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Effects of Biofilm Age and Composition on Oyster Larval Setting

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EFFECTS OF BIOFILM AGE AND COMPOSITION ON OYSTER LARVAL SETTING

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

EFFECTS OF BIOFILM AGE AND COMPOSITION ON OYSTER LARVAL SETTING

By Alaina A. Hart, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Director: Bonnie L. Brown, Ph.D. Professor, Department of Biology

The lack of success in restoring oyster, *Crassostrea virginica*, populations to Chesapeake Bay and its tributaries has raised many questions about why many restoration efforts have failed. A number of studies have focused on the larval stage of oysters and considered the variables that impact oyster setting behavior in an effort to understand why oyster populations have not recovered. Studies that have examined setting surfaces suggest that biofilms promote oyster larval settlement; however, similar studies with barnacle larvae have found an inhibitory relationship. The present study utilized field-produced biofilms of different ages to determine if natural biofilms inhibit or promote setting of larval oysters. Several aspects of the biofilms where analyzed including biomass, chlorophyll a

concentration, percent organic matter, bacterial cell counts, and bacterial community composition. Larval setting was found to increase as biomass and age of biofilm increased. No effects of chlorophyll a concentration, percent organic matter, bacterial cell counts, or bacterial community composition were detected. The predator *Stylochus elipticus* was observed to have a profound effect on newly set larvae.

A new method for enumerating bacterial cells was explored to promote high throughput analysis of biofilm specimens. This method involves applying bacterial suspensions to bio-adhesive slides with subsequent staining and was compared to the standard method of enumeration on filters. The bio-adhesive slide procedure allowed processing of ten times more specimens per slide, resulted in lower background fuorescence, and higher bacterial counts than the standard filter method. The method promoted high throughput while yielding more accurate counts than filters when compared to dilution curves and was found to be useful for direct enumeration of bacteria in laboratory cultures, wastewater, sediments, and biofilms.

Effects of biofilm age and composition on oyster larval setting

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ABSTRACT

The lack of success in restoring oyster, *Crassostrea virginica*, populations to Chesapeake Bay and its tributaries has raised many questions about why many restoration efforts have failed. A number of studies have focused on the larval stage of oysters and considered the variables that impact oyster setting behavior in an effort to understand why oyster populations have not recovered. Studies that have examined setting surfaces suggest that biofilms promote oyster larval settlement; however, similar studies with barnacle larvae have found an inhibitory relationship. The present study utilized field-produced biofilms of different ages to determine if natural biofilms inhibit or promote setting of larval oysters. Several aspects of the biofilms where analyzed including biomass, chlorophyll a concentration, percent organic matter, bacterial cell counts, and bacterial community composition. Larval setting was found to increase as biomass and age of biofilm increased. No effects of chlorophyll a concentration, percent organic matter, bacterial cell counts, or bacterial community composition were detected. The predator *Stylochus elipticus* was observed to have a profound effect on newly set larvae.

INTRODUCTION

Oyster populations are an important ecological and economic resource to the coastal United States. Both aspects have suffered over the past century as natural oyster populations have been impacted by human activity. Since the mid-1950s, published accounts of the consequences of anthropogenic effects on oyster populations in the Chesapeake and Delaware Bays have been common. In Chesapeake Bay, oyster landings peaked in 1884 and then began to drastically decrease as overfishing reduced the number of oysters and fishing practices destroyed reefs (Rothschild et al. 1994). Unstable oyster populations were further affected in the mid-1950s as two diseases, MSX and Dermo, began to appear in oysters growing in the coastal mid-Atlantic waters of the U.S (Ewart et al. 1993). The introduction of these diseases into the Chesapeake Bay population caused already low landings to become commercially non-existent (Rothschild et al. 1994).

More recently, a number of fishing bans and other severe regulatory actions have failed to remedy the decline of naturally occurring oysters. For Chesapeake Bay in particular, steps to rehabilitate the oyster population have been, by and large, unsuccessful (Hargis et al. 1999). Scientists, harvesters, and aquaculturists involved with the oyster industry have attempted to recreate reefs artificially; attempts that have largely failed except in a few isolated instances (Nestlerode et al. 2007). It is unclear why artificial oyster reefs have failed to thrive. Possibilities include high siltation (Hargis et al. 1999), low dissolved oxygen (Lenihan et al. 1998, Baker et al. 1992), and reduced numbers of viable larvae due to environmental changes affected by climate as well as

reduced numbers of adult oysters (Kimmel et al. 2007). However, anecdotal evidence exists that larvae are in fact present in locations of Chesapeake Bay (Southworth et al. 2008), including Little Wicomico River where this study will be performed, but that for some unknown reason, the larvae do not set in locations or on surfaces where they would otherwise be expected to set.

In oysters and other mollusks, metamorphosis of larvae is preceded by behaviors known as settlement and setting. Settlement occurs when larvae reach the pediveliger stage at approximately two-weeks-old and involves searching for and temporary attachment to hard substrates enhanced by chemical cues (Tamburri et al. 1992). Once an appropriate surface is found, the larvae form a strong attachment with the use of their foot. Oyster larvae are commonly referred to as "spat" once they have permanently set on a surface. Research has clearly demonstrated that adult oysters and recently attached spat release chemical cues to induce further settlement (Tamburri et al. 1992, Veitch et al. 1971). As a result of these chemical cues, larvae that are ready to set undergo gregarious settlement (Veitch et al. 1971). Research has also shown that biofilms associated with oyster shells and other substrates produce similar chemical cues, with specific bacterial species and substances identified as important to enhancing settlement (Zhao et al. 2003, Tamburri et al. 1992, Fitt et al. 1989, Weiner et al. 1985). Surfaces covered in biofilm have been found to be the preferred setting substrate for oysters over clean substrates (Tamburri et al. 2008).

The fact that oyster larvae are found in the water column of Little Wicomico River and other tributaries to Chesapeake Bay (Southworth et al. 2008) but do not set raises the question, "what is happening to the larvae?" One possibility is that larvae do

settle and metamorphose but don't survive. Another alternative is that viable larvae can no longer find suitable setting substrates in this tributary. If it is the substrate affecting settlement, perhaps it is specific characteristics of the biofilm covering these substrates that is inhibiting settlement. To date, few studies have examined biofilms for potentially inhibitory effects on oyster settlement. The mechanism by which biofilms may inhibit setting is unknown, but these studies suggest that bioconcentration of heavy metals and other contaminants by biofilms may affect this stage of an oyster's life cycle (Labare et al. 1997, Chang et al. 1996).

As eutrophication of coastal waters continues, the importance of the microbial consortium and its role in changing the environment of the Bay is being recognized. Recent research on barnacles has revealed that increasing both the age of the biofilm and bacterial cell densities inhibits barnacles from setting (Lau et al. 2003, Olivier et al. 2000). Specific bacterial species within biofilms also have been shown to produce compounds that inhibit or kill barnacle larvae, with aged biofilms having a more toxic effect (Holmstrom et al. 1992). If biofilms can inhibit crustacean metamorphosis, then it is reasonable to assume that biofilms may be a factor in the lack of mollusk settlement in Chesapeake Bay. This study was designed to investigate the question of whether the biofilm that naturally forms on hard surfaces in a typical tributary of Chesapeake Bay (Little Wicomico River) is preventing oyster larvae from setting at the time of metamorphosis. Various aspects of the biofilms were examined, in particular age, biomass, and microbial composition, to determine their impact on oyster larval setting.

MATERIALS AND METHODS

Study site. Biofilms were retrieved from five sites in Little Wicomico River, Virginia, hydrologic unit number 02070011, (Site A- 37N 53.83', 76W 17.92'; Site B- 37N 53.57', 76 W 15.99'; Site C- 37N 54.19', 76W 17.59'; Site D- 37N 54.60', 76W 18.92'; Site E-37N 53.24', 76W. 14.89'; Figure 1). Little Wicomico River is a small shallow (2 m MLW) tributary of Chesapeake Bay located below the mouth of Potomac River. Historically, oyster grounds were located in this moderate salinity (10-15 ppt) tributary as were processing facilities. However presently, the potential for restoring oysters to this tributary is considered at best modest because of low occasional natural spat settlement and consistent risk of MSX and Dermo diseases (VIMS 2009).

Biofilm collectors. Biofilm collectors consisted of two separate suspension mechanisms for ceramic tiles with a floating buoy. The bottom collector (sediment depth ranging from 2-3m) consisted of a $0.5m^3$ cubical enclosure constructed of 3cm-opening stainless steel mesh inside which ceramic tiles were suspended from nylon rope. The surface collector, suspended approximately 1m below the surface, consisted of a 1m long PVC pipe suspended horizontally with nylon rope from which ceramic tiles were attached as in the bottom collectors. Surface collectors were attached to the same line as each bottom collector and buoyed with a foam float. Each 116 cm^2 ceramic tile was attached to 3mm diameter braided polypropylene cord using stainless steel clips and identified by a code consisting of a series of holes drilled along the perimeter of the tile.

Spatial and temporal sampling. Biofilms were allowed to develop naturally to different ages in several experimental trials conducted between 2008 and 2009. Both surface and bottom biofilm collectors were deployed at sites A, B, D and E. At site C,

only a surface collector was deployed because water depth was insufficient (approximately 1 m MLW) to accommodate both surface and bottom collectors. In Trial 1, collectors were deployed at all five sites. Collectors for Trials 2 and 3 were deployed at sites A, C, D and E. Trial 4 collectors were deployed at sites A and E, with four surface collectors and four bottom collectors per site. Tiles were added to collectors one at a time, every seven days, with a total of four tiles per collector. Collectors were deployed for 4-week intervals so that at the end of each trial, the youngest biofilms were one week old and the oldest were 4 weeks old. Trial 1 collectors were deployed from April to May of 2008, Trial 2 from June to July of 2008, Trial 3 from August to September of 2008, and Trial 4 from April to May of 2009.

Oyster larval setting experiment. At the end of each biofilm collection period, all tiles were removed from the collectors, placed into individual ziplock bags, and transported at ambient temperature (approximately 18^{o}C) to the University of Maryland's Horn Point Laboratory in Cambridge, Maryland. There, the tiles were randomly placed into a 2.5 x 1 x 0.5m deep setting tank rigged to allow tiles to be suspended midwater across the tank. The larval setting experiment was conducted the same day the tiles were collected from the natural environment for Trials 2, 3 and 4. Tiles from Trial 1 were held at 4°C for seven days before being transported to Horn Point Laboratory. Clean, non-biofouled surfaces were not utilized in these trials because the purpose of these trials was to evaluate the effect of biofilm age only, not to determine the effect of biofilm presence/absence on oyster larval setting.

Live *Crassostrea virginica* Gmelin (the eastern oyster) pedivelliger larvae were placed in the setting tank containing each set of experimental tiles for each trial. The

water temperature in the tank was adjusted in spring trials to be as similar as possible to the environment where the tiles were collected but high enough to be conducive to the setting experiment. The tank with tiles suspended was aerated prior to introduction of the larvae to establish adequate circulation. Oyster larvae were examined by microscopy to verify that they displayed pre-settlement characteristics, including the presence of an eyespot and movement of the foot. Approximately 1 million larvae ready for settlement were acclimated to the tank conditions by being placed in a bucket of the tank water for five minutes after which they were transferred to the setting tank in several small volume additions. Cultured single-cell algae (*Chaetoceros* sp.) were added to the tank as a food source for the oyster larvae.

For Trials 1 and 2, larvae were given approximately two days to set, determined by observing several tiles for adequate numbers of spat (approximately 100 larvae per tile observed). After that time, the tank was flushed and the tiles were left for approximately five weeks with ambient water (containing wild phytoplankton) constantly running. This period allowed oyster larvae on the tiles to grow to a diameter of approximately 0.5 cm, which facilitated counting. Only larvae that set on the unglazed side of each tile were counted, not including the outer 1 cm to avoid any edge effects possibly due to handling or the coding of the tiles. For Trials 3 and 4, tiles were removed after adequate set was determined at approximately 48 hrs after introduction of larvae to the tank. Upon removal, each tile was placed into individual ziplock bags and transported on ice to the Ecological Genetics Lab at Virginia Commonwealth University. Tiles were then dried for at least 24 hr in a gravity convection incubator at 60^{o}C after which larvae on the unglazed side of each tile were counted using a dissecting microscope.

Biofilm sampling. During the period of acclimation in the setting tank and prior to the setting experiment, each tile was serially removed and the biofilm from the glazed side of the tile was sampled to facilitate evaluation of how the biofilm changed with age and placement in the water column. Three separate samples were collected from the glazed side of each tile and archived at -20^{o}C for later analysis. For biomass and ash free dry weight, biofilm covering 25% of the tile area was scraped using a scalpel. For both bacterial community composition tests and chlorophyll a analysis another 25% was collected. Approximately 10-50cm^{2} of the remaining area was scraped and archived for enumeration of the bacterial cells, depending on the level of biofouling. Sampling for Trial 1 took place prior to transporting the tiles to Horn Point Laboratory.

Biomass and organic matter determination. Biofilms were dried to completion in a gravity convection incubator at 60^{o}C to estimate total dry weight of biomass per tile. Organic matter was determined by placing up to 0.4 g of dried biofilm in pre-weighed metal boats and holding at 450^{o}C for 6 hrs in an Isotemp® Programmable Muffle Furnace (Fisher Scientific) to allow combustion of the organic material. A final sample weight was recorded and subtracted from the dry weight to determine the weight of the organic content and percent organic content.

TRFLP to examine community composition. Bacterial community composition was estimated using "community fingerprinting" accomplished by terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997). DNA was extracted using a PowerSoil^{TM} DNA Isolation Kit (Mo Bio Laboratories, Inc.) per the manufacturer's instructions. DNA concentrations were determined using a Nanadrop 8000 Spectrophotometer (Thermo Scientific). A portion of the 16S rRNA gene was then

amplified via PCR using bacteria-specific primers 27F and 1492R (Lane 2001). Total volume of each PCR reaction was 50 μl containing 1 μl DNA template at the appropriate dilution, 5 U AmpliTaq DNA Polymerase (Applied Biosystems), 1.5 mM $MgCl_2$ solution (Applied Biosystems), 0.1 volume of GeneAmp 10X PCR Buffer II (Applied Biosystems), 20 μg BSA, 1mM each dNTP (Applied Biosystems), 0.3 μM primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 0.3 μM primer 27F (5'-AGAGTTTGATCMTGGCTAG-3'). The 27F primer was fluorescently labeled at the 5' end with FAM. Most templates were run at the original elution concentration; some required 10^{-1} dilution. Thermal cycling for PCRs was performed in a BioRad iCycler for 95 ^{o}C for 5 min, 35 cycles of 94 ^{o}C for 1 min, 49 ^{o}C for 1 min, 72 ^{o}C for 2 min, and 72 ^{o}C for 8 min. PCR products were purified using a MinElute 96 UF™ PCR purification kit (Qiagen) then split into equal 5-7 μl aliquots for restriction digestion with *Msp*I and *Hha*I. Amplicons were digested with 20 U *Msp*I and *Hha*I at 37 ^{o}C for 6 hr followed by 65 ^{o}C for 20 min. Digested amplicons were purified using the MinElute 96 UF PCR purification kit (Qiagen) and resolved using capillary electrophoresis in a MegaBACE 1000 fluorescent genotyper. Map Marker 400 Rox ladder (Bioventures) was included with each sample, injection was at 3000V for 100 seconds, and run time was 100 min at 10,000V. Following electrophoresis, T-RFLP peaks were viewed and scored as present/absent using Fragment Profiler (Ver 1.2).

Chlorophyll-a determination. Subsamples of approximately 0.1-0.25 g from each biofilm were extracted in 10 volumes of 90% acetone buffered with $MgCO_3$. Samples were held for 24 hr at -20^{o}C then diluted (factors determined empirically using representative samples). Chlorophyll-a concentration was analyzed using a TD-700

Fluorometer (Turner Designs) and final results were recorded as μg chlorophyll-a per g of biofilm.

Bacterial Enumeration. An accurately weighed portion (c.a. 0.05g optimized to ensure that roughly 100 cells were visible per microscopic field of view at a magnification of 1000X) of each biofilm sample was added to 1 ml of sterile filtered PBS. Samples were vortexed with 1mm diameter acid washed glass beads for ten minutes (2500 rpm) to liberate the cells from the organic and inorganic substrate particles. The vortexed solutions were gravity filtered through Miracloth™ to remove large sediment particles, macroflora, and macrofauna. From the resulting suspension, 20 μl was applied to individual wells on 24 well (5mm) Diagnostic Printed Slides (ES-230B, Thermo Scientific). Slides were dried at 37°C after which samples were stained with 20 μl of a 0.003X solution of BacLight™ bacterial stain (Invitrogen) for 15 min. This stain was utilized because it provided greater resolution of the total number of bacteria (regardless of whether they were live or dead) in biofilms than was obtained using other stains such as DAPI and Acridine Orange. After rinsing twice with filtered deionized water, an antifade solution was applied to each well, a cover slip was applied to the slide, and the material was viewed under oil immersion using epifluorescence microscopy. Live bacteria (green fluorescent) and dead bacteria (red fluorescent) cells were viewed separately with fluorescein and Texas Red bandpass filter sets on an Olympus BX-41 and enumerated by counting from an accurately ruled grid subdivided into 100 squares of equal area. Five fields were counted per well and the results averaged for each biofilm specimen. The total number of cells per g of biofilm was determined.

Statistical Analyses. Each trial was statistically analyzed separately. Oyster counts and bacterial cell counts were transformed by ln(Y+1) to preserve constant variance. Data for biofilm characteristics (biomass, chlorophyll a, % organic matter) were not transformed. To determine if there were differences among biofilms of varying ages and from different placements in the water column of Little Wicomico River, significant differences were assessed for biomass, chlorophyll a, % organic matter, and number of bacterial cells using two-way ANOVA with interaction. In the case where multiple interactions were indicated, the Tukey-Kramer multiple comparison procedure was evaluated at the 0.05 significance level (SPSS). To determine if oyster larvae set preferentially on different biofilms from Little Wicomico River, a two way ANOVA with interaction was performed on placement (top/bottom), site, and age of tile. To determine whether a relationship exists among biofilm characteristics and observed differences in oyster setting, regressions of transformed oyster count and biofilm characteristics were performed.

For T-RFLP analysis, data of T-RFLP peaks were coded as presence (1) or absence (0) in a binary matrix. Each T-RFLP peak was assumed to represent the genetic signal of at least one unique operational taxonomic unit (OTU), with the understanding that there can be multiple OTUs/organisms that generate the same fluorescent peak (Liu et al. 1997). To assess the overall community genetic similarity among biofilm samples, Jaccard similarity coefficients was calculated (Legendre and Legendre, 1998). Non-metric multidimensional scaling (NMDS) was performed using the matrix of Jaccard similarity coefficients as the data and producing two-dimensional plots of how the samples were related (Legendre and Legendre, 1998). NMDS analysis was accomplished

using the computer program PAST (obtained from http://folk.uio.no/ohammer/past/index.html; Hammer et al. 2001). The significance of differences detected among groups was tested as outlined by Clarke (1993) using ANOSIM (ANalysis Of SIMilarities), a non-parametric test customarily applied to ecological taxa-in-samples data.

RESULTS

Biomass, Time, and Placement. The interaction of time deployed (number of weeks) and placement was deemed statistically insignificant ($0.708<P<0.965$), as was the main effect of placement ($0.516<P<0.734$). Hence, placement of the tile in the water column of the river surveyed had essentially no effect on the amount of biofilm biomass. The amount of time a tile was deployed was statistically significant ($0.001<P<0.0306$) indicating that the age of the biofilm is a significant contributor to biomass. Multiple comparisons found that tiles placed for four weeks had significantly higher biomass than those placed for one to three weeks for Trials 3 and 4. Trial 1 had no significant difference between the tiles in the amount of biomass build up.

Biofilm Characteristics, Time, and Placement. Vertical placement in the water column was determined to be a significant contributor ($0.001< P <0.009$) to chlorophyll a concentrations with top tiles having a higher mean ($202.3< \bar{x} <219.5$) compared to bottom tiles ($54.1< \bar{x} <62.3$). The interaction of time and placement was also deemed significant ($P =0.029$) for Trial 4 with two week and three week old top tiles having

higher means compared to other times and placements. Time and placement and time alone were not statistically significant ($0.428 < P < 0.836$) for Trials 1 and 3.

Time and placement and the interaction of time and placement were statistically insignificant ($0.098 < P < 0.976$) to predicting percent organic (OM) and bacterial cell counts in most cases. There was a marginally significant relationship between bacterial cell counts and time ($P = 0.060$) and a significant relationship ($P = 0.042$) for cell counts and placement for Trial 3. Cell counts for bottom tiles were higher than top tiles ($\bar{x}$ = 22.00 and 20.76, respectively).

Oyster Set *versus* Time, Placement, and Site. Time, placement and site were significant predictors ($0.001 < P < 0.0279$) of oyster set in most trials (Table 1). Oyster counts increased as the age of biofilms increased in all trials with the exception of Trial 1 (Figure 2). Oyster counts were also higher on bottom tiles ($4.0993 < \bar{x} < 4.5706$) than top tiles ($2.6514 < \bar{x} < 4.1882$) for Trials 1 and 3. Multiple comparisons showed that there were significant differences between sites and oyster count for all trials except Trial 1. For Trial 3, site A had a higher amount of oyster set and Site C had a lower amount of oyster set compared to all other sites. For Trial 4, site E had a higher amount of oyster set compared to site A.

Oyster Set and Biofilm Characteristics. We found significant relationships ($0.001 < P < 0.0279$) for oyster set *versus* biomass dry weight for Trial 3 and 4, chlorophyll a for Trial 1 only, and % OM for Trial 4 only (Table 1). Trial 3 illustrated that for every one-unit increase in biomass dry weight we expect a 1.6 unit increase in the log count of oyster set. A non-linear regression model was fit to oyster set *versus* biomass resulting in a significant positive relationship ($P = 0.078$, $R^2 = 0.29$) (Figure 3).

For Trial 4, for every one-unit increase in biomass dry weight we expect a 0.965 unit increase in oyster set. A significant positive relationship (P = 0.078, R^2=0.572) was found for oyster set *versus* biomass when fit with the same non-linear regression model (Figure 3). A significant negative relationship was found for chlorophyll a *versus* oyster set only for Trial 1 (R^2 = 0.191) where a one-unit increase in chlorophyll a predicts a 0.005 unit decrease in oyster set. A negative relationship was also found between % OM and oyster set in Trial 4 (R^2 = 0.537, Figure 4) where site A had significantly higher %OM than site E ($\bar{x}$ =0.24 and 0.14, respectively; P<0.001), and a one-unit increase in % OM predicted a 15.93 unit decrease in oyster set. We found no relationship (0.078< P <0.953) for oyster set *versus* biomass dry weight for Trial 1, chlorophyll a for Trial 3 and 4, % OM for Trial 3, and bacterial cell counts for all trials (Table 1).

TRFLP. NMDS revealed for Trials 1 and 3 that sites closer to the mouth of the river (Sites E and B) have a bacterial community profile that is different from sites further upstream (Sites A, C and D, Figure 5). No significant differences were observed between top and bottom tile communities (sites and times pooled; 0.23<P<0.78) or among biofilms of different ages (sites and depths pooled; 0.08< P <1.00). At one site (site D in Trial 3), the NMDS analysis indicated a different bacterial community profile for one-week and two-week old biofilms compared to three-week and four-week old biofilms (Figure 5).

Stylochus. Larval set for trial 2 was observed several days after initiating the growth period by which time almost 100% larval mortality occurred. This high mortality rate was a result of predation by the flatworm *Stylochus ellipticus*, determined by viewing the presence of many flatworms and predation of spat. At the time, it was unknown if the

source of *S. ellipticus* was just from the running of ambient water or if the flatworm was present in the biofilms. It was later determined during biofilm sampling for Trial 3, that *S. ellipticus* was present in the biofilms upon removal from the Little Wicomico River. Predation was reduced as a significant factor in Trials 3 by removing the tiles immediately post setting (within 48 hours of adding larvae to the setting tank) and by transporting the tiles from Horn Point Lab to VCU on ice.

DISCUSSION

The expectation that oysters would preferentially set on less-biofouled tiles was grounded in prior research where larval oysters have been found to prefer to set on clean hard substrates compared to soft substrates such as sediments (Tamburri et al. 2008). We found, however, that oyster larvae set best on tiles covered by a greater amount of biofilm, sediment, and other components, i.e., tiles that were biofouled. Tiles from the Little Wicomico River had a number of colonizing organisms including bacteria and barnacles that appear to have influenced the settlement behavior of larvae. Set increased with increasing age and biomass of biofilms, suggesting that larvae prefer to set on established communities of micro- and macrofauna. Although the colonization of experimental tiles by barnacles was not a part of this experiment by design, observations were made about the setting behavior of oyster larvae in response to the presence of barnacles. High numbers of oyster larvae were noted to set both upon barnacles and in free spaces between adjacent barnacles, even on tiles where little or no exposed tile surface remained. Barnacles are known to colonize substrates quickly (Butler 1955) and

appear to play a role in forming a surface that is suitable for oyster settlement. Studies have suggested that barnacles do in fact facilitate settlement of oyster larvae (Barnes 2008, Osman et al. 1989), potentially by providing a calcareous surface that is the preferred setting surface for oysters (Tamburri et al. 2008), inducing set through chemical cues (Barnes 2008, Osman et al. 1989), and creating a microcurrent environment that is conducive to oyster settlement (observed in this study but not measured).

Of the remaining biofilm characteristics analyzed in this study (chlorophyll a concentration, percent organic matter, bacterial cell counts, and bacterial community composition profile) little insight was gained regarding the role biofilms play in oyster larval setting behavior. In most trials, these variables did not change significantly over time or with placement in the water column, with the exception of greater chlorophyll a concentrations on top *versus* bottom tiles. These factors had little or no observed impact on the numbers of spat set on each tile. One exception was the inverse relationship observed between percent organic matter and larval set observed in only Trial 4 (lower set at the higher percentage of organic matter), but a similar effect was not observed in Trial 3, even though tiles deployed during that trial exhibited levels of organic matter (17-29 % OM) similar to Trial 4. The strong relationship cannot be discounted and warrants further investigation.

This study supports previous findings that biofilms enhance the settlement and setting of oyster larvae and provides evidence that may be of use in aquaculture and restoration of oysters. Prior studies have shown that settlement is induced by chemical cues released by bacteria present in biofilms in a manner similar to inducement by adult oysters and newly set spat (Zhao et al. 2003, Tamburri et al. 1992, Fitt et al. 1989,

Weiner *et al*. 1985). Tamburri *et al*. (2008) also found that oysters preferred to set on biofilms *versus* clean surfaces. The results from the present study expand the existing findings by demonstrating that older biofilms are preferred over younger biofilms as setting surfaces. In general, as long as a biofilm totaling roughly 200 g DW/m^2 was present (Figure 3), oysters were likely to set in higher numbers.

This begs the question, why are there not large numbers of oysters in Little Wicomico River? Aside from the stochastic nature of oyster spawning, development, and proximity at metamorphosis to optimum setting surfaces, *Stylochus ellipticus* may be a factor that warrants greater consideration. This predator became an important variable in the current study because of the presence of the flatworm in biofilms for two of the trials and almost complete mortality of spat during one of these trials. It is not known whether the numbers of *Stylochus* were higher in our enclosed setting and rearing tank than they would be in the natural environment, however, *Stylochus* can be collected from tributaries of Chesapeake Bay year round and have been found to reproduce in spring, summer and early autumn, with greater reproduction during the cooler temperatures of spring and autumn (Chintala et al. 1993). We did not observe *Stylochus* or have predation occur in spring trials (Trials 1 and 4; biofilms grown from April through the end of May) but did observe *Stylochus* and predation during summer trials (Trials 2 and 3; biofilms grown between June and early September). Trial 2 indicated that mortality of spat by *Stylochus* can be devastating within the first week of setting. Past studies have suggested that although adult *Stylochus* do feed on larger juvenile oysters, the greatest effect of *Stylochus* predation is on newly set spat and seed (Kingsley-Smith et al. 2009, Newell et al. 2000, Landers et al. 1970). In fact, Newell et al. (2000) suggested that predation on

newly set spat by the smallest life stage of *Stylochus* has a more profound effect on natural oyster populations than predation of adult oysters by any other natural predator. In the current study, predation on newly set spat by the early stages of *Stylochus* was viewed microscopically on tiles from Trial 3 within the first two days of setting. These results implicate the need for continued study into the distribution and life history of *Stylochus* in Chesapeake Bay and provide continued insight regarding what could be happening to naturally set oysters in the Bay and its tributaries.

ACKNOWLEDGMENTS

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Table 1. Probability values for significant difference between oyster set *versus* biofilm age (weeks), placement of collectors in water column (top vs. bottom), location within Little Wicomico River (site), and four biofilm characteristics. No data were collected for Trial 2.

	Trial 1	Trial 3	Trial 4
Age	0.2996	**0.0279**	**0.0014**
Placement	**0.0071**	**0.0199**	0.265
Site	0.078	**0.012**	**<0.001**
Biomass dry weight	0.345	**0.001**	**0.005**
Bacterial cell count	0.359	0.214	0.123
Chlorophyll a	**0.003**	0.128	0.574
% organic matter	N/A	0.953	**<0.001**

Figure 1. Little Wicomico River, Virginia showing sites where biofilm collectors were deployed.

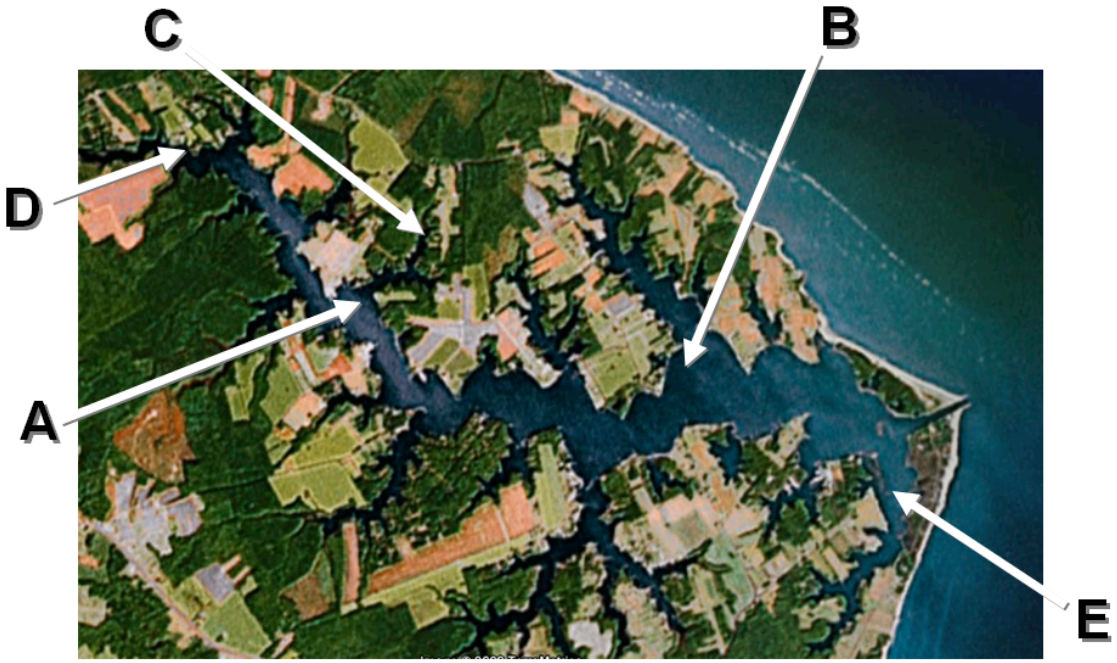


Figure 2. Oyster set (ln(Set Count+1)) *versus* age of biofilm (weeks) for (A) Trial 3 and (B) Trial 4.

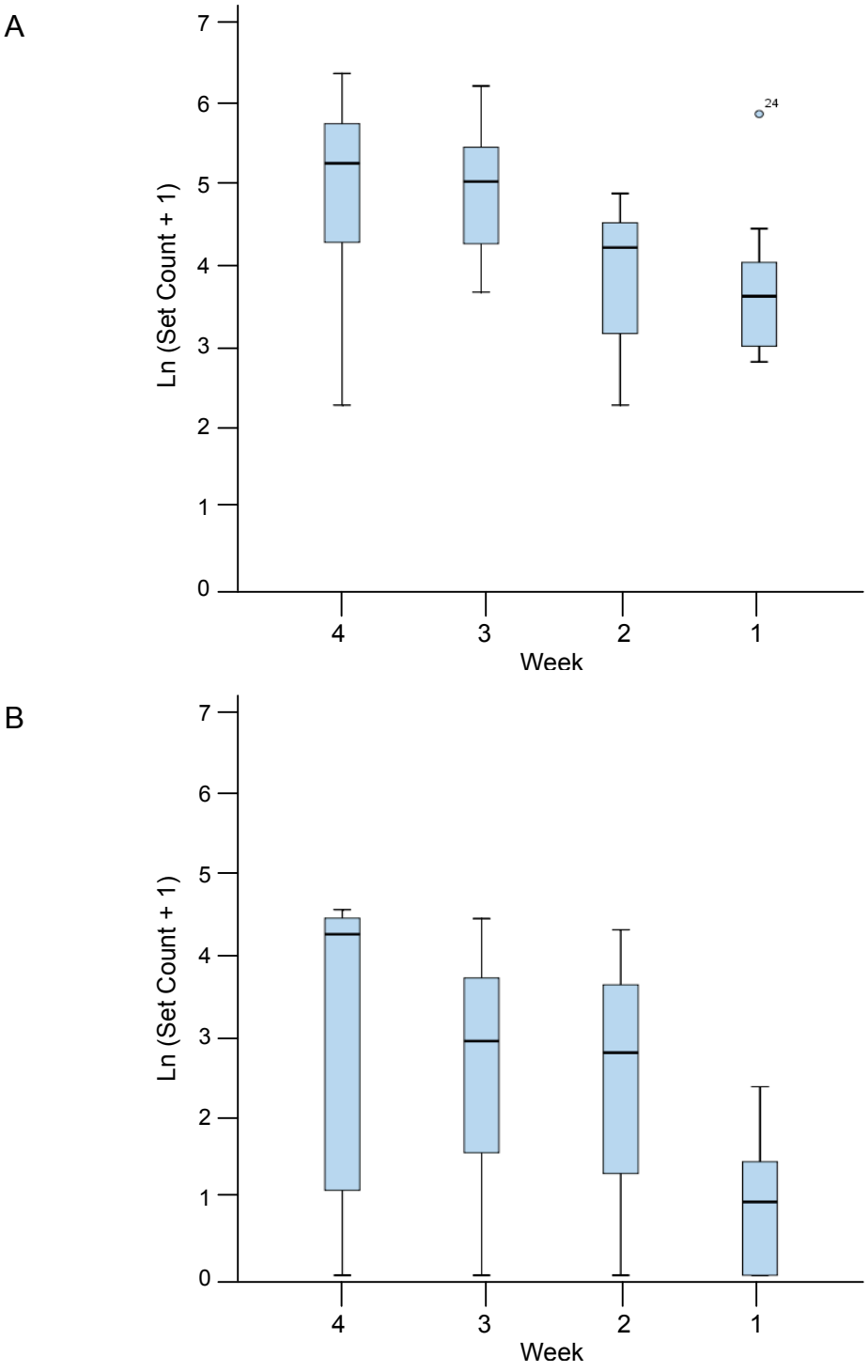
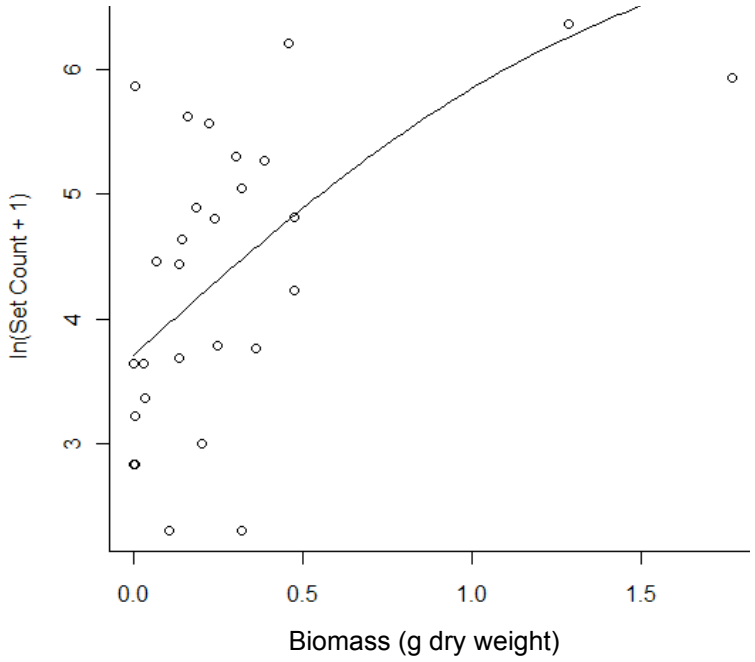


Figure 3. Regressions of oyster set (ln(Set Count+1)) *versus* biofilm biomass dry weight (g) for (A) Trial 3 and (B) Trial 4. Scatter plots are fit with the non-linear regression model $\ln Count = \frac{\alpha}{1+e^{\beta x}}$.

A



B

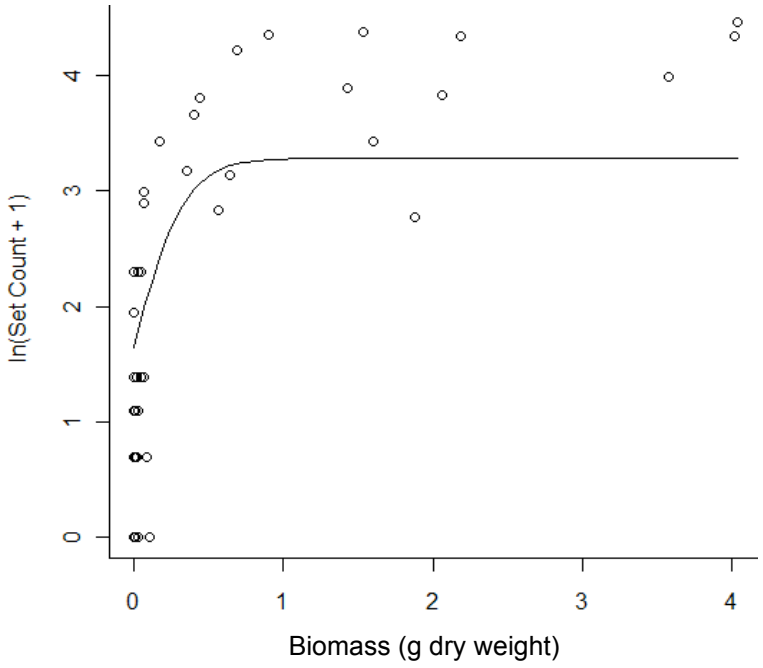


Figure 4. Regression of oyster set (ln(Set Count+1)) versus percent organic matter (OM) for Trial 4.

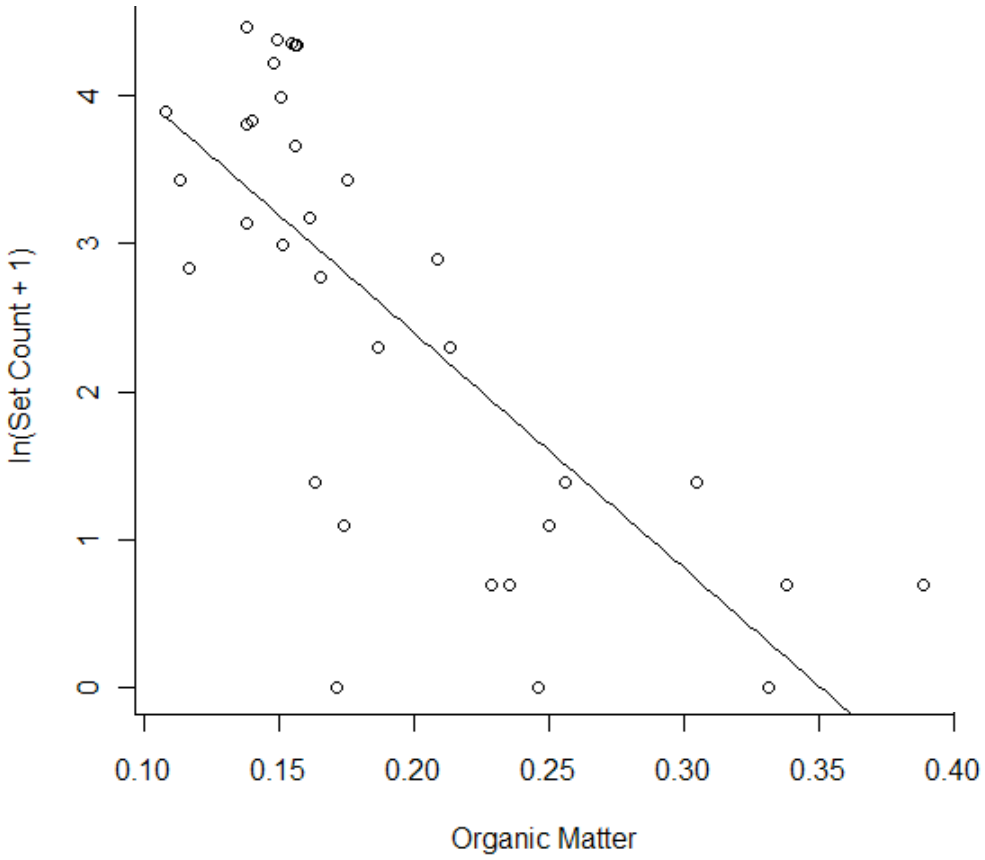
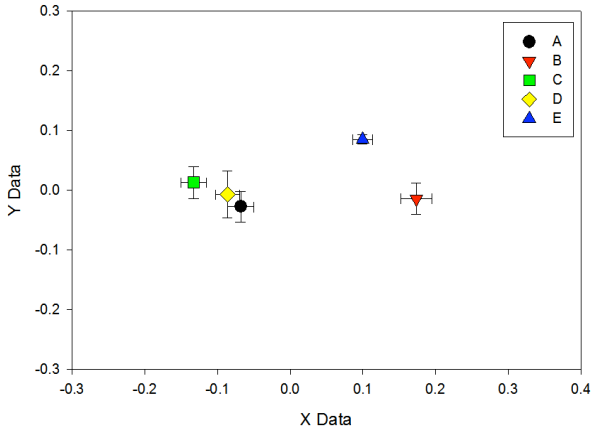
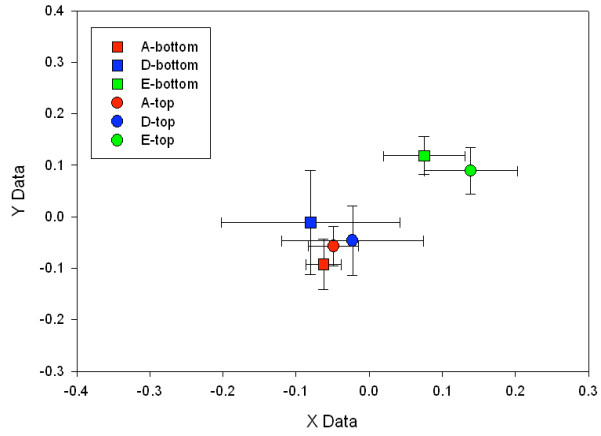


Figure 5. Non-metric multidimensional scaling graphs for TRFLP analysis of biofilms from (A) Trial 1 with times and depths pooled (B) Trial 3 with times pooled and (C) Trial 3 with depths pooled. Letters in the key correspond to sites.

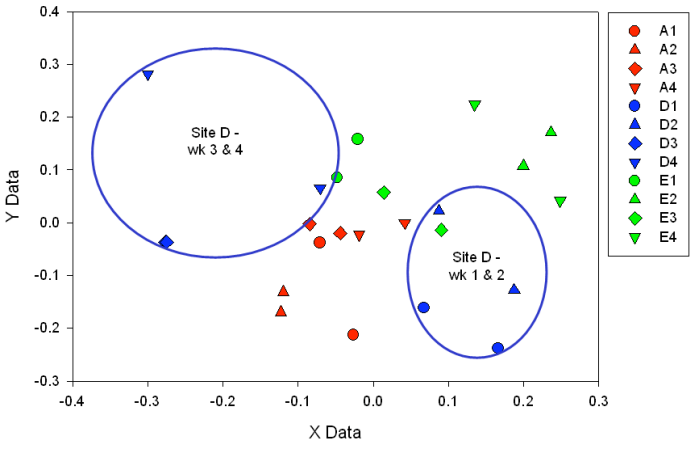
A



B



C



A method for enumerating bacterial cells on bio-adhesive coated slides

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Summary

Direct enumeration of bacteria is generally performed by dispersal of samples in aqueous solution, epifluorescent staining, filtration through black polycarbonate membrane filters, and counting under UV illumination. The intermediate filtration step is often time consuming making routine estimation of bacterial densities in large numbers of samples impractical. We therefore investigated the utility of capturing and viewing stained bacterial suspensions on bio-adhesive slides as compared to the standard method of enumerating bacteria on filters and found that the two platforms exhibited both quantitative and qualitative differences. Cultured-cell total counts using slides were 10% lower than the standard filter method. Dead cell counts were six times higher on slides than on filters. Overall congruence was weakly positive (R^2=0.17). In replicate trials, bacterial cell counts exhibited roughly equal variation on filters and slides (CV=11% and 15%, respectively). When counting environmental samples, slides yielded higher total counts (by a factor of 5). Qualitatively, bio-adhesive slides required less sample handling, longer drying time, and resulted in lower background fluorescence. The use of bio-adhesive slides promoted high throughput while yielding more accurate counts than filters when compared to dilution curves and was found to be useful for direct enumeration of bacteria in laboratory cultures, wastewater, sediments, and biofilms

Keywords: bio-adhesive slides, cell-count, bacteria

Introduction

Methods for enumerating bacteria using polycarbonate filters and fluorescence microscopy have been used for several decades beginning with the use of Acridine Orange (AO) and nuclepore filters stained with Irgalan black for direct couting of bacteria in aquatic samples (Hobbie et al. 1977). This procedure has developed over the years to be utilized for examining many types of environmental samples, including soils and sediments, and tested with several other stains, including 4',6-diamidino-2-phenylindole (DAPI). One limitation with this approach is that environmental samples must be manipulated prior to filtration in order to separate bacteria from detritus, reduce the masking of bacteria, and reduce the likelihood that one will mistake detritus for bacterial cells. There are multiple ways suggested for how to achieve this separation with different types of environmental samples (Bolter et al. 2002, Kepner et al. 1994, Schallenberg et al. 1989). Despite these techniques, AO and DAPI and less popular stains were found to have high background staining of detritus in certain types of environmental samples (Bolter et al. 2002, Yu et al. 1995). These direct count methods also involve the processing of a single sample at a time, which can become problematic when dealing with large numbers of samples. The technique described here was developed to replace filters with bio-adhesive slides to increase throughput and reduce the materials needed to examine environmental samples.

The use of slides to enumerate bacteria has its own issues including clumping of bacteria and edge effects as a result of drying slides prior to microscopy. Several types of slides were considered for this technique, which utilize different adhesive coatings to attract biological material. Slides used for final tests were 24-well Diagnostic Printed

Slides (Thermo Scientific) made with coatings to both attract cells and keep samples within wells. These slides have commonly been used for biomedical and molecular studies with Eukaryotic cells and tissues (Ray et al. 2000, Piyathilake et al. 1995) but no published reports are available for use of these slides for bacterial enumeration.

The combined use of SYTO 9 and propidium iodide counter-staining is a relatively recent method for enumerating bacterial cells. Boulus et al. (1999) used a Live/Dead (L/D) staining technique on drinking water promoting simultaneous viable cell and total cell counts. Counts were found to be comparable to AO and slightly higher than DAPI with batch cultures and drinking water. Live/Dead staining appears to not be a commonly used stain for environmental samples. One of the few mentions of its use involved staining a mixture of E. coli cells and soil samples to take fluorescence emission readings to determine proper stain concentrations and the effect of soil particles on readings (Pascaud et al. 2009). We choose to use Live/Dead stain for this technique because in preliminary tests with staining biofilm and soil samples, Live/Dead exhibited lower background fluorescence compared to AO and DAPI. Live/Dead stain was used on both filters using a standard method and on slides with batch cultures as well as biofilm samples as a representative environmental sample.

Materials and Methods

Samples

Cultures of *Nitrosomonas europaea* and *Paracoccus denitrificans* were harvested at OD of 0.03-0.60 at 670 nm and combined into a stock mixture that was then split into various dilutions: 2X (spun at 2500 x g for two min and resuspended in one-half volume of cell

culture media) and 0.5X (an equal volume of media added). The three resulting dilutions (0.5X, 1X, and 2X) were then applied to either filters or bio-adhesive slides as described below for enumeration. To field test the method, 0.006g of biofilms that had developed on ceramic tiles were suspended in 1 mL of sterile filtered PBS in 1.7 mL microfuge tubes and shaken for 10 min at 2,500 rpm with a quantity of glass beads equal to roughly 250 uL. After settling at room temperature for 5 min, the solutions were passed through Miracloth ™ (average pore size 22-25 μ, Calbiochem) and then enumerated using either filters or bio-adhesive slides. Other samples tested included primary-treated waste water and wetlands sediment, both shaken, settled, and pre-filtered as described above prior to enumeration using either filters or bio-adhesive slides.

Staining

Aqueous cell suspensions (both cultured and environmental) were stained in 1 mL aliquots using 3 uL of LIVE/DEAD BacLight™ Bacterial stain in microcentrifuge tubes (pre-made 2:1 mixture of SYTO 9 and propidium iodide; Invitrogen). For bioadhesive slides, 20 uL of the cell suspension with stain was added immediately to appropriate wells and allowed to dry in the dark. For filters, the cell suspension was allowed to incubate for approximately 10 minutes in the dark at room temperature prior to filtration.

Bio-adhesive slides

A number of brands of adhesive coated slides, including poly-d lysine slides prepared in house, were tested initially for adhesion of cultured cells and environmental samples. Excell Adhesion™ coated slides (Thermo Scientific) printed with hydrophobic septa

among "wells" were found to perform best and were used for all validation tests. For the current study, slides with 5mm diameter wells (cat # ES-230B) were used. Freshly stained cell suspensions were vortexed for 30 sec in a Vortex Genie II after which 20 uL of the supernatant was immediately applied to appropriate wells on the bio-adhesive slide. The slides were held at 37^{o}C in the dark until dry (approximately 10-15 min) after which they were briefly washed with filtered deionized water, twice for cultured cells and thrice for environmental samples, then dried in the dark. The area of each well on slides used in this experiment was 19.6 mm^2.

Filtration

A 1 mL aliquot of the prestained material was vortexed for 30 sec and immediately passed through a prewetted 0.2μ Millipore Isopore™ polycarbonate black membrane filter as is typically done for enumerating aquatic microbial specimens (modification of Kepner and Pratt 1994). Prior to disassembly, filters were washed once with filtered deionized water then dried in the dark at room temperature. Filters were arranged in groups of three on clean microscope slides for viewing. The effective filtration surface area in this experiment, excluding the border, was 380 mm^2.

Microscopy

Antifade solution (BacLight™ mounting oil) was applied to both media, filters and bio-adhesive slides, prior to application of a cover slip and the material was viewed under oil immersion using epifluorescence microscopy. Live (green fluorescent) and dead (red fluorescent) bacterial cells were viewed separately with fluorescein and Texas Red

bandpass filter sets, respectively, on an Olympus BX-41. Enumeration was accomplished by counting from an accurately ruled eyepiece graticule (0.0004 mm^2 total grid area) subdivided into 100 smaller squares of equal area. Five grids (all 100 squares) were counted then averaged for each sample (live and dead cells counted separately). The total numbers of live and dead cells per mL were determined for filters as

$$\frac{\text{(average cell count per grid) (effective filtration area)}}{\text{(grid area) (volume of cells filtered) (dilution)}}$$

The total numbers of live and dead cells per mL were determined for bio-adhesive slides as

$$\frac{\text{(average cell count per grid) (area of a well)}}{\text{(grid area) (volume of cells applied) (dilution)}}$$

Statistical Analyses

Data for split samples counted from filters and from bio-adhesive slides were compared to determine whether the novel counting method yielded counts similar to the standard accepted method using linear regression (SigmaStat). The resulting R^2 values were considered as an indication of congruence of the two methods. Data from the dilution curves for each platform were plotted and the resulting R^2 values were considered as an indication of the precision and potential utility of each method. To determine significance of the relationship between dilution and cell count, paired t-tests were used where assumptions of normality and equal variance held true, otherwise the Mann-Whitney rank sum test was used (SigmaStat).

Results

Validation with bacterial cultures

Filters and slides performed differently on a number of criteria. For cultured cells, counts obtained from filters were higher than counts made on bioadhesive slides from the same mixed culture solutions by a factor of 0.10 (Table 1). Total cell counts on the two platforms differed significantly (P=0.004) and overall congruence between the two platforms was only weakly positive ($R^2 = 0.17$; Figure 1). The weak concurrence was due primarily to two factors: counts of dead cells were much lower on filters than slides and total counts were highly variable for any particular dilution on both platforms (average coefficient of variation 11% and 15% for filters and slides, respectively). An increase in cell concentration was reflected as an increase in live cell counts on both platforms ($R^2 = 0.29$ and 0.92 for filters and slides, respectively) and cell counts appeared to plateau on filters at higher cell concentrations. However, for dead cells, filters resulted in a decreased cell count with increased cell concentration (inverse relationship $R^2 = 0.36$) whereas bioadhesive slides showed the expected positive relationship ($R^2 = 0.55$). Background fluorescence was higher for filters than for bioadhesive slides (Figure 2 A,B) effectively quenching the fluorescent signals of bacteria, especially dead cells.

Analysis of environmental samples

Filters and slides also produced dissimilar results when enumerating environmental samples. Although significantly different (T=45; P<0.001), total cell counts per gram biofilm enumerated on both platforms were positively correlated ($R^2 = 0.42$). Counts obtained from filters were 2-10 times lower than counts made on slides from the same

samples (Table 2). Matrix fluorescence and detrital fluorescence were higher for filters than slides (Figure 2 C,D), reducing the bacterial signals and making it more difficult to distinguish bacteria from detritus on filters. A number of other biofilm components, including diatoms and algal cells were easier to identify on slides compared to filters. Sediment samples yielded total cell counts 13 times higher on slides than filters, affected greatly by the ability to detect dead cells on slides. Waste water samples yielded total cell counts 1.2 times higher on slides than filters.

Conclusions

Although bio-adhesive slide counts were not directly comparable to filter counts, the slides had more favorable qualities. Filters exhibited slightly higher counts than slides for cultured cells but much lower counts of dead cells than slides, possibly as a result of cells being lost during the filtration process (Kepner et al. 1994) and as a result of the ease of counting the red-fluorescent cells against the lower background on slides. For the environmental samples, the use of bio-adhesive slides successfully identified cells that were not readily discernible on filters, reduced the processing time and materials, and increased throughput (up to 24 samples per slide for the brand of slides used in this trial) while simultaneously increasing the ease of counting. Cells were easier to discern on slides, which in turn reduced the time taken to make counts. As noted in the current study, Bloem *et al*. 1995 saw less background staining and less fading of fluorochromes in soil smears on slides compared to filters. These benefits are notable for environmental samples, which should be analyzed as soon as possible after collection (Bolter et al. 2002) and to reduce fading of the stain. Finally, bio-adhesive slides combined with

Live/Dead staining provide a rapid and convenient means of determining the initial concentration and viability of bacterial cells in cultures or environmental samples, promoting more accurate enumeration by the standard filtration method.

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Table 1. Average bacterial cell counts when enumerated using polycarbonate filters and bio-adhesive slides. Replicates are listed in order of more dilute (0.5) to more concentrated (2). SE for filters ranged from 38.51-90.26 and 0.83-2.8 for live and dead counts, respectively. SE for slides ranged from 33.79-75.79 and 2.18-7 for live and dead counts, respectively.

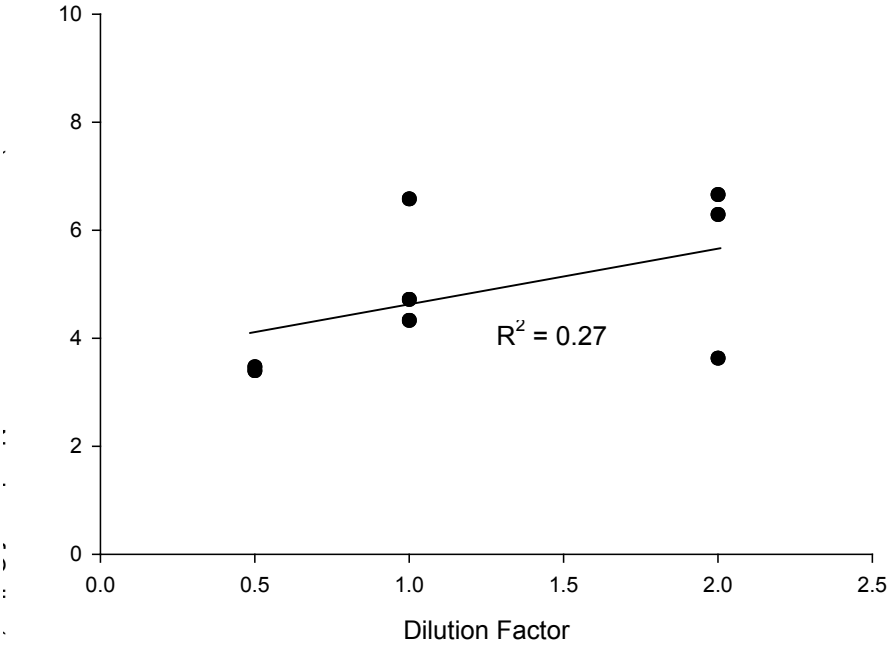
	Live (x 10^8)		Dead (x 10^6)		Total (x 10^8)	
Dilution	Filter	Slide	Filter	Slide	Filter	Slide
0.5	3.28	1.16	0.12	0.00	3.40	1.16
0.5	3.44	1.91	3.01	3.84	3.47	1.95
1	4.70	3.78	1.72	2.99	4.72	3.81
1	6.50	2.45	0.73	7.26	6.58	2.52
1	4.33	3.18	0.00	21.77	4.33	3.40
2	6.65	5.96	1.29	22.62	6.66	6.19
2	3.63	7.17	0.00	21.34	3.63	7.38
2	6.28	8.22	1.29	12.80	6.29	8.35

Table 2. Total bacterial cell counts for nine biofilm samples when enumerated using polycarbonate filters and bio-adhesive slides. Samples were not counted in replicate so no SE is available.

	Total (x 10^{10})	
Bioifilm	Filter	Slide
1	2.42	5.53
2	0.89	4.88
3	0.74	7.80
4	1.96	11.02
5	2.88	14.83
6	0.94	6.22
7	1.76	7.87
8	1.09	4.66
9	1.45	9.62

Figure 1. Relationship between total cell count and dilution factor for polycarbonate filters (A) and bio-adhesive slides (B). Dilution factor refers to original cell culture (1.0) made more dilute (0.5) and more concentrated (2.0).

A



B

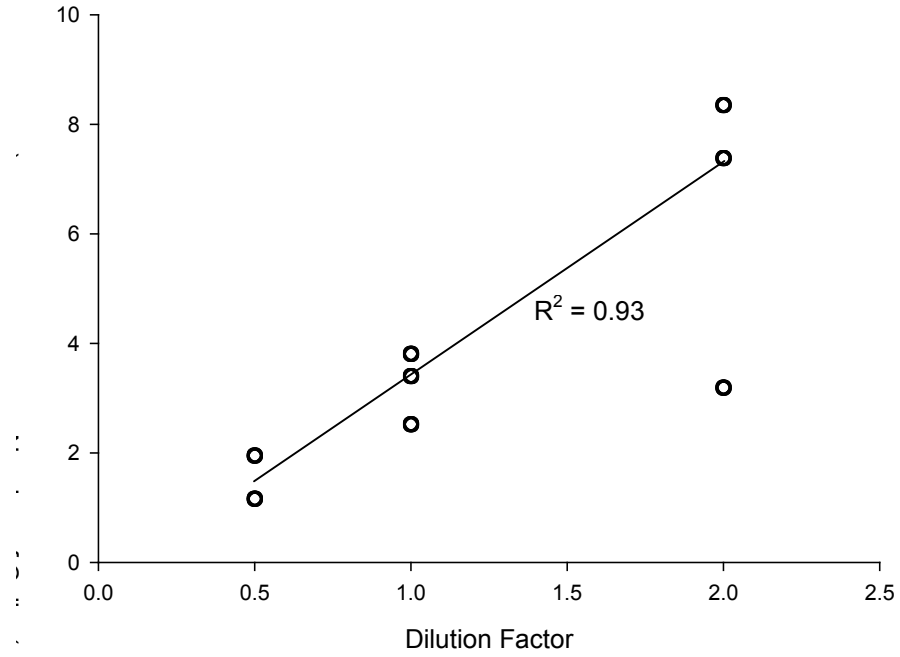
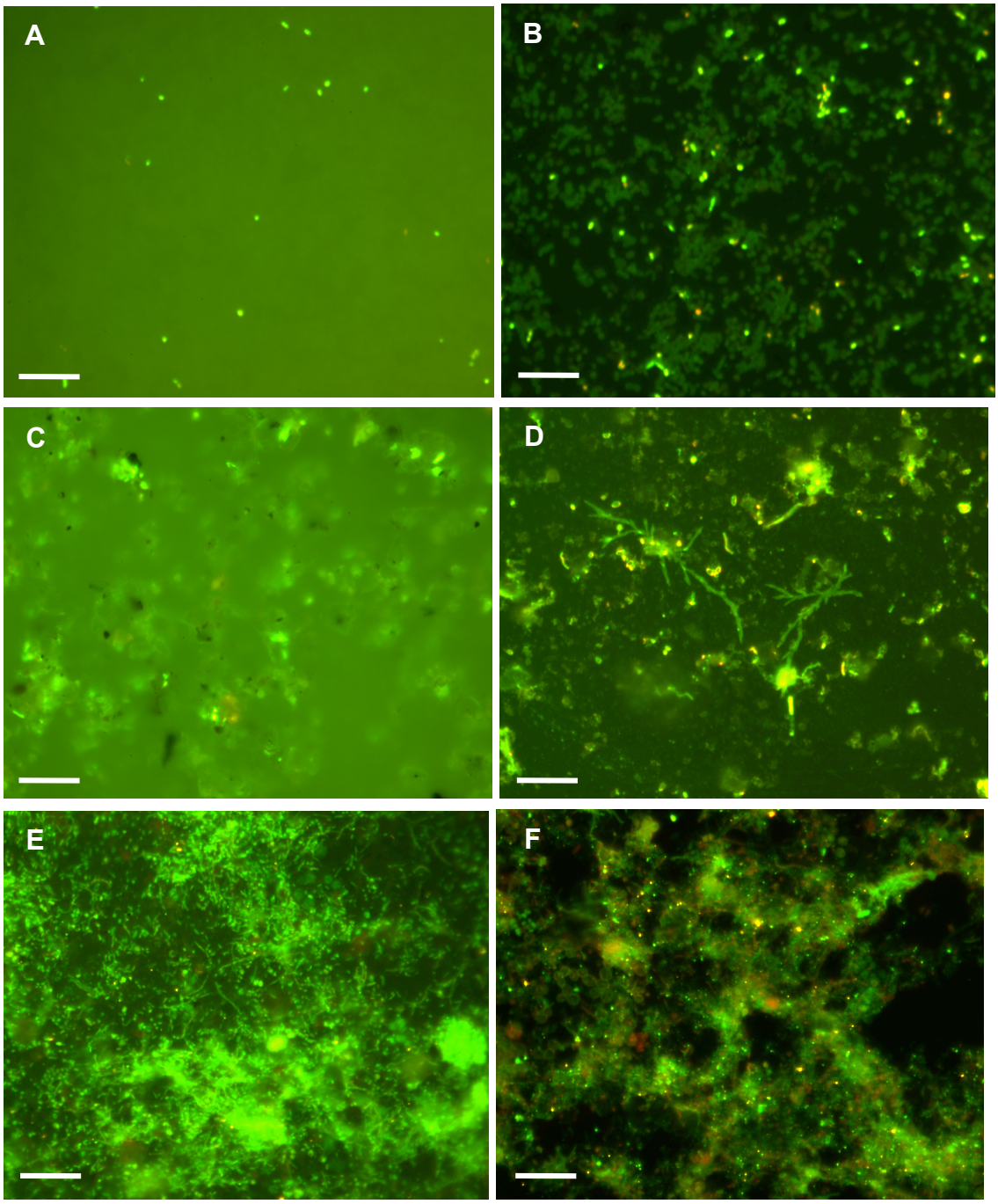
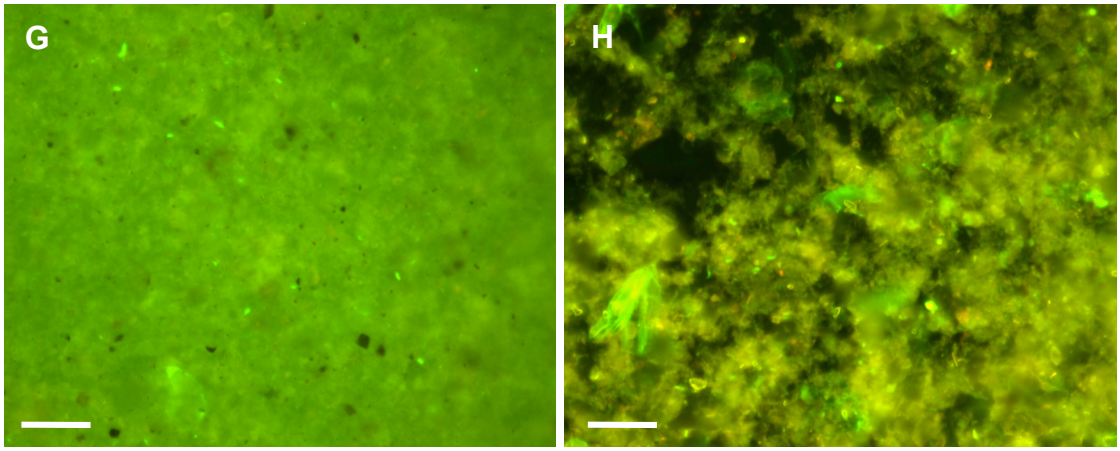


Figure 2. Comparative photomicrographs of epifluorescent enumeration using Live/Dead staining (SYTO 9 and propidium iodide) from filters and bio-adhesive slides, respectively, of cultured cells (A, B), biofilms (C, D), wastewater (E, F), and sediments (G, H). All photos 1000x; bar is 10 µ.




G
H

Vita

Alaina Ashley Hart was born on May 8, 1986 in Torrejon, Spain. She lived in several cities around the U.S. before moving to Poquoson, Virginia where she graduated from Poquoson High School in 2004. Alaina moved to Richmond, Virginia in 2004 to attend Virginia Commonwealth University and graduated in 2007 with a B.S. in Biology. She remained at VCU to pursue a M.S. degree in Biology and began her research in the spring of 2008 in the Ecological Genetics Lab. After graduation, Alaina plans to pursue teaching science at the high school level.