TEMPORAL VARIATION IN AN INITIAL MARINE BIOFILM COMMUNITY AND

ITS EFFECT ON LARVAL SETTLEMENT OF THE TUBEWORM *Hydroides elegans*

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ABSTRACT

Planktonic larvae of many invertebrates settle preferentially on surfaces covered by bacterial biofilms. The polychaete tubeworm *Hydroides elegans* is induced to settle by biofilms and is the primary colonizer of newly submerged surfaces in the succession of macrofouling invertebrates in Pearl Harbor, Hawai'i. This study examines culture-independent community composition, as well as densities of bacteria, and how these aspects of marine biofilms affect settlement preferences of *H. elegans*. Settlement assays of *H. elegans* were conducted on naturally formed biofilms of increasing age from Pearl Harbor, Hawai'i. Denaturing Gradient Gel Electrophoresis (DGGE) and epifluoresence microscopy were used to identify community composition and densities of bacterial biofilms. This study showed that increased densities of bacteria rather than dominant species composition are likely responsible for the primary colonization of submerged surfaces by *H. elegans* in Pearl Harbor.

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INTRODUCTION:

The larvae of many sessile marine invertebrates are known to possess the ability to find suitable substrates on which to settle (Crisp, 1974). Patterns of settlement, such as succession in marine biofouling communities, indicate that there may be underlying mechanisms for this ordered recruitment (Chalmer, 1982). Settlement cues often direct settlement preferences of these larvae and can be biological in origin (Crisp, 1974; Hadfield and Paul, 2001; Steinberg et al., 2002). Bacterial biofilms are widely known to act as a cue and have been shown to induce settlement in many invertebrate larvae from multiple phyla (Hadfield et al., 1994; Hamer et al., 2001; Hofmann and Brand, 1987; Negri et al., 2001; Pawlik, 1992; Unabia and Hadfield, 1999; Webster et al., 2004).

Hydroides elegans is an appropriate model organism to study the induction of settlement by bacterial biofilms. This serpulid polychaete is the primary invertebrate colonizer of newly submerged surfaces in sub-tropical and tropical harbors and estuaries around the world (Hadfield et al., 1994). *H. elegans* has separate sexes, and starts to produce gametes at about 3 wk. in laboratory culture. Larvae must settle and take advantage of a 4 to 6 wk. period when surfaces are initially submerged before bryozoans, ascidians, and sponges overgrow and out compete them for space (Holm et al., 2000). Unlike other fouling polychaete species (Jensen and Morse, 1984; Toonen and Pawlik, 1996), *H. elegans* is not induced to settle by conspecific animals (Walters et al., 1997) but does settle in response to bacterial biofilms (Hadfield et al., 1994). Possible advantages to settling on surfaces covered by bacterial biofilms include an indication of food, preferable habitat, and likelihood that the surface will be submerged

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long enough for the animal to mature and reproduce. Specific settlement cues for *H. elegans* have yet to be identified, but studies have shown that the cue is either bound to the biofilm surface in the exopolysacharides or on the bacterial cells themselves (Huang and Hadfield, 2003).

Natural biofilms are composed of many microbial species and their extracellular matrices attached to a surface (Costerton and Stewart, 2001). Rather than conglomerations of cells and slime, biofilms are instead organized communities complete with functional microcolonies and channels to perform complex metabolic processes (Costerton et al., 1999). Bacterial succession on newly submerged surfaces is known to occur in multiple habitats (Jackson et al., 2001; Kelly and Chistoserdov, 2001; Rao, 2003), but how these natural microbial assemblages influence macrofouling settlement is not yet completely understood.

Single species biofilms, developed from bacteria previously isolated from natural multi-species biofilms, were shown to induce varying rates of settlement of *H. elegans* (Huang and Hadfield, 2003; Lau and Qian, 2002; Unabia and Hadfield, 1999). Monospecific biofilms of *Cytophaga lytica* induced about 40% settlement of *H. elegans*, while biofilms of *Pseudoalteromonas luteoviolacea* induced settlement rates similar to natural biofilms (Huang and Hadfield, 2003). Percent settlement of *H. elegans* and cell density in a monospecific biofilm of *P. luteoviolacea* was also shown to be positively correlated (Huang and Hadfield, 2003). These results suggest that there may be a quantitative increase in the cue to settle when more cells are present, perhaps because more cells produce greater quantities of the signal (Huang and Hadfield, 2003).

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However, until the present study, it has not been shown whether variations in the species composition of biofilms play a role in *H. elegans* settlement preferences on biofilms as they age.

The current knowledge of microbially induced settlement of *H. elegans* larvae is limited to biofilms of species cultured with artificial media and natural multi-species biofilms of unknown species composition (Hadfield et al., 1994; Huang and Hadfield, 2003; Unabia and Hadfield, 1999). Artificial media can often bias estimates of species composition of natural multi-species communities due to the metabolic and growth condition requirements of some types of bacteria (Amann et al., 1995). Unculturable species present in natural biofilms may play a role in *H. elegans* larval induction.

Denaturing Gradient Gel Electrophoresis (DGGE) can be used to assess natural bacterial assemblages independent of artificial culture, and provide a greater understanding of the settlement-induction qualities of natural biofilms. DGGE is performed on a vertical polyacrylamide gel, and can be used to characterize microbial communities. Multiple 16S ribosomal gene sequences from bacterial assemblages are amplified simultaneously using target-specific primers and Polymerase Chain Reaction (PCR). A GC-rich region (GC clamp) is added to the 5′ end of the forward primer to render comparable melting behaviors among all PCR products in the test sample. A denaturing chemical gradient then separates amplified rDNA fragments into individual bands based on total GC content. Relative differences between bacterial communities can be compared by loading PCR products from different microbial assemblages into adjacent lanes of the same gel (Muyzer et al., 1993).

One advantage of using the 16S ribosomal gene sequence is that bacterial species may be identified to the group, genus, or sometimes species level. Known bacterial 16S sequences can be loaded into adjacent lanes alongside multi-species PCR products to determine if these known species are present in the community (Muyzer et al., 1993). rDNA bands can also be excised from the gradient gel, purified, sequenced, and compared with published sequence data via the Internet (Jackson et al., 2001; Riemann et al., 2000).

Although DGGE is a powerful technique, it has limitations. Bands may smear together or not separate well due to similarity of sequences, and may be difficult to isolate (Kisand and Wikner, 2003). The number of bands that are separated from a single bacterial assemblage by DGGE can give insight into the numbers of species present (Muyzer et al., 1993), although 16S sequences are known to yield multiple bands from a single species (Dahllof et al., 2000). Another drawback of DGGE is that it does not yield absolute community composition (Muyzer et al., 1993). 16S sequences from species in low abundances may be overwhelmed by more prevalent sequences and a fraction of the total species composition may go undetected. PCR amplification may also bias abundances of species in communities, making quantitative estimates inaccurate (Kisand and Wikner, 2003). Nevertheless, this technique remains a rapid, effective tool for assessing compositions of microbial communities without the limitations of artificial culture media.

The present study focused on three aspects of marine biofilms and how they may effect settlement preferences of *Hydroides elegans*. (1) DGGE was used to

determine dominant species compositions of bacteria on newly submerged surfaces in Pearl Harbor, Hawai'i, over a 2 wk. interval. (2) Temporal changes in bacterial density and community composition of biofilms were examined and correlated with settlement rates of *H. elegans*. (3) 16S rDNA from previously isolated single strains of bacteria known to induce settlement of *H. elegans* were loaded in adjacent lanes alongside biofilm bacterial assemblages on DGGE gels to determine if these species were present in natural biofilms.

MATERIALS & METHODS:
Biofilm Site and Collection

Glass microscope slides were cleaned with 2-propanol, UV sterilized, and attached to plastic frames with plastic cable ties. Slides were positioned vertically along each frame and spaced approximately 2 cm apart from each other. Nylon rope was used to suspend the frames from a pier on Ford Island, Pearl Harbor, Hawai'i, between about one half and two meters below the water surface depending on tide. Preliminary experiments to examine variance in community composition of 1 wk. old biofilms along the pier length using DGGE revealed no differences in band patterns. A total of 7 frames were built, each holding 18 slides. One frame was submerged initially, and an additional frame was submerged every two days after the previous frame until all 7 frames were in the water. Fourteen days after submerging the first frame, all 7 frames were removed from the water at the same time in order to decrease variance in subsequent experiments. Biofilms therefore ranged in age from 2 to 14 days old in intervals of two days.

Hydroides elegans Larval Culture

Hydroides elegans naturally settles onto newly submerged biofilmed surfaces near Ford Island, Pearl Harbor, Hawaiʻi, year round (Carpizo-Ituarte and Hadfield, 1998). Plastic screens were submerged in the water at Ford Island for about 4 wk. to recruit *H. elegans*. Adults were transported to the Kewalo Marine Laboratory of the University of Hawaiʻi at Mānoa, Honolulu, Hawaiʻi, and kept in sea tables for up to 14 days with continuously flowing unfiltered sea water. Calcareous tubes of about 15 adult worms were gently broken to induce spawning in a common dish filled with about 10 ml of 0.22 μm filtered sea water (FSW). Several males were removed before completely spawning to reduce polyspermy of eggs. Zygotes were transferred into 2 L beakers filled with FSW shortly after the 2 cell stage. Larvae were maintained at a density of 5-10 ml^{-1} and a temperature of 22-24°C. *Isochrysis galbana* was provided as food throughout larval culture at a density of 10^{-5} cells ml^{-1}. On days 2 through 4 of culture, larvae were sieved from the water with a 41 μm mesh and transferred into fresh FSW, algae, and clean beakers to decrease exposure to biofilms prematurely. Larvae became competent to settle and metamorphose on day 5 of laboratory culture.

H. elegans Settlement Assays

Six replicate slides of each biofilm age were exposed to about 100 larvae of *H. elegans* in 95-mm diameter sterile petri dishes and 6 ml of FSW. A negative control treatment, which consisted of larvae exposed to clean glass slides, was included. Larvae were exposed to a 10-mM CsCl$_2$ pulse for 2 hours, followed by immersion in FSW and exposed to clean glass slides for 22 hours to serve as a positive control for

metamorphic competence (Carpizo-Ituarte and Hadfield, 1998). The percentage of
larvae in each dish that settled onto the biofilmed surface was determined 24 hours after
initial exposure. Settlement was defined as adherence of the larvae to the substratum,
formation of a primary proteinacious tube, and initial formation of branchial rudiments.
Animals that had recruited to the slides in the field could be distinguished from newly
settled larvae in the experiment by the large size of the animals and length of their
calcareous tubes. Field recruited animals were not included in subsequent analyses.

Bacterial Density Estimates

Bacteria on six replicate slides of each biofilm age were stored at 4°C for up to
1 month in 3.7% formalin in FSW. Bacteria on these slides were stained using the
nuclear stain 4'-6-Diamidino-2-phenylindole (DAPI 1μg ml^{-1}) and counted using an
epifluorescence Zeiss Axiophoto Microscope (Zeiss, Oberkochen, Germany). On each
slide, bacteria were viewed at 1000X magnification under the microscope and counted
in six circular fields measuring 0.245 mm in diameter. Areas were chosen using a grid
on the stage of the microscope and randomly generated numbers from the website
www.randomizer.org. Bacteria were counted in each field, averaged for each slide, and
averaged over all replicate slides for each biofilm age to provide bacterial density
estimates.

DNA Extraction

Six replicate slides for each biofilm age were used for biofilm DNA extraction.
Biofilms from two slides were pooled into a 1.5 ml centrifuge tube by scraping them
from the surface using a sterile razor, creating 3 replicates per age group. The tubes

were stored at 4°C for 1 to 2 hours before DNA extraction. Genomic DNA from biofilms was extracted using an UltraClean Microbial DNA Kit (Mo Bio Laboratories Inc.) according to the manufacturer's instructions.

The bacteria *Pseudoalteromonas luteoviolacea* and *Cytophaga lytica* were previously isolated and employed to induce settlement of *H. elegans* in this laboratory (Huang and Hadfield, 2003). Single colonies from each species were grown to log phase in half-strength seawater-tryptone (SWT) broth (Boettcher and Ruby, 1990), and DNA was extracted using an UltraClean Microbial DNA Kit (Mo Bio Laboratories Inc.) according to the manufacturer's instructions.

PCR Amplification

The Polymerase Chain Reaction (PCR) was used to amplify a ~480-bp fragment of the 16S ribosomal gene. Universal bacterial forward and reverse primers were complementary to positions 341 to 357 with a 40-bp GC clamp (underlined) (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3') (Muyzer et al., 1993), and position 758 to 776 (5'-CTACCAGGGTATCTAATCC-3') (Juck et al., 2000) (*Escherichia coli* numbering). 25 µl reactions were carried out with final concentrations of 1.5 mM $MgCl_2$, 1X reaction buffer ($MgCl_2$-free), 800 µM PCR Nucleotide Mix, 0.025 u/µl Taq DNA Polymerase (Promega, Madison, WI, USA), Molecular Biology Grade Water (Eppendorf, Westbury, NY, USA), and 1-5 µl template in 200 µl thin walled PCR tubes (USA Scientific, Ocala, FL, USA). PCR reactions were carried out with an initial denaturing step at 94°C for 2 minutes, followed by 40 cycles of a 45 second denaturing

step at 94°C, a 45 second annealing step at 55°C, and a 1 minute extension step at 72°C. A final extension step of 6 minutes at 72°C was carried out. The 16S ribosomal gene fragment from the previously isolated bacteria, *Pseudoalteromonas luteoviolacea* and *Cytophaga lytica* (Huang and Hadfield, 2003), were amplified using PCR with the same protocols described above. Each PCR reaction was carried out with a negative control where the volume of template DNA was replaced with an equal volume of sterile water.

Each PCR reaction product was run on a 1% agarose gel to verify fragment length and quantify DNA. A Kodak Gel Logic 100 Imaging System and Kodak 1D Image Analysis Software (Kodak, Rochester, NY, USA) were used to take and analyze digital images of the agarose gels stained with ethidium bromide (0.5 μg mL^{-1}). DNA was quantified by comparing band intensities of PCR products on the gel to a DNA mass curve created using exACTGene DNA ladder (Fisher Scientific, Hampton, NH, USA) that was loaded into an adjacent lane. DNA amplified by PCR was purified of excess primers and reagents, and concentrated into 15μl sterile water using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Denaturing Gradient Gel Electrophoresis (DGGE)

Gradient 8% polyacrylamide gels (Acrylamide-N,N'-methylenebisacrylamide) containing 40-60% denaturant, from top to bottom, were poured in triplicate. 100% denaturant is defined as a 7 M urea and 40% vol/vol formamide solution. Gels were poured using a multi-gel caster (4-place caster), a 150 ml gradient maker, and mini-

peristaltic pump (CBS Scientific, Del Mar, CA, USA). A total of 210 ng of DNA from each of the 3 replicate purified PCR products of each biofilm age were loaded into each of the three gradient acrylamide gels. The three gels were loaded into a Denaturing Gradient Gel Electrophoresis System (CBS Scientific, Del Mar, CA, USA) containing 1X TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) at 60°C and run at 200V for 8 hours. Gels were stained in 1X Cybr Gold solution (Molecular Probes, Eugene, OR, USA) in 1X TAE buffer for 30 minutes. Digital images of the gels were taken using a Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY, USA) under UV illumination. The PCR-DGGE process was repeated in 3 trials with the same genomic DNA for each biofilm age, for a total of 9 gels, to determine reproducibility of banding patterns.

Statistical Analyses

(1) The null hypothesis that percent settlement did not differ between biofilms of different age was tested using a nonparametric Kruskal-Wallis Test, because the assumption of homogeneity of variance for an Analysis of Variance (ANOVA) was not met. (2) The null hypothesis that bacterial densities did not differ in biofilms of different age was tested using an ANOVA. Bacterial densities were natural log transformed to meet the homogeneity of variance assumption of an ANOVA. (3) Tukey's Pairwise Comparison was performed to determine statistical differences between bacterial densities of biofilms of different age. All of the above tests were done using the statistical program SPSS (Student Version 10.0). (4) An ANOVA was used to determine whether settlement of *H. elegans* and bacterial densities are

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correlated. (5) An ANOVA was also used to determine if *H. elegans* settlement is

correlated with natural log transformed bacterial densities in biofilms of different age.

Each correlation was tested for significance using an ANOVA in Microsoft Excel.

Hypotheses were tested to determine if the presence or absence of specific

bands appearing in DGGE gels and percent settlement on biofilms of different age were

correlated. A binary matrix was created to represent the presence or absence of bands

in the DGGE gels. Bands that appeared at a similar distance down the gel in different

lanes, corresponding to biofilms of different age, are thought to have similar sequences.

Therefore bands that ran the same distance down the gel in different lanes were given a

value of 1, while the absence of a band at the same distance down the gel was given a 0.

The Pearson Correlations and Pearson Probabilities matrix of DGGE band patterns was

used to test the hypothesis that the appearance of a single band, or set of bands at a

specific distance down the gel, could significantly correlate with settlement rates of *H.

elegans*.

If a succession of bacterial species did occur in the biofilms, and one of these

species was producing a positive or negative settlement cue for *H. elegans*, then bands

in DGGE gels corresponding to this bacterial species and settlement on these biofilms

should be correlated. Bacterial species creating a cue should appear in biofilms over a

specific period of time when percent settlement of *H. elegans* was high, for a positive

cue, or low, for a negative cue. To test this hypothesis, hypothetical band patterns,

representing bacterial species that produce positive or negative cues, were created to

determine how bands could have been arranged to explain settlement rates. If bands

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that appear in biofilms at the beginning of the 2-14 day study and percent settlement on

biofilms of different age are negatively correlated, these bands may represent species

that inhibit settlement. Likewise, a positive correlation between bands that appear in

the latter part of the study and *H. elegans* settlement on biofilms of different age could

represent species that induce settlement. Therefore, hypothetical bands appearing for 2,

4, 6, 8, or 12 days in a row, in patterns that would indicate either positive or negative

cues for settlement, were correlated with *H. elegans* settlement on biofilms between 2

and 14 days old. The above statistics were performed using the StatPlus add-in (Berk

and Carey, 2004) and Microsoft Excel.

RESULTS:
Settlement on Biofilms of Sequential Age

Mean percent settlement by *H. elegans* increased as biofilm age increased

(Kruskal-Wallis Test, $p < 0.001$) (Fig. 1). The $CsCl_2$ positive control produced high

settlement rates (91%), indicating that the larvae were normally competent. Settlement

in the absence of a biofilm averaged 2%. Mean settlement increased from 16 to 60%

on biofilms between 4 and 6 days old. Settlement rates subsequently increased linearly

from 60 to 100% on biofilms 6 to14 days old.

Bacterial Biofilm Densities

Mean densities of bacteria in the biofilms ranged from 547 to 4446 cells mm^{-2}

(ANOVA, $p < 0.001$) (Fig. 2). Mean bacterial density appeared to increase as the

biofilm age increased from 2 to 10 days and then decreased between 10 and 14 days.

However, statistical analysis (Tukey's Pairwise Comparison) revealed that densities of

bacteria in the biofilms were not significantly different between days 8 and 14, nor

between days 2 and 6. Thus, the only significant increase in biofilm density occurred

between days 6 and 8.

Correlation Between Settlement and Bacterial Densities

Percent settlement of *H. elegans* on biofilms of different age and bacterial

density gave a significant positive correlation (ANOVA, R^2=0.62, p<0.05) (Fig. 3A).

A stronger relationship was found between percent settlement of *H. elegans* on biofilms

of different age and natural log transformed bacterial density (ANOVA, R^2=0.82,

p<0.01) (Fig. 3B).

PCR-DGGE Analysis

Band patterns varied across replicates and across trials on DGGE gels (Fig. 4).

Seven bands appeared consistently in all 3 replicate DGGE gels, in all biofilms of each

age, and in the 3 repeated DGGE trials (Fig. 4 & 5). Other bands appeared in some

replicates (e.g., 8,10, 12, and 14 day-old biofilms in replicate 2, trial 2) (Fig. 4 & 5) and

not in others. The appearance of bands varied even between trials of the same replicate,

where the same genomic DNA was used. When the binary matrix representation of

these bands and settlement of *H. elegans* on biofilms of different age were correlated,

there were no significant relationships (Table 1, Part A, Pearson Correlations).

H. elegans settlement and hypothetical bands appearing between 6 and 14 days,

8 and 14 days, as well as 10 and 14 days produced significant positive correlations

(Table 1, Part B, Pearson Correlations). *H. elegans* settlement and hypothetical bands

appearing between 2 and 4 days, 2 and 6 days, as well as 2 and 8 days produced

significant negative correlations (Table 1, Part C, Pearson Correlations).

When 16S rDNA amplified from the single species *P. luteoviolacea* and *C.*

lytica was loaded into lanes adjacent to the biofilm bacteria, migration rates of the

bands from the single species closely matched some dominant bands within the biofilm

bacterial community (Fig. 4 Trial 3, & Fig. 5).

DISCUSSION:

The community composition of newly formed bacterial biofilms was studied

using DGGE to determine how temporal changes in bacterial community composition

might effect settlement preferences of *H. elegans*. Replicate DNA extractions, repeated

PCR amplifications, and DGGE gels indicated that there was a set of bands that

appeared in all biofilms of all ages in all three replicates and between all 3 trials. There

were other transient bands that appeared in only one or two replicates, and sometimes

changed in intensity or did not appear at all in repeated trials of PCR-DGGE with the

same genomic DNA.

The presence of transient bands did not seem to correlate with larval settlement

rates for three possible reasons. (1) Patchy bacteria with high settlement inducing

properties, as represented by transient bands in the DGGE gels, may cause *H. elegans*

to settle on some slides and not others, increasing variances between replicates.

However, this was not seen in the settlement assay (Fig. 1). (2) Neither any single

band, nor combinations of bands represented by a binary matrix correlated significantly

with settlement. (3) Settlement of *H. elegans* and the appearance of specific

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hypothetical bands in biofilms of different ages correlated significantly only when these bands occurred early or late in the study period (Table 1, Parts B & C). These hypothetical band patterns were not seen in any of the DGGE gels.

Although the bands that appeared consistently in all DGGE gels cannot be shown to be the primary inducers of settlement in *H. elegans*, the relative stability of the bacterial community and strong correlation between bacterial density and settlement is evidence that the increase in percent settlement of *H. elegans* as biofilms age is due to increasing bacterial density of species that are present throughout the study period. This relationship has also been shown in previous experiments where increases in densities of rod shaped bacteria in natural biofilms correlated with settlement of *H. elegans* (Hadfield et al., 1994). Settlement was also shown to be directly related to densities of bacteria in monospecific biofilms (Huang and Hadfield, 2003).

The correlation between settlement and bacterial densities in biofilms supports the general theory (Crisp, 1974; Hadfield and Paul, 2001; Steinberg et al., 2002) that biofilms allow *H. elegans* to fill their ecological niche in marine biofouling communities. *H. elegans* must take advantage of an initial 0-6 wk. period before it is smothered by other invertebrate species colonizing a surface (Holm et al., 2000). It must use bacterial biofilms to find a suitable, non-toxic substrate, where food is readily available, and which will remain submerged until the animal reaches reproductive maturity (Holm et al., 2000; Huang and Hadfield, 2003). To find a substrate with these specifications, *H. elegans* must be able to receive a cue from one or multiple species of bacteria in biofilms on newly submerged surfaces. *H. elegans* was shown to settle in

response to monospecific biofilms of several species of bacteria from disparate groups (Huang and Hadfield, 2003; Lau and Qian, 2002; Unabia and Hadfield, 1999). It follows that specific bacterial species may be present in biofilms residing in suitable, non-toxic, food rich waters that produce cues for settlement. A specific density of these bacteria may indicate that the surface is likely to be submerged long enough for *H. elegans* to mature and reproduce.

The correlation between bacterial densities and *H. elegans* settlement indicate that bacterial cells or their specific extracellular polymeric substances provide the cue for settlement. While settlement rates on the negative control, 2-, and 4-day old biofilms were low, there was a 43% increase in settlement between those biofilms and biofilms that were 6 days and older (Fig. 1). A possible explanation for this sudden increase in settlement is the phenotypic change of bacteria from planktonic to biofilm states (Costerton, 1995). Gene expression has been shown to be drastically different between these two states, invoking a suite of separate genes that are expressed based on non-attached or attached conditions (Costerton, 1995; Costerton and Stewart, 2001). It has also been shown that *H. elegans* does not settle in response to broth culture extracts or diffusible substances through 12 to 14 kDa dialysis tubing from *P. luteoviolacea* (Huang and Hadfield, 2003). It is probable that bacterial genes encoding cues for *H. elegans* settlement are expressed only in the biofilm state, and furthermore, only when bacteria have reached a threshold level. Quorum sensing allows bacteria to coordinate expression of genes based on the density of signal molecules, and therefore density of similar bacteria around them (Costerton, 1995; Parsek and Greenberg, 2000). Specific

products resulting from biofilm formation may appear in sufficient quantity once the

bacterial threshold density has been reached, in order to cause this spike in settlement

between 4 and 6 day old biofilms.

In contrast to biofilm cells or substances, bacterial species composition may still

play a role in settlement of *H. elegans*. It has been shown that the bacterium *P.*

luteoviolacea induces higher rates of settlement than *C. lytica* in monospecific biofilms,

even when in dramatically lower densities (Huang and Hadfield, 2003). Unculturable

biofilm bacteria in low densities, but with high settlement inductive qualities, may have

a significant impact on *H. elegans* settlement, and not appear on DGGE gels. An

inherent limitation of the DGGE technique is that only relative species compositions

may be determined. Bacterial species in low densities may not amplify in the PCR

process or may be overwhelmed by DNA from more prevalent bacterial species

(Kisand and Wikner, 2003; Muyzer et al., 1993). The 16S rDNA fragment amplified

by PCR may also be too short to determine species level differences between sequences

(Kisand and Wikner, 2003). A study on settlement induction of larvae of a

Scleractinian coral showed that relative densities of *Alphaproteobacteria,*

Betaproteobacteria, and *Gammaproteobacteria* between bacterial biofilms of 2, 4, and

8 wk. old did not differ (Webster et al., 2004). However, percent metamorphosis was

significantly different between biofilms of 4 and 8 weeks old, suggesting that specific

bacterial species in older biofilms produce the settlement cue (Webster et al., 2004).

Modification to the PCR-DGGE technique could provide further insight into

changes in bacterial communities. It has been shown that a higher resolution of

bacterial communities may be observed using group-specific primers, nested PCR, and

DGGE (Boon et al., 2002). Less prevalent, but relatively influential bacteria elucidated

by this modification to the DGGE technique may show more detailed changes in the

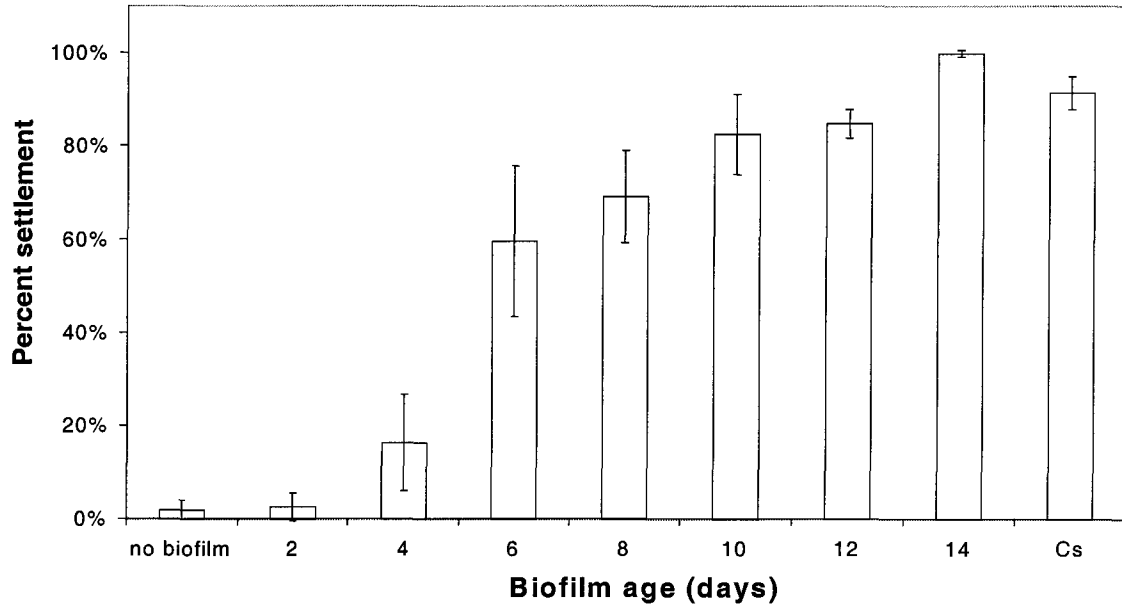biofilm community over time, and how they effect settlement of macrofouling

invertebrates.

Figure 1. Percent settlement of larvae exposed to biofilms 2 to14 days old. Bars
indicate mean percent settlement of six replicates +/-1 SD (Kruskal-Wallis
Test, p<0.001). A 10 mM Cs positive control was used to determine if larvae
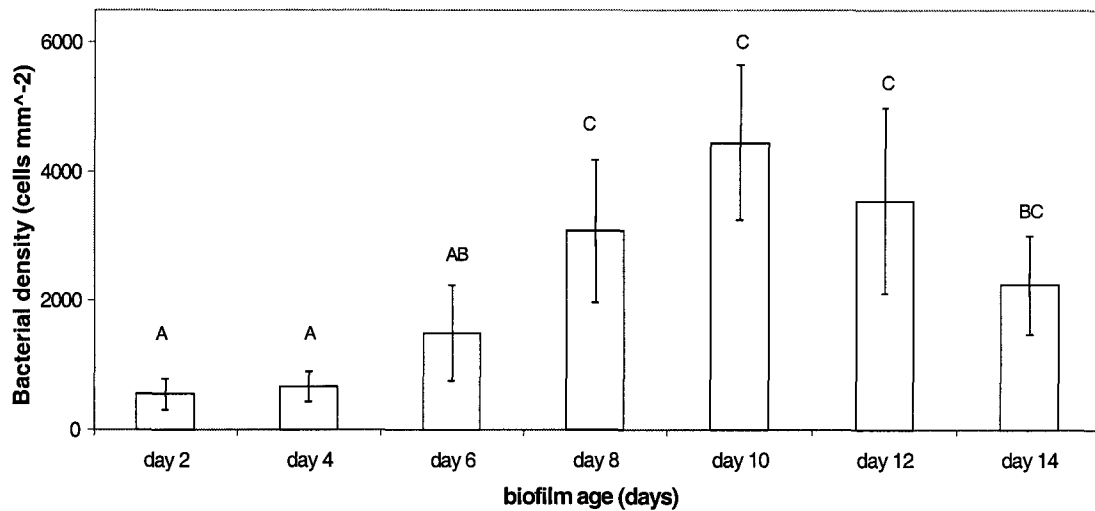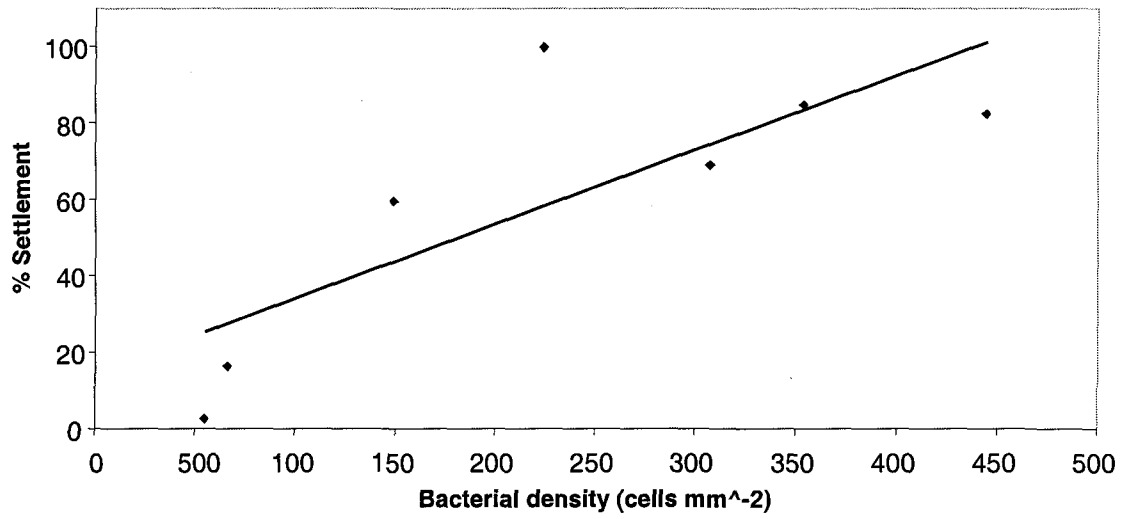of *H. elegans* were competent.

Figure 2. Bacterial cell density in 2 to 14 day old biofilms. Bars indicate mean cell
density per square millimeter of six replicates +/-1 SD. Letters above bars
indicate significant differences between biofilms of different age using a One-
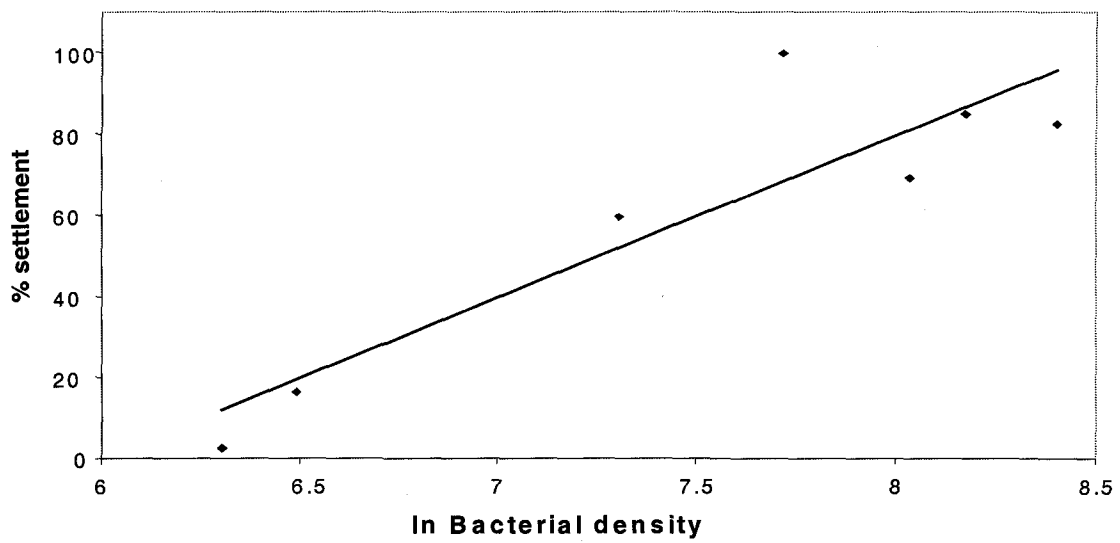Way ANOVA (p<0.001) and Tukey's Pairwise Comparison.

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A.

B.

Figure 3. A. Percent larval settlement versus bacterial density (R-square = 0.62, p<0.05). B. Percent larval settlement versus natural log transformed bacterial density (R-square = 0.82, p<0.01).

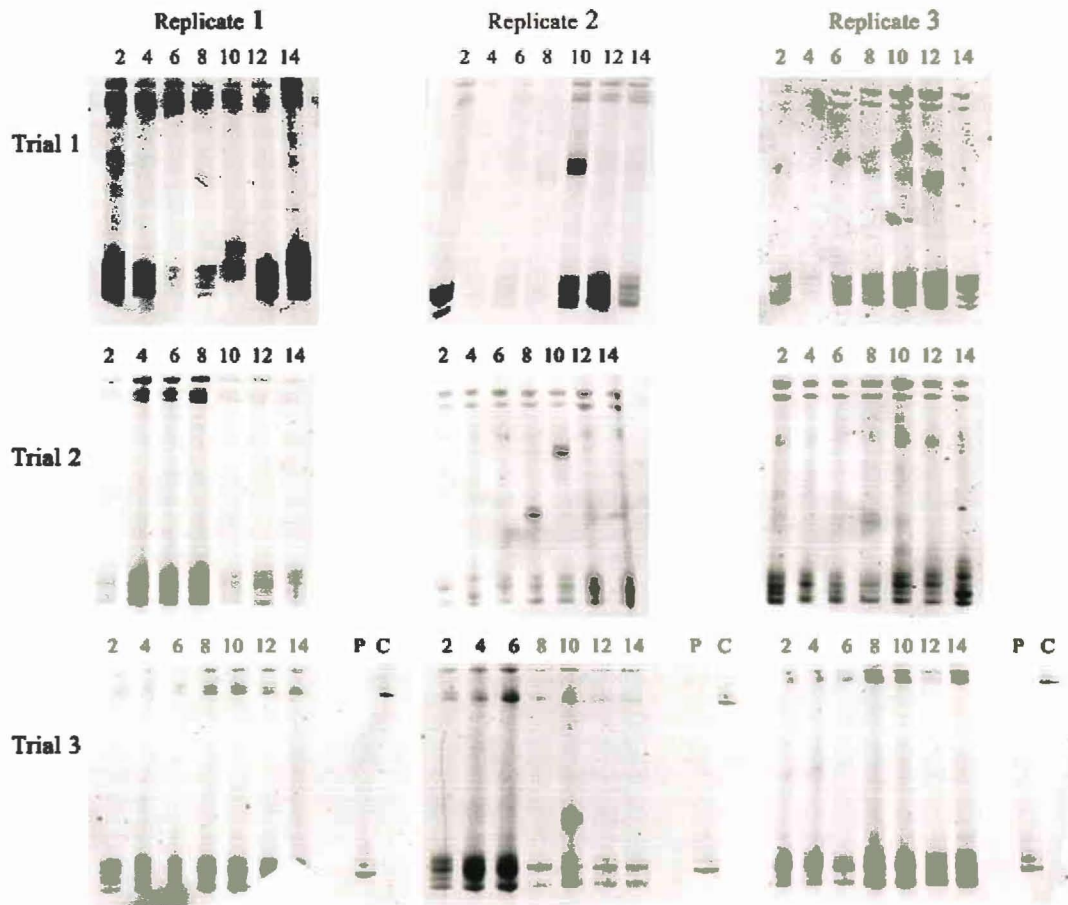Figure 4. DGGE profiles of PCR amplified 16S rDNA fragments from replicate bacterial biofilms from Pearl Harbor, Hawaiʻi and trials of PCR-DGGE of the same DNA. Numbers above lanes indicate age of biofilms in days at time of DNA extraction. rDNA from the bacterial species *Pseudoalteromonas luteoviolacea* (P) and *Cytophaga lytica* (C) were extracted, amplified, and loaded alongside biofilm DNA.
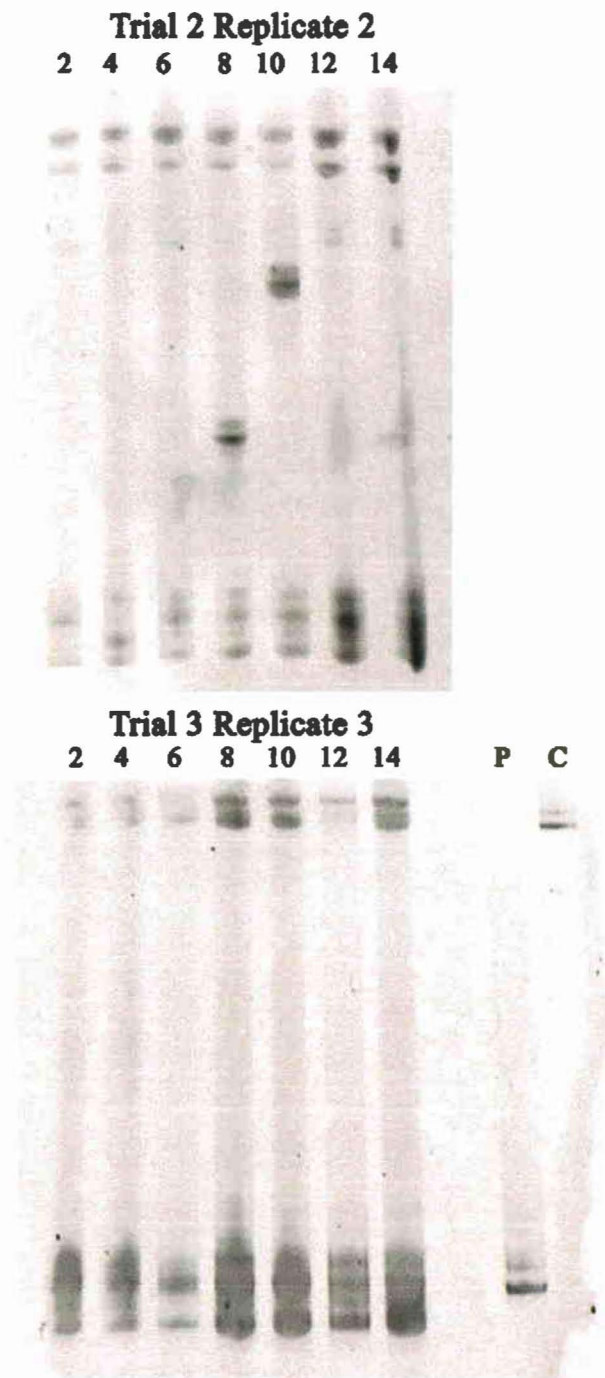
Figure 5. DGGE profiles of PCR amplified 16S rDNA fragments from replicate
bacterial biofilms from Pearl Harbor, Hawai'i. Numbers above lanes indicate
age of biofilms in days at time of DNA extraction. rDNA from the bacterial
species *Pseudoalteromonas luteoviolacea* (P) and *Cytophaga lytica* (C) were
extracted, amplified, and loaded alongside biofilm DNA.

Table 1. Binary representation of transient band patterns in DGGE gels. Zero or one indicate the absence or presence of a band respectively. Pearson correlatons and probabilities of mean percent settlement vs. band patterns are indicated by row. A. Band patterns observed in experimental DGGE gels. B. Hypothetical band patterns representing positive cues that could account for settlement rates by *H. elegans*. C. Hypothetical band patterns representing negative cues that could account for settlement rates by *H. elegans*.

	Biofilm age	2	4	6	8	10	12	14	Pearson correlations	Pearson probabilities
	Mean % settlement	3	16	60	69	82	85	100		
A.	Band patterns in	0	0	0	1	0	0	0	0.12	0.80
	experimental	0	0	0	0	1	0	0	0.28	0.54
	DGGE gels	0	0	0	0	0	0	1	0.49	0.26
		0	0	0	0	0	1	1	0.62	0.14
B.	Hypothetical	0	0	0	0	1	1	1	0.76	0.05*
	positive cues	0	0	0	1	1	1	1	0.85	0.02*
		0	0	1	1	1	1	1	0.93	0.00*
		0	1	1	1	1	1	1	0.68	0.09
C.	Hypothetical	1	0	0	0	0	0	0	-0.68	0.09
	negative cues	1	1	0	0	0	0	0	-0.93	0.00*
		1	1	1	0	0	0	0	-0.85	0.02*
		1	1	1	1	0	0	0	-0.76	0.05*
		1	1	1	1	1	0	0	-0.62	0.14
		1	1	1	1	1	1	0	-0.49	0.26

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