Table 2

Effect of biofilm type (wildtype, mutant and no biofilm controls) on cyprid settlement determined using PERMANOVA analyses for *V. anguillarum*, *A. hydrophila* and *Sulfitobacter* sp. using three separate batches of cyprids.

		PERMANOVA						PAIR-WISE TESTS			
Batch	Bacterium	Source	df	SS	MS	F	*P* (perm)	Unique perms	Groups	*t*	*P* (perm)	Unique perms
1	*A. hydrophila*	Biofilm	2	5369.6	2684.8	33.35	0.001***	998	WT, Mutant	2.83	0.003***	998
		Res	234	18838	80.505				WT, No biofilm	2.62	0.007***	997
		Total	251	28612					Mutant, No biofilm	0.47	0.875	998
1	*Sulfitobacter*	Biofilm	2	28059	14030	116.59	0.001***	998	WT, Mutant	4	0.001***	997
		Res	234	28164	120.36				WT, No biofilm	5.58	0.001***	999
		Total	251	69209					Mutant, No biofilm	1.83	0.031**	997
2	*Sulfitobacter*	Biofilm	2	20875	10438	105.79	0.001***	999	WT, Mutant	6.55	0.001***	998
		Res	469	46273	98.662				WT, No biofilm	6.43	0.001***	997
		Total	485	140010					Mutant, No biofilm	0.65	0.714	999
1	*V. anguillarum*	Biofilm	2	11999	5999.5	12.631	0.001***	999	WT, Mutant	3.8	0.001***	999
		Res	51	24224	474.98				WT, No biofilm	4.81	0.001***	999
		Total	53	36223					Mutant, No biofilm	149	0.121	998
2	*V. anguillarum*	Biofilm	2	34534	17267	31.724	0.001***	999	WT, Mutant	7.21	0.001***	999
		Res	106	57695	544.29				WT, No biofilm	5.13	0.001***	999
		Total	108	92229					Mutant, No biofilm	0.77	0.569	999
3	*V. anguillarum*	Biofilm	2	8129.3	4064.6	10.665	0.001***	999	WT, Mutant	4.21	0.001***	999
		Res	69	26298	381.13				WT, No biofilm	3.25	0.001***	993
		Total	71	34427					Mutant, No biofilm	0.59	0.675	993

Asterisks indicate significant P values (* = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$).

Table 3

Effect of cyprid batch and *V. anguillarum* biofilm type on cyprid settlement after 7 days incubation as determined using PERMANOVA analyses of two crossed, fixed factors: cyprid batch and biofilm type (signal-producing, signal-deficient and no biofilm controls).

PERMANOVA						
Source	df	SS	MS	F	*P* (perm)	Unique perms
Batch	2	97829	48915	9.2449	0.001***	999
Biofilm	2	43689	21844	4.1286	0.002***	998
Batch x Biofilm	4	39877	9969.1	1.8842	0.036**	999
Res	162	857140	5291			
Total	170	1038500				

PAIR-WISE TESTS			
Groups	*t*	*P* (perm)	Unique perms
WT, Mutant	1.9139	0.017**	999
WT, No biofilm	2.8794	0.002***	998
Mutant, No biofilm	1.1039	0.286	999

Asterisks indicate significant P values (* = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$).