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Effects of initial surface wettability on biofilm formation and subsequent settlement of *Hydroides elegans*

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Hydroides elegans is a major fouling organism in tropical waters around the world, including Pearl Harbor, Hawaii. To determine the importance of initial surface characteristics on biofilm community composition and subsequent colonization by larvae of *H. elegans*, the settlement and recruitment of larvae to biofilmed surfaces with six different initial surface wettabilities were tested in Pearl Harbor. Biofilm community composition, as determined by a combined approach of denaturing gradient gel electrophoresis and fluorescence *in situ* hybridization, was similar across all surfaces, regardless of initial wettability, and all surfaces had distinct temporal shifts in community structure over a 10 day period. Larvae settled and recruited in higher numbers to surfaces with medium to low wettability in both May and August, and also to slides with high wettability in August. Pearl Harbor biofilm communities developed similarly on a range of surface wettabilities, and after 10 days in Pearl Harbor all surfaces were equally attractive to larvae of *Hydroides elegans*, regardless of initial surface properties.

Keywords: *Hydroides elegans*; larval settlement; biofouling; biofilm; wettability; hydrophobicity; tube worm

Introduction

All surfaces immersed in seawater are rapidly colonized by a range of micro- and macroorganisms. Colonization generally begins with an assortment of microbial organisms, which compose the biofilm, followed by settlement of various marine invertebrates and algae. Many invertebrates and algae are stimulated to settle by biofilms and individual components of biofilms, such as specific species of bacteria and diatoms. Biofilms have been shown to have inhibitory, inductive and neutral effects on macrofouling (Zobell and Allen 1935; Crisp and Ryland 1960; Holmström et al. 1996; Wieczorek and Todd 1998; Unabia and Hadfield 1999). Invertebrate and algal propagules can also detect differences between surfaces with different bacterial community composition (Johnson and Sutton 1994; Patel et al. 2003; Huggett et al. 2006; Chiu et al. 2008), suggesting that the specific composition of bacterial species within biofilms can serve as important indicators of suitable settlement sites.

The physical properties of a surface can affect both biofilm composition and larval settlement. The inherent characteristics of a surface such as roughness, color, surface wettability and topography have been shown to affect the ability of both micro- and macroorganisms to attach (Dillon et al. 1989; James and Underwood 1994; Ista et al. 2004, Guenther and de Nys 2007; Scardino et al. 2008). In particular, surface wettability has been shown to be important for the settlement of algal spores (Greer et al. 2003), bryozoans and barnacles (Rittschof and Costlow 1989b; Roberts et al. 1991; Hung et al. 2008) and some microbial communities (Cooksey and Wigglesworth-Cooksey 1995), but it does not affect settlement of hydroids (Roberts et al. 1991), and its effect may be short lived (Holm et al. 1997). However, little is known about the effect of surface wettability on settlement of many taxa, and, importantly, the interaction between the physical characteristics of a surface, such as wettability, biofilm formation and subsequent larval settlement has been little studied for most organisms.

The accumulation of organic carbon and nitrogen on a clean surface during submersion for just 24 h in seawater can modify the physical and chemical properties of a surface, and favor the settlement of microorganisms (Bhosle 2004; Jain and Bhosle 2009). Biofilm adhesion begins with the adsorption of proteins and macromolecules to a surface, followed by the adhesion of cells. The tertiary structure of proteins is shaped by hiding, or burying their hydrophobic cores, whereas the external structure remains hydrophilic (Genzer and Efimenko 2006). By unfolding and exposing their interiors which have different characteristics to the exterior of the molecule, or by remaining folded, proteins can adsorb to both hydrophobic and hydrophilic surfaces (Genzer and

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Efimenko 2006). Some strains of environmental bacteria, such as the estuarine bacterium MI-1A, are able to undergo transition between attachment mechanisms, enabling them to attach to surfaces with a range of wettabilities within periods of time as short as 5 min (Weineck and Fletcher 1995). Despite the plastic nature of both cells and proteins to attach to surfaces with different wettabilities, Rittschof and Costlow (1989a) found that experimentally manipulated surfaces maintained their various wettabilities for up to several days in the marine environment. One explanation for this is that the conditioning layer of macromolecules and cells may transmit the underlying surface chemistry (Dexter et al. 1975). Thus subsequent settlement and attachment by invertebrates and algae is likely to be a response to both surface chemistry and the composition of the biofilm on a surface.

Settlement of marine invertebrates and algae onto man-made structures is termed biofouling. Marine biofouling increases global shipping costs by increasing drag and thereby decreasing speed and increasing fuel costs (Townsin 2003; Schultz 2007). Fouling also impacts the aquaculture industry by reducing product quality, increasing the load on nets and cages, as well as facilitating structural deterioration. Many attempts have been made to create surfaces that physically repel, or inhibit settlement of the larvae of fouling organisms. Despite efforts aimed at designing and testing surfaces that repel or inhibit biofouling, at present none have been both environmentally friendly and successful in reducing biofouling over long periods in the field.

The tube-building serpulid polychaete *Hydroides elegans* is one of the major problem organisms from warm-temperate to tropical biofouling communities. It is a model organism for biofouling studies, and is known to settle in response to bacterial biofilms and single strains isolated from biofilms, with preference for older biofilms (Hadfield et al. 1994; Unabia and Hadfield 1999; Lau and Qian 2001; Huang and Hadfield 2003; Lau et al. 2003; Shikuma and Hadfield 2006; Nedved and Hadfield 2009). The genetic similarity of populations of *H. elegans* throughout most oceans suggests that there has been regular and consistent transport of adults and larvae around the world on ships' hulls and in ballast water (Otani et al. 2007; Pettengil et al. 2007). Previous studies have shown that older biofilms induce higher amounts of larval settlement of *H. elegans* than younger biofilms (Hadfield et al 1994; Shikuma and Hadfield 2006). In this study, larval settlement of *H. elegans* was investigated on manipulated surfaces in a field and laboratory study. Test surfaces covering a range of wettabilities were evaluated for differences in biofilm-bacterial composition over time and whether subsequent settlement of *H. elegans* was affected.

Methods

Preparation and deployment of slides

Glass microscope slides were treated to create surfaces with six different wettabilities following the methods described by Gerhart et al. (1992). Two treatments, trimethylsilyl (TMS) and dimethyldichlorosilane (DMS), created low surface wettability; two treatments, aminopropyltriethoxysilane (APS) and 3-chloropropyltrimethoxysilane (CLPRS), created intermediate wettability; and two treatments, trimethylaminopropyltrimethoxysilane (QAP) and untreated glass (G), created high surface wettability.

Fifteen replicates of each treatment were placed in frames in a randomized complete block design, and the frames were deployed 2 m below the low tide mark at a pier at Ford Island, Pearl Harbor, Hawaii. This experiment was performed twice. For the first experiment, three frames were deployed on 29 April 2006 and one frame was retrieved after immersion for 3, 6 and 10 days. For the second experiment, four frames were deployed on 4 August 2006, and one frame was retrieved after each of 1, 3, 6 and 10 days submersion.

Contact angle measurements

Contact angles were determined by goniometry from sessile drops of HPLC grade water. Water was applied by allowing a 2 μl drop to form on the tip of a micropipettor, then raising a platform supporting the treated slide until it had contacted the drop. The platform was then lowered until the drop detached from the pipette tip, and a backlit image of the drop recorded within 1–2 s. Images of the drops were captured using a KSV Instruments CAM 100 Optical Contact Angle Meter. The instrument's software was used to fit the Young-Laplace equation to the drop, and calculate the contact angle where the fitted curve intersected the surface of the treated slide. All contact angles reported are advancing angles, and represent mean values of a minimum of 9 measurements (3 readings from 3 replicates of each treated slide).

Recruitment

Frames were collected and slides were gently removed and placed in Petri dishes containing 0.22 μm filtered seawater (FSW). The number of *H. elegans* on slides was counted under a dissecting microscope.

H. elegans *larval culture*

Adult *H. elegans* were collected from Ford Island, Pearl Harbor, Hawaii, and maintained in sea tables at the Kewalo Marine Laboratory of the University of

Hawaii at Manoa, Honolulu, HI. Adult tubes were gently broken near their apertures to induce spawning. Developmental stages were maintained at a density of 5–10 larvae ml^{-1} in 2 l of FSW at room temperature (22–24°C). After 2 days, larvae were collected on a 0.41 μm mesh, transferred to fresh FSW and fed daily until they became metamorphically competent at ~5 days. The alga *Isochrysis galbana* Tahitian strain was provided as food for the planktotrophic larvae at a concentration of 10^5 cells ml^{-1}. Larval rearing methods are described in detail in Nedved and Hadfield (2009).

Settlement assays

For each day that frames were collected, 10 slides were cut into four pieces, and one quarter of each from each treatment was used in a settlement assay. Slide pieces were placed in 5 ml Petri dishes with 3 ml of FSW, and ~50 competent larvae were added to each dish. The percentage metamorphosis was recorded after 24 h.

Community analysis

Denaturing gradient gel electrophoresis

After field-recruited *H. elegans* had been counted, the remaining whole five replicate slides from each treatment were held at −80°C. These slides were used for analysis of bacterial community composition with denaturing gradient gel electrophoresis (DGGE). The biofilm from each slide was scraped off the surface using a sterile scalpel blade, and genomic DNA was extracted using an Ultra Clean Microbial DNA Kit (Mo Bio) following the manufacturer's instructions. A nested PCR protocol was used, following Boon et al. (2002). The first round of PCR used the universal bacterial primers P63F and R1378R, targeting the 16S rRNA gene, with PCR conditions: 95°C for 10 min, 95°C for 1 min, 53°C for 1 min, 72°C for 2 min, 30 cycles and a final extension step of 72°C for 12 min. The second round of PCR used the universal primers P338F and P518R, which amplified a smaller fragment of the 16S rRNA gene for DGGE analysis. A 5′ GC clamp was added to P338F, and the PCR conditions were the same as the first round. The final concentrations of the different components of the mastermix were as follows: 5 pmol of each primer, 2.5 mM of each dNTPS, 1.5 mM MgCl, 0.025 U μl^{-1} taq polymerase (Promega, Madison, WI), and DNase and RNase free water (Sigma). During the first PCR round, 1 μl of genomic DNA was added to 24 μl mastermix, and in the second PCR round, 1 μl of the first round product was added to 24 μl of the second round mastermix. After each PCR, the size of the PCR product was checked on a 1% agarose gel stained with ethidium bromide.

Gradient 8% (w/v) polyacrylamide gels (acrylamide-*N*,*N*_-methylenebisacrylamide) containing 40–90% denaturant, from top to bottom, were poured. One hundred percent denaturant is defined as a 7 M urea and 40% (v/v) formamide solution. Two gels were poured simultaneously using a multi-gel caster, a 40 ml gradient maker, and miniperistaltic pump (CBS Scientific). Approximately half of each of the 25 μl PCR reactions was loaded into gels. The gels were loaded into a DGGE system (CBS Scientific) containing 1 × TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) at 60°C and run at 100 V for 4 h. Gels were stained in 1 × Sybr Gold solution (Molecular Probes) in 1 × TAE buffer for 30 min. Digital images of the gels were taken using a Kodak Gel Logic 100 Imaging System (Kodak) under UV illumination. Initially, five replicates within each treatment, at each time point, were run together on gels to assess the within treatment variability. Then representative replicates of each treatment were chosen to run on a single gel together, to compare community composition between the various treatments. For slides that were submerged for 10 days, no PCR product was generated, and therefore day-10 communities were not assessed using DGGE.

Community analysis

Fluorescent in situ *hybridization*

After field recruitment of *H. elegans* had been recorded, the remaining 75% of the 10 slides used for settlement assays were fixed in 4% paraformaldehyde suspended in phosphate buffered saline (PBS) for 8 h at 4°C and transferred to 1:1 ethanol : PBS at −20°C until further processing. All probes were labeled with either fluorescein or one of the indocarbocyanine fluorochromes Cy3 or Cy5, synthesized by Thermo Electron (Germany). Replicate wells ($n = 6$) were created on each slide using a DakoCytomation Pen (DakoCytomation Pty Ltd). Hybridization solution (8 μl of 0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% sodium dodecyl sulfate [SDS]), and the optimal percentage of formamide for each probe (see Table 1) were mixed with 1 μl of the appropriate fluorescently labeled oligonucleotide and applied to each well of the slide. Slides were incubated in 50 ml polypropylene tubes at 46°C for 3 h. After hybridization, slides were removed and gently rinsed immediately in pre-warmed wash buffer containing 20 mM Tris-HCl, 0.01% SDS and 0.225 M NaCl at 48°C for 15 min. Slides were then rinsed in fresh water to remove excess salts, air dried and mounted in 3:1 PBS:glycerol. The slides were examined using a Zeiss LSM 510 scanning confocal

Table 1. List of fluorescence *in situ* hybridization probes used in this study.

Probe	Formamide %	Sequence	Specificity
EUB388	20	GCTGCCTCCCGTAGGAGT	All bacteria
Non EUB	20	ACTCCTACGGGAGGCAGC	Negative control
ALF968	20	GGT AAG GTT CTG GCG GTT	Alphaproteobacteria
BET42a	35	GCC TTC CCA CTT CGT TT	Betaproteobacteria
Gam42a	35	GCC TTC CCA CAT CGT TT	Gammaproteobacteria
HGC69A	25	TAT AGT TAC CAC CGC CGT	Actinobacteria
LGC354	35	YSGAAGATTCCCTACTGC	Some Firmicutes
PLA46	30	GACTTGCATGCCTAATCC	Planctomycetales
ARC915	20	GTGCTCCCCCGCCAATTCCT	Archea
CF319	35	TGGTCCGTGTCTCAGTAC	*Cytophaga-Flavobacterium* of Bacteroidetes

microscope (Zeiss, Germany) equipped with the appropriate laser and filter combinations. Digital images of optical sections of the preparations were produced using the LSM 510 imaging software (Version 4) and single plane projections of these images, which have a greater depth of focus, were produced using the same software package.

Enumeration of bacterial density on slides

Three replicate one-quarter pieces of slides that had been fixed with 4% paraformaldehyde and stored as described above were used to determine the total density of bacteria on slides. Pieces were air dried, then 50 μl of PBT (0.5% Triton in 1 $\times$ PBS) were added to the surface of the slide and left to incubate at room temperature for 20 min. Slides were rinsed in 1 $\times$ PBS and 50 μl propidium iodide (1:200 dilution in PBT) were added to each piece and incubated in the dark at room temperature for 20 min. Slides were again rinsed in 1 $\times$ PBS, allowed to dry, and either stored at 4°C until examination or mounted immediately in 1 part glycerol 3 parts 1 $\times$ PBS for enumeration via microscopy, using a Zeiss LSM 510 scanning confocal microscope (Zeiss, Germany) as described above for FISH.

Data analysis

Settlement assays, recruitment in the field and bacterial densities were analyzed using SYSTAT (Wilkinson 1997). Data for analyses of variance (ANOVAs) were checked for normality and heterogeneity of variance using frequency histograms of residuals and plots of residuals versus means, respectively. Arcsine square root transformations were performed where appropriate. DGGE gels were analyzed to identify differences in bacterial community composition on slides using Non-metric Multidimensional Scaling (NMDS). NMDS iteratively seeks the best position of n entities on k dimensions that minimize stress of the final configuration, and has been effectively used to explore and graphically represent relationships in microbial communities. Following NMDS, a one-way analysis of similarity [ANOSIM, PRIMER 6, (Clarke and Warwick 1994)] was conducted with wettability treatment as the factor. ANOSIM computes a test statistic (R), where $R = 1$ if all replicates within a treatment are more similar to each other than any replicates from different treatments. R is approximately zero if the null hypothesis is true that similarities between and within treatments are the same. A Monte Carlo simulation where the Bray-Curtis matrix is randomly rearranged allows comparison between simulated and observed R-values and also determines the significance level at which the null hypothesis can be rejected. FISH data were analyzed using principal components analysis (PCA) (Clarke and Warwick 1994). Data were normalized by subtracting the mean and dividing by the standard deviation, prior to PCA.

Results

Manipulation of surface hydrophobicity

The treatments applied to the glass slides were successful in producing slides with a range of surface wettabilities (Figure 1). The DMS and TMS treatments resulted in the most hydrophobic surfaces, with the highest contact angle measurements, while the QAP treatment and untreated glass resulted in the most hydrophilic surfaces, with the lowest contact angle measurements. The APS and CLPRS treatments resulted in contact angle measurements that were between the two extremes. These treatments, therefore, gave two hydrophobic surfaces, two hydrophilic surfaces, and two surfaces of intermediate wettability.

Field recruitment to manipulated surfaces

After only 1 day in the field, there was very low recruitment to all slides, and no differences were seen in recruitment of *H. elegans* (Figure 2B). After 3 days in the field, recruitment of *H. elegans* was significantly higher on the slides treated with DMS and APS for

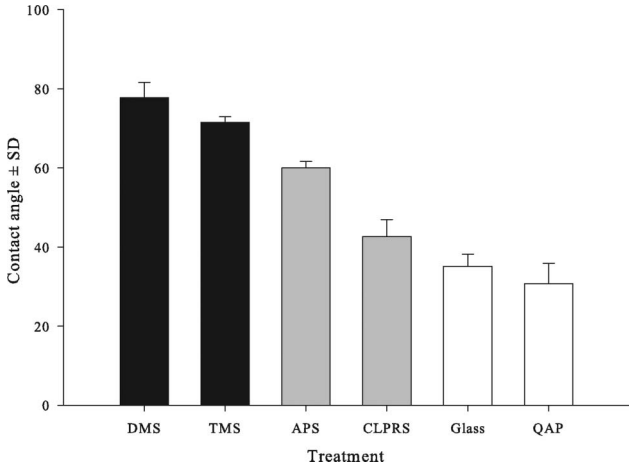


Figure 1. Contact angle measurements of the surfaces of glass slides treated with trimethylsilyl (TMS), dimethyldichlorosilane (DMS), aminopropyltriethoxysilane (APS), 3-chloroproplyltrimethoxysilane (CLPRS), trimethylaminopropyltrimethoxysilane (QAP) and untreated glass (Glass).

both trials (Figure 2), and during the August trial a high number also recruited to slides treated with QAP. DMS, APS and QAP treated slides encompassed the range of wettabilities that were deployed in the field. The remaining slides, which were treated with CLPRS and TMS, and untreated glass, all had lower numbers of recruits during both trials. After 6 days and 10 days in the field, for both trials, slides treated with APS and DMS still had significantly higher numbers of recruits than all other surfaces, but QAP treated slides had comparable number of recruits to the remaining slides.

Settlement on manipulated surfaces

Manipulated surfaces were collected from the field after 3, 6 and 10 days during May, and after 1, 3, 6 and 10 days during August and used in laboratory settlement assays with larvae of *H. elegans*. Patterns of larval settlement of *H. elegans* measured on manipulated surfaces were similar to those observed for recruitment in the field. After immersion for 1 and 3 days during August, there was very low settlement on all slides. After 3 days immersion during May there was a significantly higher settlement response to slides treated with APS, whereas after 6 days, slides treated with APS (during August) and DMS (during May) received the highest numbers of settlers (Figure 3). The remaining slides all received low larval settlement after 3 and 6 days immersion, with slides treated with CLPRS having the lowest settlement during August, after 6 days immersion. After 10 days in Pearl Harbor, all surfaces received high amounts of larval settlement during both trials.

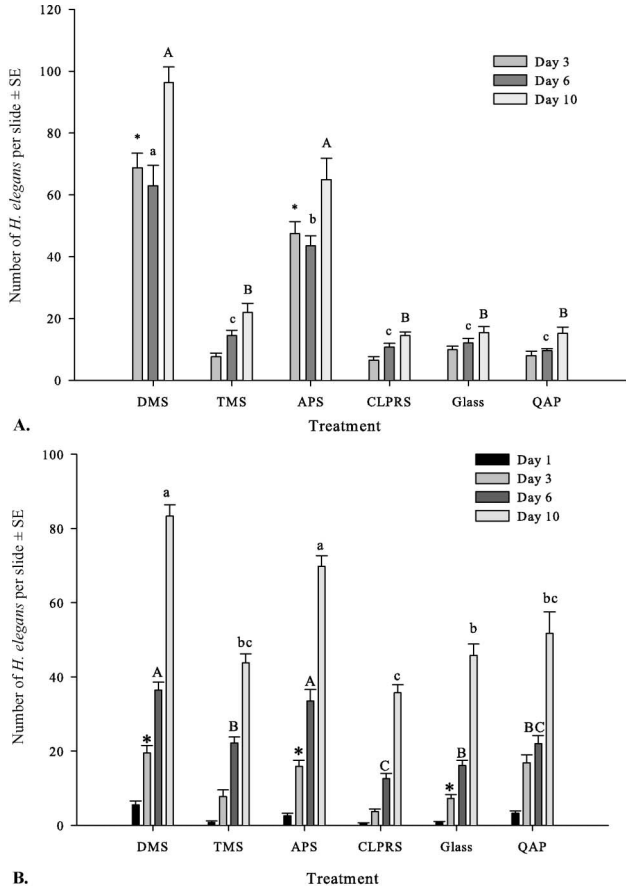


Figure 2. Recruitment of *H. elegans* to slides with manipulated wettability, during May (A) and August (B). Numbers are mean ($n = 15$) $\pm$ SE. Letters and symbols above bars indicate the results of a one-way ANOVA (A: Day 3 $p < 0.001$, $F\text{-ratio}_{5,54}$: 42.343; Day 6 $p < 0.001$, $F\text{-ratio}_{5,54}$: 86.618; Day 10 $p < 0.001$, $F\text{-ratio}_{5,54}$: 52.779; B: Day 1 $p < 0.001$, $F\text{-ratio}_{5,54}$: 10.009; Day 3 $p < 0.001$, $F\text{-ratio}_{5,54}$:13.629 Day 6 $p < 0.001$, $F\text{-ratio}_{5,54}$: 17.458; Day 10 $p < 0.001$, $F\text{-ratio}_{5,54}$: 23.338).

Bacterial cell counts on manipulated surfaces

For both trials, the total number of bacteria attached to slides increased during the period they were left in Pearl Harbor (Figure 4). During May, there were no differences in the densities of bacteria between the six different treatments after both 3 and 6 days in Pearl Harbor. After 3 days, all slides had a cell density between 1484.17 ($\pm$827.77) and 2724.40 ($\pm$760.28) cells mm^{-2}, and after 6 days, all slides had a density between 2664.34 ($\pm$515.31) and 5757.98 ($\pm$1476.73) cells mm^{-2}. After 10 days, there were higher numbers of bacteria present on slides treated with QAP, but all slides had high number of bacteria present, ranging from 8259.31 ($\pm$1554.14) cells mm^{-2} for untreated glass slide, to 16452.16 ($\pm$1260.27) cells mm^{-2} for slides treated with QAP (Figure 4A).

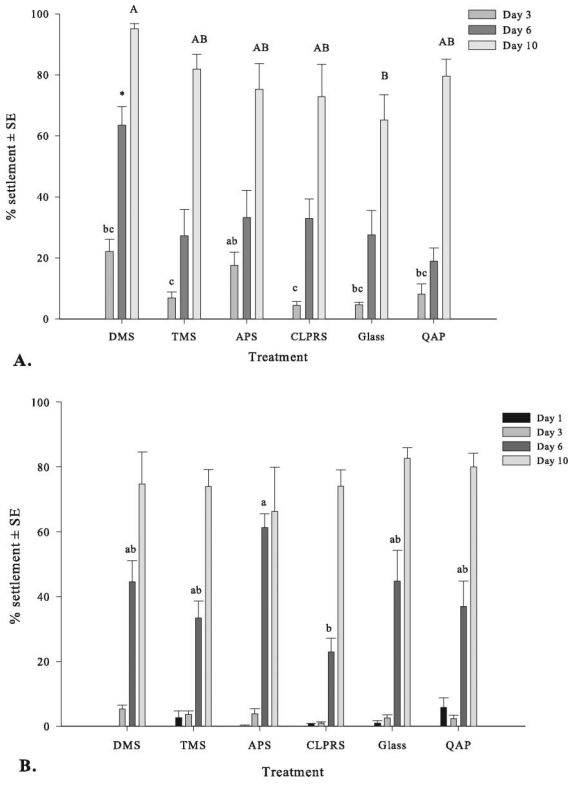


Figure 3. Percentage of laboratory settlement of larvae of *H. elegans* on slides with manipulated wettability, during May (A) and August (B). Numbers are percentage means ($n = 20$ larvae) $\pm$ SE. Letters and symbols above bars indicate the results of a one-way ANOVA (A: Day 3 $p < 0.001$, F-ratio$_{5,54}$: 6.292; Day 6 $p < 0.05$, F-ratio$_{5,54}$: 4.485; Day 10, ns F-ratio$_{5,54}$: 2.027. B: Day 1 ns, F-ratio$_{5,54}$: 1.856; Day 3 $p < 0.05$, F-ratio$_{5,54}$:3.168; Day 6 $p < 0.01$, F-ratio$_{5,54}$: 3.601; Day 10 ns, F-ratio$_{5,54}$: 0.454).

During August, after 1 day in the field, slides treated with CLPRS had the lowest number of bacterial cells (528.55 $\pm$ 115.85 cells mm^{-2}), and slides treated with QAP (2757.45 $\pm$ 793.22 cells mm^{-2}) and DMS (2041.24 $\pm$ 423.83 cells mm^{-2}) had the highest number of cells. After 3 days in Pearl Harbor, bacterial densities were similar to those after only 1 day, with maximum cells found on slides treated with QAP (3422.57 $\pm$ 532.66), and the lowest cell densities of 1104.2 $\pm$ 219.67, found on slides treated with DMS. After 6 days in the field, no differences were seen in the number of cells on the various treatments, and the number of bacteria per mm^2 ranged from 2840.84 $\pm$ 300.16, on slides treated with CLPRS to 4436.93 $\pm$ 395.15 on slides treated with TMS. After 10 days in the field, a much greater density of bacteria was found on all slides (between 14024.93 $\pm$ 2248.88 and 12720.75 $\pm$ 2818.65), with no differences in cell density between the treatments (Figure 4B).

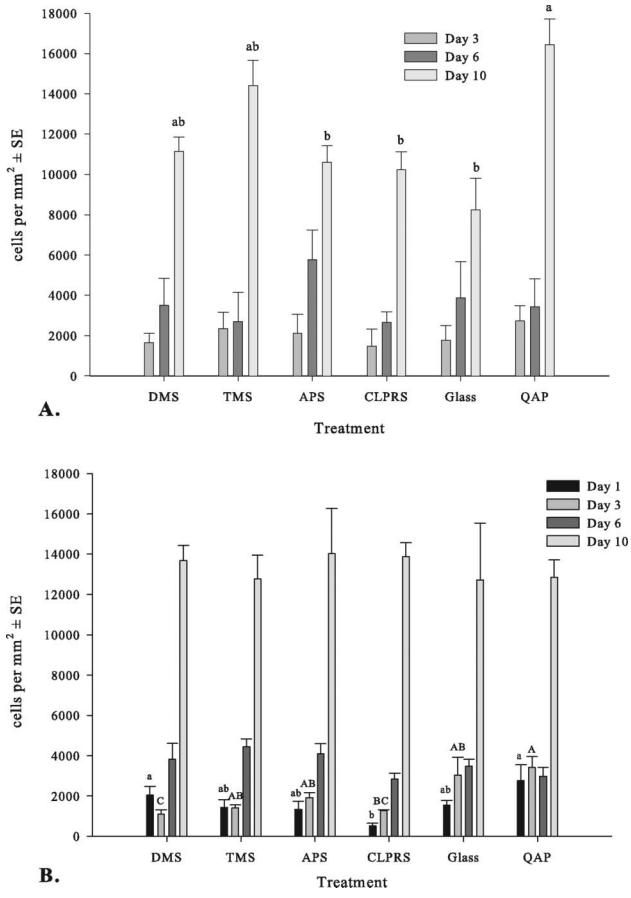


Figure 4. Total densities of bacteria on slides during A. May and B. August. Numbers are percentage means ($n = 3$) $\pm$ SE. Letters and symbols above bars indicate the results of a one-way ANOVA (A: Day 3 ns, F-ratio$_{5,12}$: 0.782; Day 6 ns, F-ratio$_{5,12}$: 2.379; Day 10, $p < 0.05$ F-ratio$_{5,12}$: 7.594. B: Day 1 $p > 0.05$, F-ratio$_{5,12}$: 5.531; Day 3 $p < 0.05$, F-ratio$_{5,12}$: 6.759; Day 6 ns, F-ratio$_{5,12}$: 1.824; Day 1- ns, F-ratio$_{5,12}$: 0.176).

Community analysis – DGGE

A representative DGGE gel of all samples from Day 1 during the August trial is shown in Figure 5. NMDS plots from all DGGE gels are shown in Figure 6. ANOSIM was performed on all NMDS plots. R-values were interpreted as follows: $R > 0.75$ as well separated, $R > 0.5$ as overlapping, but clearly different, and $R < 0.25$ as barely separable at all, in accordance with the PRIMER-manual (Clarke and Warwick 1994). For all days and for both trials no differences between community composition of bacteria on the manipulated surfaces were found, with the exception of Day 3 for the August trial (Figure 6D), which had an R value of 0.818. For Day 3 of the August trial, the bacterial community composition on slides treated with CLPRS (C) was found to be

significantly different from both TMS (T) and untreated glass (G).

Community analysis – FISH

There were no differences in the numbers of bacteria that bound the various probes in either trial, with two exceptions: Actinobacteria-positive cells on slides treated with DMS (May trial, Day 3, Figure 7A),

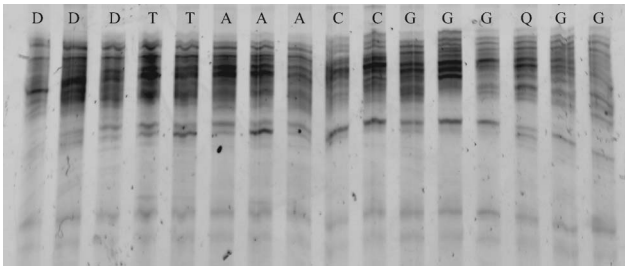


Figure 5. Photograph of a representative denaturing gradient electrophoresis gel of 1-day old biofilms from the August trial. From left to right: Lanes 1–3 = slides treated with DMS (D); lanes 4–5 = slides treated with TMS (T); lanes 6–9 = slides treated with APS (A); lanes 10–11 = slides treated with CLPRS (C); lanes 12–15 = untreated glass slides (G); lanes 16–18 = slides treated with QAP (Q).

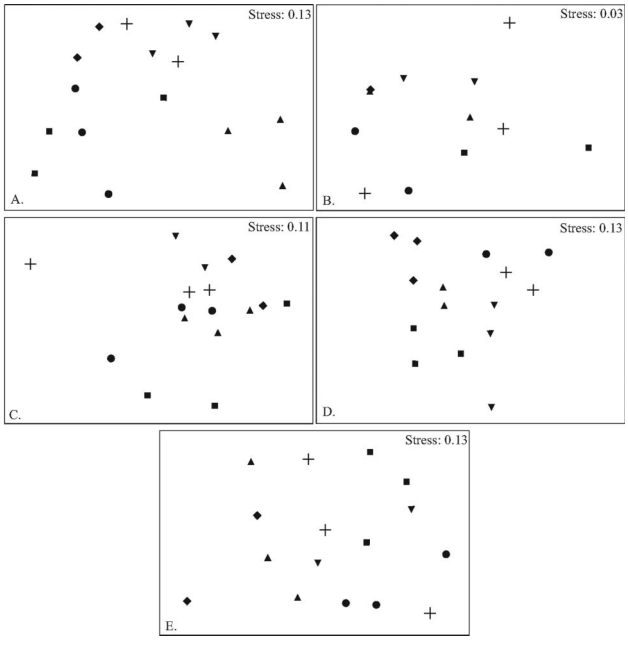


Figure 6. Multi-dimensional scaling plots showing differences between samples run on DGGE gels. Samples were from (A) Day 3 (May trial); (B) Day 6 (May trial); (C) Day 1 (August trial); (D) Day 3 (August trial); (E) Day 6 (August trial). ▲ = slides treated with DMS; ▼ = slides treated with TMS; ■ = slides treated with APS; ♦ = slides treated with CLPRS; ● = untreated glass; + = slides treated with QAP.

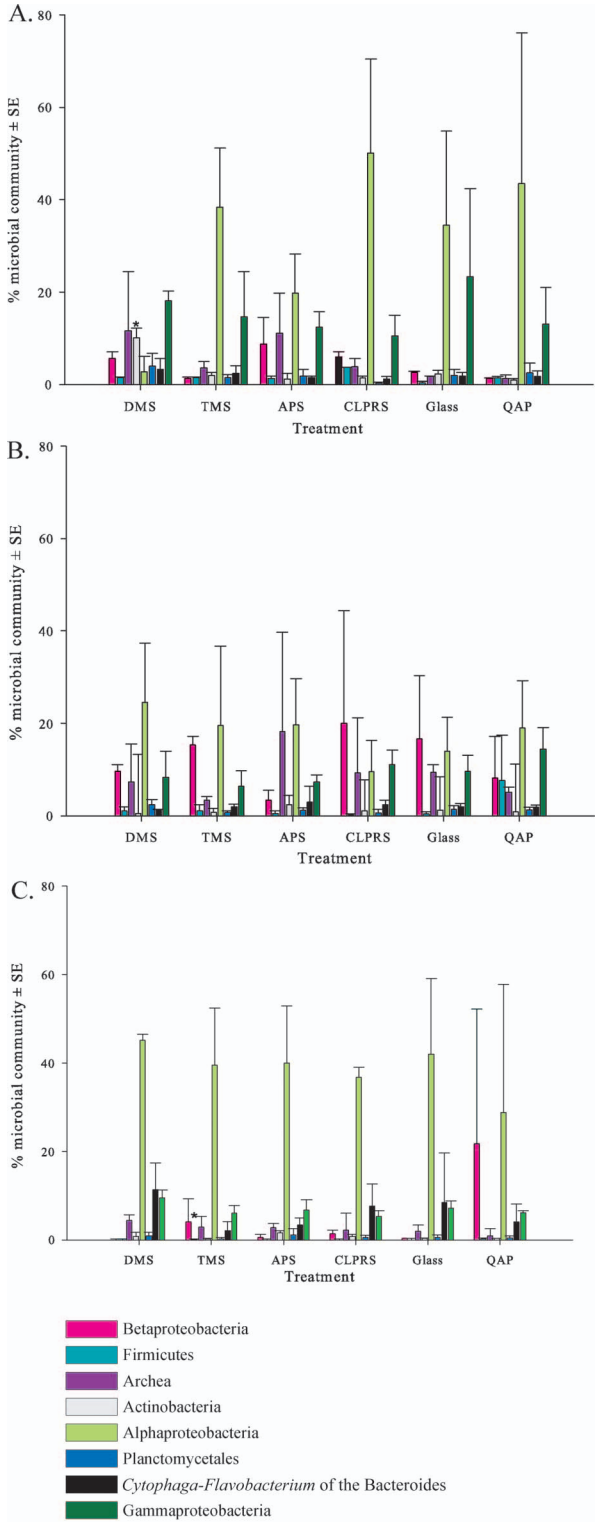
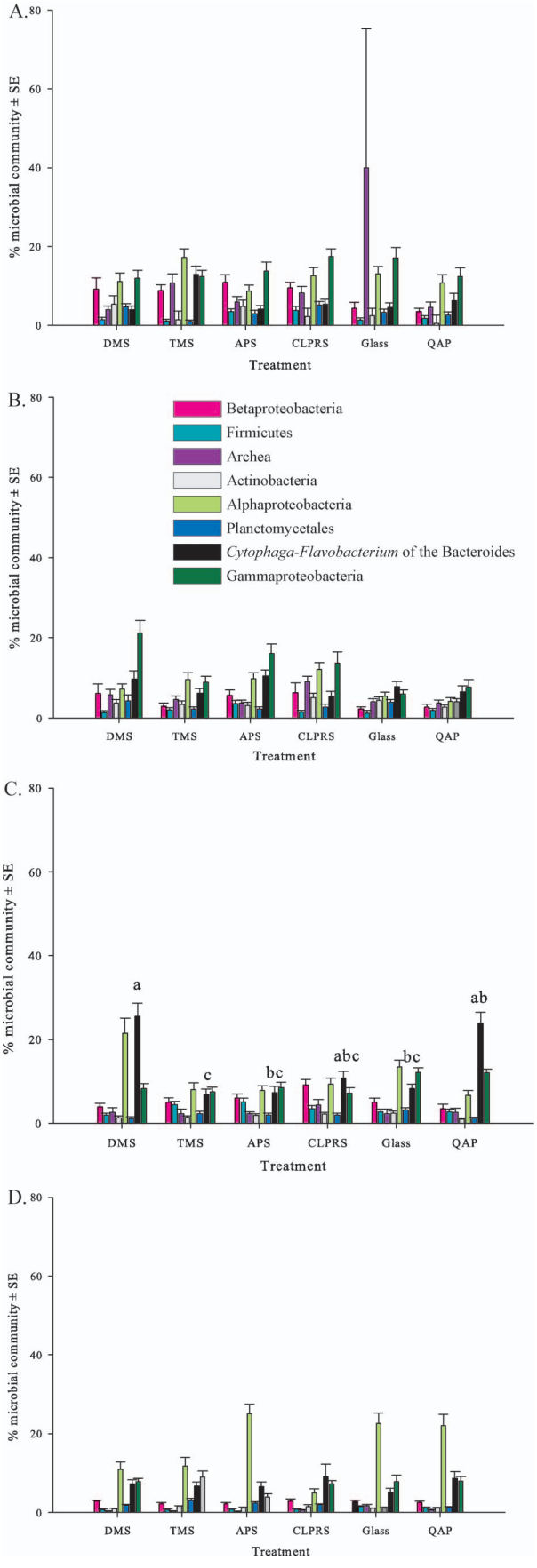


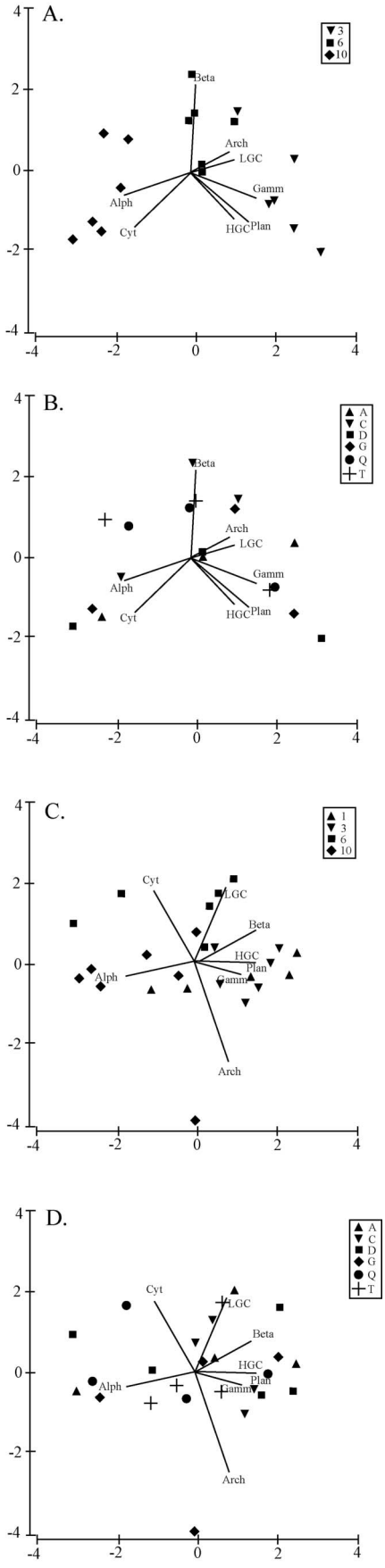
Figure 7. Results of FISH for May trial after (A) 3 days; (B) 6 days; (C) 10 days submersion at Pearl Harbor. One-way Analysis of variance revealed no differences between numbers of bacteria for any of the groups on any surfaces except for Actinobacteria on Day 3, which were higher on surfaces treated with DMS than all other surfaces, and Firmicutes on day 10, which were higher on surfaces treated with TMS than all other surfaces.



and the *Cytophaga-Flavobacterium* of Bacteroidetes-positive cells on certain slides treated with DMS and QAP in the August trial (Day 6, Figure 8C). Specifically, for the May trial, there were higher numbers of Actinobacteria-positive cells found on slides treated with DMS (10.04% $\pm$ 2.21%) than on any other treatment (between 0.94% $\pm$ 0.38% and 2.24 $\pm$ 0.77%). For the August trial there were higher numbers of *Cytophaga-Flavobacterium* of Bacteroidetes-positive cells on slides treated with DMS (25.29% $\pm$ 3.15%) than all slides (between 6.78% $\pm$ 1.37% and 10.75% $\pm$ 1.67%) except those treated with QAP (23.91% $\pm$ 2.56%), and slides treated with QAP had higher numbers of *Cytophaga-Flavobacterium* of Bacteroidetes-positive cells than those treated with TMS (6.78% $\pm$ 1.37%). There were no differences found for any other groups, as detected by analysis of variance (Figures 7 and 8).

PCA based on percentages of bacteria labeled by FISH probes revealed that there were differences between the various sampling dates, but not between the various surface treatments, in the community composition of bacteria found in biofilms (Figure 9). For the May trial, PC1 accounted for 47.3% of the variation, and PC2 accounted for 21.5% of the variation (Figure 9A,B). For the August trial, PC1 accounted for 35.1% of the variation, and PC2 accounted for 18.0% (Figure 9C,D). During the May trial, Alphaproteobacteria, Actinobacteria and Planctomycetales contributed the highest amounts to PC1, and PC2 is a weighted combination of Firmicutes, Gammaproteobacteria and Archea (Figure 9A,B). During the August trial, all groups except for Archea and *Firmicutes* contributed roughly equally and highly to PC1, while PC2 was mostly explained by Firmicutes, *Cytophaga-Flavobacterium* of Bacteroidetes and Archea (Figure 9C,D). For both trials, principal component analysis showed clear separation of community composition between days, but not between surfaces. This result is supported by both the NMDS and ANOSIM analyses. No differences are seen between the communities on manipulated surfaces, while differences are seen in communities of different ages.

Figure 8. Results of FISH for August trial after (A) 1 day; (B) 3 days; (C) 6 days; (D) 10 days submersion at Pearl Harbor. One-way Analysis of variance revealed no differences between numbers of bacteria for any of the groups on any surfaces except for Day 6, when *Bacteroides* were higher on surfaces treated with DMS and QAP than TMS, and higher on surfaces treated with DMS than on surfaces treated with APS and untreated glass surfaces.



Discussion

The results presented here indicate that the formation of a biofilm community on hard surfaces in Pearl Harbor, Hawaii, was not influenced by the initial wettability of a surface. Bacterial density was consistent across all surfaces for all time points during both trials, and the bacterial community composition of the biofilms formed across the six surface treatments was also largely indiscernible using two different DNA fingerprinting methods. Hung et al. (2008) also observed that similar marine biofilms (both density and composition) formed on surfaces that had a range of wettabilities. The advantages for bacteria of being surface bound in a community rather than planktonic are well known (reviewed by Dunne 2002), and adhesion of some bacteria to surfaces can be strongly correlated with surface properties (Dexter et al. 1975; Dexter 1979; Fletcher and Loeb 1979; Pringle and Fletcher 1983; van Pelt et al. 1985; Cooksey and Wigglesworth-Cooksey 1995; Weineck and Fletcher 1995; Allion et al. 2006; Jain and Bhosle 2009). However, no effect of wettability on bacterial biofilm formation was observed in this study. This may be due to the ability of some bacteria to develop different attachment mechanisms, depending on the stage of bacterial growth, enabling them to attach to a range of surface wettabilities (Paul and Jeffrey 1985; Shea et al. 1991). There is also evidence that the attachment of certain species of bacteria can enhance the attachment ability of other bacteria and that some bacterial cells have multiple mechanisms of adhesion and thus can utilize whichever mechanism is appropriate for the surface properties of the substratum contacted (Shea et al. 1991). These mechanisms may all contribute to the similar colonization of surfaces by marine biofilms in Pearl Harbor. It is clear that a combination of rapid processes occurs on all surfaces that are immersed in seawater, and that the adsorption of inorganic material and the attachment of proteins can affect the attachment of bacterial cells. In the present study, the community composition of the marine biofilm on all surfaces was largely the same across all surfaces,

Figure 9. Principal component analysis of FISH results of biofilms from May (A and B) and August (C and D), graphed with age of biofilms as the factor (A and C), and surface as the factor (B and D). Biplots of the FISH results were overlaid as vectors. Abbreviations: Alph = Alphaproteobacteria; Arch = Archea; Beta = Betaproteobacteria, Cyto = *Cytophaga-Flavobacterium* of Bacteroidetes; Gamm = Gammaproteobacteria; HGC= Actinobacteria; LGC = Firmicutes; Plan = Planctomycetales. A and C: Day 1 ▲; Day 3 ▼; Day 6 ■; Day 10 ♦; B and D: A = APS; ▲; C=CLPRS, ▼; D = DMS, ■; G = glass, ♦; Q = QPS, ●; T = TMS, +.

suggesting that these rapid processes enable similar colonization by communities of marine bacteria in a dynamic environment regardless of initial surface wettability.

The data presented here suggest that the biofilm community was similar across all surfaces for each time point, yet larvae of *H. elegans* exhibited preferences for some surfaces both in laboratory and field settlement trials. *H. elegans* will not settle in the absence of a biofilm, and as such, effects of wettability cannot be directly tested on these larvae. However, previous studies indicate that the wettability of surfaces that have been manipulated using silane treatments remains stable for up to 6 days (Rittschof and Costlow 1989a). Despite similar biofilm formation, initially, and up to 6 days following immersion, larvae settled in highest numbers on surfaces treated with DMS and APS. These treatments resulted in surfaces having low to medium wettability, and larvae settled on these in higher numbers than on all other surfaces. Only low settlement was observed on treatments with comparable contact angle readings, namely TMS and CLPRS. Surfaces treated with QAP, which had the highest wettability, received variable amounts of settlement during the first 6 days of the trial. Qian et al. (2000) also tested larval settlement preferences of *H. elegans* on biofilmed surfaces with a variety of wettabilities, and observed settlement preferences that were not correlated with surface wettability. Other organisms show strong preferences to hydrophobic *vs.* hydrophilic surfaces. Larvae of *Balanus amphitrite* settle in higher numbers on glass, which is hydrophilic, than on polystyrene, which is hydrophobic, and bryozoans and the cnidarian *Cyanea* show the opposite trend (Rittschof et al. 1984; Rittschof and Costlow 1989a,b; Roberts et al. 1991). The bacterium *Cobetia marina* (Ista et al. 2004), spores of the green alga *Ulva* sp. (Dillon et al. 1989; Ista et al. 2004), and spores of the brown alga *Hincksia irregulatus* (Greer et al. 2003) all show strong attachment preferences for hydrophobic surfaces. Why is *H. elegans* is less selective than these other organisms? An obvious difference is that *H. elegans* requires the presence of a biofilm to settle, while these other organisms do not. However, previous studies have shown that silane treated surfaces maintain differences in surface wettability for up to 6 days submersion in seawater in the field (Rittschof and Costlow 1989a), suggesting that *H. elegans* was being presented with surfaces that had a range of wettabilities in the present study, but still did not show any clear preferences for surfaces with either low or high wettability. One explanation is that inductive bacterial species, such as *Pseudoalteromonas luteoviolacea* (Huang and Hadfield 2003), might be faster to colonize some of the surfaces (such as DMS and APS), and that *H. elegans* is responding to specific species of bacteria within the biofilm. The FISH probes that were used in this study did not provide sufficiently high resolution to determine the presence and absence of specific species of bacteria. Therefore, it is not possible to separate the effects of initial surface chemistry and the possible effects of subsequent biofilm formation at a species-specific level. However, it is clear that the silane treatments DMS and APS, either in combination with early biofilm formation, or by themselves, had a positive effect on larval settlement of *H. elegans*, over untreated glass.

The age of the biofilm has been shown to be strongly associated with larval settlement of *H. elegans*, with older biofilms inducing higher amounts of larval settlement than younger biofilms (Hadfield et al 1994; Jin and Qian 2005; Shikuma and Hadfield 2006; the present study). This pattern has also been observed for coral larvae (Webster et al. 2004), mussel larvae (Bao et al. 2007) and barnacle cyprids (Hung et al. 2008). Principal component analysis indicated that the community composition of biofilms changed over time, as is expected through the process of succession observed in many communities. Initially, Gammaproteobacteria were present in higher percentages, and, in general, the percentage of Gammaproteobacteria decreased over the 10 day period. Alphaproteobacteria were always present in high percentages, and in both May and August, the Alphaproteobacteria were the dominating group present in 10 day old biofilms. Principal component analysis indicated that for both trials, members of the Archea, Gammaproteobacteria and Planctomycetales were most strongly associated with young, 3 day old biofilms, while the Alphaproteobacteria were most strongly associated with 10 day old biofilms. This shift in community composition over time may be responsible for the higher amounts of settlement on older surfaces. In contrast to the present results, Webster et al. (2004) were unable to detect differences in the composition of biofilms of different ages, despite settlement preferences by coral larvae for older biofilms. As a biofilm ages, its cell density also increases, and Huang and Hadfield (2003), Lau et al. (2005), Jin and Qian (2005) and Shikuma and Hadfield (2006) all attributed the increase in settlement to an increase in density of the biofilm. However, cell density does not appear to be the only requirement of *H. elegans* for larval settlement to occur, since monospecies films of some bacteria do not induce, while others do, even though the densities are the same (Huang and Hadfield 2003). The correlation of larval settlement with bacterial density was also seen in the present study, with older dense biofilms inducing the highest levels of larval settlement. This relationship

may be due to higher overall bacterial densities or due to the increase of specific inductive species within the biofilm. The settlement of *H. elegans* on slides that had been immersed for up to 10 days was independent of the presence of already settled worms, as previous studies have shown that larvae of *H. elegans* do not settle gregariously (Walters et al. 1997).

One factor not examined here, which may potentially influence patterns of settlement and recruitment, are changes in the diatom community within biofilms. Certain species of marine diatoms may induce settlement of significant numbers of *H. elegans* (Harder et al. 2002), but it is unclear what role these diatom species play in mixed biofilms in the field. Chiu et al. (2008) observed that with the addition of nutrients to a marine system, the biofilm-diatom community changed, and the density of both bacteria and diatoms increased. This change was reflected by a change in settlement of the barnacle *B. amphitrite* and the slipper limpet *Crepidula onyx*. However, no change was observed in settlement of *H. elegans*. There were diatoms present in the biofilm communities that developed on the slides in the present study, but their presence was not quantified or correlated with either surface properties or larval settlement of *H. elegans*. Diatoms have been shown to influence larval settlement of various marine invertebrates, including some species of abalone (Roberts 2001), barnacles (Patil and Anil 2005) and sea cucumbers (Ito and Kitamura 1997), and they may have played an undetected role in the interactions that were observed in this study. The influence of bacteria on larval settlement of *H. elegans* has been well demonstrated, but the influence of diatoms remains unclear.

This study revealed that the bacterial community composition of biofilms that accumulated on surfaces over a 10 day period of immersion in Pearl Harbor was similar, regardless of initial surface wettability. The bacterial community undergoes a process of succession, with the initial community having a distinctly different composition than older biofilms. The settlement and recruitment of *H. elegans* onto surfaces immersed for up to 6 days does differ on surfaces with different initial surface wettability. However, these differences do not appear to be related to the composition or density of the biofilms, and they do not follow a predictable pattern with relation to surface wettability. Larval settlement of *H. elegans* appears to require a minimum density of bacteria, and neither larval settlement nor bacterial attachment is reduced by the manipulation of surface wettability. Rather, the ability of bacterial cells to attach to a range of surfaces, and the pressure for *H. elegans* to settle and reproduce rapidly, result in the equal and high recruitment of *H. elegans* to all surfaces.

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References

Allion A, Baron J, Boulange-Petermann L. 2006. Impact of surface energy and roughness on cell distribution and viability. Biofouling 22:269–278.

Bao W-Y, Satuito CG, Yang J-L, Kitamura H. 2007. Larval settlement and metamorphosis of the mussel *Mytilus galloprovincialis* in response to biofilms. Mar Biol 150:565–574.

Bhosle NB. 2004. Chemical and molecular characterization of biofilm on metal surfaces. In: Ramaiah N, editor. Marine microbiology: facets and opportunities. Dona Paula, Goa, India: National Institute of Oceanography. p. 225–239.

Boon N, Windt WD, Verstraete W, Top EM. 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol Ecol 39:101–112.

Chiu JMY, Zhang R, Wang H, Thiyagarajan V, Qian P. 2008. Nutrient effects on intertidal community: from bacteria to invertebrates. Mar Ecol Prog Ser 351: 41–50.

Clarke KR, Warwick RM. 1994. Change in marine communities: an approach to statistical analysis and interpretation. Plymouth: Plymouth Marine Laboratory.

Cooksey KE, Wigglesworth-Cooksey B. 1995. Adhesion of bacteria and diatoms to surfaces in the sea: a review. Aquat Microb Ecol 9:87–96.

Crisp DJ, Ryland JS. 1960. Influence of filming and of surface texture on the settlement of marine organisms. Nature 4706:119.

Dexter SC. 1979. Influence of substratum critical surface tension on bacterial adhesion – *in situ* studies. J Colloid Interface Sci 70:346–354.

Dexter SC, Sullivan JDJ, Williams J III, Watson SW. 1975. Influence of substrate wettability on attachment of marine bacteria to various surfaces. Appl Microbiol 30:298–308.

Dillon PS, Maki JS, Mitchell R. 1989. Adhesion of *Enteromorpha* swarmers to microbial films. Microb Ecol 1989:39–47.

Dunne WM. 2002. Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev 15:155–166.

Fletcher M, Loeb GI. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl Environ Microbiol 37:67–72.

Genzer J, Efimenko K. 2006. Recent developments in superhydrophobic surfaces and their relevance to marine fouling: a review. Biofouling 22:339–360.

Gerhart DJ, Rittschoff D, Hooper IR, Eisenman K, Meyer AE, Baier RE, Young CM. 1992. Rapid and inexpensive quantification of the combined polar components of surface wettability: applications to biofouling. Biofouling 5:251–259.

Greer SP, Iken K, Mclintock JB, Amsler CD. 2003. Individual and coupled effects of echinoderm extracts and surface hydrophobicity on spore settlement and germination of the brown alga *Hincksia irregularis*. Biofouling 19:315–326.

Guenther J, de Nys R. 2007. Surface microtopographies of tropical sea stars: lack of an efficient physical defence mechanism against fouling. Biofouling 23:419–429.

Hadfield MG, Unabia C, Smith CM, Michael TM. 1994. Settlement preferences of the ubiquitous fouler *Hydroides elegans*. In: Fingerman R, Nagabhushanam R, Sarojini R, editors. Recent developments in biofouling control. New Delhi: Oxford & IBH. p. 65–74.

Harder T, Lam C, Qian P-Y. 2002. Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229:105–112.

Holm ER, Cannon G, Roberts D, Schmidt AR, Sutherland JP, Rittschof D. 1997. The influence of initial surface chemistry on development of the fouling community at Beaufort, North Carolina. J Exp Mar Biol Ecol 215:189–203.

Holmström C, James S, Kjelleberg S. 1996. Inhibition of common fouling organisms by marine bacterial isolates with special reference to the role of pigmented bacteria. Biofouling 10:251–259.

Huang S, Hadfield MG. 2003. Composition and density of bacterial biofilms determine larval settlement of the polycheate *Hydroides elegans*. Mar Ecol Prog Ser 260:161–172.

Huggett MJ, Williamson JE, de Nys R, Kjelleberg S, Steinberg PD. 2006. Settlement of the common Australian sea urchin *Heliocidaris erythrogramma* in response to bacteria from the surface of coralline algae. Oecologia 149:604–619.

Hung OS, Thiyagarajan V, Qian P. 2008. Preferential attachment of barnacle larvae to natural multi-species biofilms: does surface wettability matter? J Exp Mar Biol Ecol 361:36–41.

Ista LK, Callow ME, Finlay FA, Coleman SE, Nolasco AC, Simons RH, Callow JA, Lopez GP. 2004. Effect of substratum surface chemistry and surface energy on attachment of marine bacteria and algal spores. Appl Environ Microbiol 70:4151–4157.

Ito S, Kitamura H. 1997. Induction of larval metamorphosis in the sea cucumber *Stichopus japonicus* by periphytic diatoms. Hydrobiologia 358:281–284.

Jain A, Bhosle NB. 2009. Biochemical composition of the marine conditioning film: implications for bacterial adhesion. Biofouling 25:13–19.

James RJ, Underwood AJ. 1994. Influence of colour of substratum on recruitment of spirorbid tubeworms to different types of intertidal boulders. J Exp Mar Biol Ecol 181:105–115.

Jin T, Qian P-Y. 2005. Amino acid exposure modulates the bioactivity of biofilms for larval settlement of *Hydroides elegans* by altering bacterial community components. Mar Ecol Prog Ser 297:169–179.

Johnson CR, Sutton DC. 1994. Bacteria on the surface of crustose coralline algae induce metamorphosis of the crown-of-thorns starfish *Acanthaster planci*. Mar Biol 120:305–310.

Lau SK, Qian PY. 2001. Larval settlment in the serpulid polycheate *Hydroides elegans* in response to bacterial films: an investigation of the nature of putitative larval settlement cue. Mar Biol 138:321–328.

Lau SCK, Harder T, Qian P. 2003. Induction of larval settlement in the serpulid polycheate *Hydroides elegans* (Haswell): role of bacterial extracellular polymers. Biofouling 19:197–204.

Lau SCK, Thiyagarajan V, Cheung SCK, Qian P-Y. 2005. Roles of bacterial community composition in biofilms as a mediator for larval settlement of three marine invertebrates. Aquat Microb Ecol 38:41–51.

Nedved BT, Hadfield MG. 2009. *Hydroides elegans* (Annelida: Polychaeta): a model for biofouling research. In: Flemming HC, Venkatesan R, Murthy SP, Cooksey KE, editors. Marine and industrial niofouling, Springer Series on Biofilms. Berlin: Springer. p. 203–217.

Otani M, Oumi T, Uwai S, Hanyuda T, Prabowo TRE, Yamaguchi T, Kawai H. 2007. Occurrence and diversity of barnacles on international ships visiting Osaka Bay, Japan, and the risk of introduction. Biofouling 23: 277–286.

Patel P, Callow ME, Joint I, Callow JA. 2003. Specificity in the settlement; modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. Environ Microbiol 5:338–349.

Patil JS, Anil AC. 2005. Influence of diatom exopolymers and biofilms on metamorphosis in the barnacle *Balanus amphitrite*. Mar Ecol Prog Ser 301:231–245.

Paul JH, Jeffrey WH. 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*. Appl Environ Microbiol 50:431–437.

Pettengil JB, Wendt DE, Schug MD, Hadfield MG. 2007. Biofouling likely serves as a major mode of dispersal for the polychaete tubeworm *Hydroides elegans* as inferred from microsatellite loci. Biofouling 23:161–169.

Pringle JH, Fletcher M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Appl Environ Microbiol 45:811–817.

Qian P, Rittschoff D, Sreedhar B. 2000. Macrofouling in unidirectional flow: miniature pipes as experimental models for studying the interaction of flow and surface characteristics on the attachment of barnacle, bryozoan and polycheate larvae. Mar Ecol Prog Ser 207:109–121.

Rittschof D, Costlow JD. 1989a. Bryozoan and barnacle settlement in relation to initial surface wettability: a comparison of laboratory and field studies. In: Ros JD, editor. Topics in marine biology. Barcelona: Instituts de Ciencias del Mar. p. 411–416.

Rittschof D, Costlow JD. 1989b. Surface determination of macroinvertebrate larval settlement. Proceedings of the 21st Engineering in Medicine and Biology Conference; 1986 September 14–19. Warsaw: Polish Academy of Sciences – Institude of Oceanology. p. 155–163.

Rittschof D, Branscomb ES, Costlow JD. 1984. Settlement and behavior in relation to flow and surface in larval barnacles, *Balanus amphitrite* Darwin. J Exp Mar Biol Ecol 82:131–146.

Roberts D, Rittschof D, Holm E, Schmidt AR. 1991. Factors influencing initial larval settlement: temporal, spatial and surface molecular components. J Exp Mar Biol Ecol 150:203–211.

Roberts R. 2001. A review of settlement cues for larval abalone (*Haliotis* spp.). J Shellfish Res 20:571–586.

Scardino AJ, Guenther J, de Nys R. 2008. Attachment point theory revisited: the fouling response to a microtextured matrix. Biofouling 24:45–53.

Schultz MP. 2007. Effects of coating roughness and biofouling on ship resistance and powering. Biofouling 23:331–341.

Shea C, Nunley JW, Williamson JC, Smith-Somerville HE. 1991. Comparison of the adhesion properties of *Deleya marina* and the exopolysaccharide-defective mutant strain DMRt. Appl Environ Microbiol 57:3107–3113.

Shikuma NJ, Hadfield MG. 2006. Temporal variation of an initial marine biofilm community and its effects on larval settlement and metamorphosis of the tubeworm *Hydroides elegans*. Biofilms 2:231–238.

Townsin RL. 2003. The ship hull fouling penalty. Biofouling 19:9–15.

Unabia CRC, Hadfield MG. 1999. Role of bacteria in larval settlement and metamorphosis of the polychaete *Hydroides elegans*. Mar Biol 133:55–64.

van Pelt AWJ, Weerkamp AH, Uyen MWJC, Busscher HJ, de Jong HP, Arends J. 1985. Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. Appl Environ Microbiol 49:1270–1275.

Walters LJ, Hadfield MD, del Carmen KA. 1997. The importance of larval choice and hydrodynamics in creating aggregations of *Hydroides elegans* (Polychaeta: Serpulidae). Invert Biol 116:102–114.

Webster NS, Smith LD, Heyward AJ, Watts JEM, Webb RI, Blackall LL, Negri AP. 2004. Metamorphosis of a scleractinian coral in response to microbial biofilms. Appl Environ Microbiol 70:1213–1221.

Weineck KM, Fletcher M. 1995. Bacterial adhesion to hydroxyl- and methyl-terminated alkanethiol self-assembled monolayers. Appl Enviro Microbiol 177:1959–1966.

Wieczorek SK, Todd CD. 1998. Inhibition and facilitation of settlement of epifaunal marine invertebrate larvae by microbial biofilm cues. Biofouling 12:81–118.

Wilkinson L. 1997. SYSTAT 7.0. Chicago, IL: SPSS Inc.

Zobell CE, Allen EC. 1935. The significance of marine bacteria in the fouling of submerged surfaces. J Bacteriol 29:230–251.