

Direct estimation of biomass on different pipe material coupons using a specific DNA-Probe

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ABSTRACT

A variety of approaches to quantify the biofilm without disruption due to detachment have been developed over the years. One basic approach is the combination of advanced microscopy with molecular staining. However many stains (4',6-diamino-2-phenylindole (DAPI), acridine orange or live-dead stains) can be non-specific when corrosion products, precipitates, and pipe material are present. In addition, some pipe materials cause high background when using epifluorescent microscopy. The new refinement discussed in this presentation used fluorescence spectroscopy to obtain the spectra from four common distribution system pipe materials: PVC, cast iron, "concrete" lined cast iron, and galvanized iron. The emission maximum for all four materials was between 500 and 550 nm, but emissions radically decreased around 575-600 nm. A molecular probe, BO-PRO3 was identified which has an emission intensity maximum at 599nm (far red), with emission intensity 200 times greater when it is bound to DNA. The BO-PRO3 has greatly reduced non-specific staining and background problems. In the preliminary experiment, using diluted waste water, a significant linear relationship was found between stained surface area/total area ratio and fixed biomass measurements from heterotrophic plate counts (HPC) on R2A medium and total direct count (TDC). In addition, the biomass on different pipe material coupons from pilot distribution systems was also correlated to the stained surface area fraction and HPC.

Key words: BO-PRO3, drinking water, confocal laser scanning microscope, epifluorescent microscopy, Raman spectroscopy.

INTRODUCTION

Monitoring the microbial water quality in a distribution system is part of the regulatory requirements that a water treatment plant must meet. Furthermore, those regulations are becoming more stringent in terms of microbiological parameters. An often-unmonitored part of water distribution systems that directly impacts the bulk liquid microbial water quality is the quantity of biofilms (LeChevallier et al., 1987; Van der Wende et al., 1989; Van der Kooij, 1992; LeChevallier et al., 1993). Detachment of bacteria from the biofilm may thus affect the water quality. Historically, most methods have relied on detaching the biofilm, suspending and homogenizing the cells, and then enumerating and/or isolating the organisms using selective media (Camper, 1996). This allows quantification and/or identification of the organisms, but only of the culturable population for the chosen selective media. A variety of approaches to quantify the biofilm without disruption due to detachment or the bias of selective media have been developed over the years. One basic approach is the combination of advanced microscopy with molecular staining. Advanced microscopy alone (e.g. Scanning Electron Microscopy) is unable to consistently differentiate cells and inorganic material based on morphology alone. Molecular stains specific to DNA, respiration, or membrane components and other molecules unique to biotic material seem to provide a mechanism to achieve this (Rodriguez et al., 1992; Schaul et al., 1993). However many stains (4',6-diamino-2-phenylindole (DAPI), acridine orange or live-dead stains) can be non-specific when corrosion products, precipitates, and pipe material are present (Hobbie et al., 1977; Porter and Feig, 1980; Lisle et al., 1998). In addition some pipe materials cause interfering background fluorescence obscuring biomass when using epifluorescent microscopy.

The new refinement discussed in this article uses Raman spectroscopy to obtain the spectra from four common distribution system pipe materials, e.g. PVC, cast iron, "concrete" lined cast iron, and galvanized iron. A molecular probe, BO-PRO3, was identified which has an emission intensity maximum at 599nm (far red), with an emission intensity 200 times greater when it is bound to DNA. So far, this seems to have greatly reduced non-specific staining and background problems. Confocal laser scanning microscope (CLSM) and computer image analysis coupled with consistent thresholding is used to build an index of stained surface area fraction, which is biotic. This is useful for relative comparisons between coupons only. After saving the image, the cells are scraped, quantitatively suspended, and homogenized. Then, a known volume of material is put into a hemacytometer so that total cell counts can be conducted on a known volume of suspension. This allows for both intact/relative comparisons of biofilms, as well as a quantitative total cell count. This report describes the application of BO-PRO3 molecular dye to directly quantify biomass on different pipe material coupons.

MATERIALS AND METHODS

Raman Spectroscopic Analysis

Raman spectra were acquired on a custom-built micro-Raman system consisting of an Olympus IX-70 inverted microscope with an Ar ion laser and spectrometer equipped with a thinned back-illuminated CCD detector. A notch filter rejected the Rayleigh line. Spectra were measured with 488 nm excitation at 10mW for laser power.

BO-PRO3 Staining

Coupons were rinsed once with Phosphate Buffer Solution (PBS) and then put into 250-mL beaker. Paraformaldehyde of 4 %, diluted 3x (17mL in 50mL of PBS) was added into the beaker and left to stand for 10 min. After the fixing step, coupons were rinsed very well with PBS twice and left to stand for 5 min. 500 μ L of BO-PRO3 (Molecular Probes, Inc.) at a final concentration of 10 μ M, was dropped on the coupon to only cover the upper surface, in order to spare some dye.

Methods for Bacteriological Enumeration

Heterotrophic Plate Counts (HPC)

Coupons colonized by biofilm were sampled and rinsed very carefully with PBS twice. For HPC, total direct count, the biofilms were detached from coupons manually using a sterile weighing spatula (sterilized by Ethanol 70%) in 4-mL of sterile PBS, and then homogenized using a tissue blender (Tissue TearorTM, Biospec products, Inc., Racine, WI, USA) at 3000 rpm for 2 min. The sample was then placed on an R2A agar plate (ref. 1826-17-1, Difco Laboratories, Detroit, Michigan, USA) and incubated for 7 days at 25°C. Each dilution series was plated in duplicate. HPCs were expressed as CFU per cm² after dividing by the surface area of coupon.

Total Direct Counts (TDC)

The total number of bacteria was determined by a hemacytometer count chamber (Hausser Scientific company, PA, USA). The same procedure used for BO-PRO3 staining was used on separate coupons. These coupons were scanned using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Thornwood, NY, USA) and the biofilms were detached from coupons in 1-mL of sterile PBS, and then vortex for 3 min. Sample of 10 μ L was taken from the 1-mL of sterile PBS and then put into the hemacytometer chamber. Microscopic examination was carried out with a Zeiss Axiolab Epifluorescent microscope (HBO 50 W mercury lamp) equipped with a digital camera. The determination of total cell number was carried out with the filter set XF41 from Zeiss (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Confocal Laser Scanning Microscopy (CLSM)

Intact BO-PRO3 stained biofilms were attached to microscope slides and placed on the stage of an inverted confocal laser scanning microscope. Using a helium/Neon laser tuned to 543 nm and an emission filter at 580 nm, serial optical sections through the entire thickness of biofilm at three randomly chosen areas of series were scanned. Data

was stored for image analysis. Using Zeiss LSM 510 software projections (single reconstructed images of each section series, representing a microscopic view with a long depth of focus) were constructed and converted to TIFF files for subsequent thresholding and quantitation.

Image Analysis

Images were analyzed by The Image Processing Tool Kit (New version 4.0, ISBN 1-928808-26-3, Reindeer Graphics, Inc., Asheville, NC, USA) which offers a comprehensive set of functions for the computer-based image processing and measurement of images. The key, and most difficult step, in most image analysis procedures is the threshold step used to separate the image into features and background. This process is usually performed manually with an original image, which provides ample room for operator differences and inconsistency. For this reason, the original image first requires some corrective processing to level the background brightness. This was done by duplicating the image. The original image was duplicated (Image -> Duplicate) and the duplicated image was manually thresholded and compared to the original image. This allowed visual determination of what features should be measured. The simple brightness threshold was established using The Image Processing Tool Kit Threshold function (select Image -> Adjust -> Threshold). This displays a histogram of the image with a single sliding marker that can be moved to separate the brightness values, which are displayed as black and white images. The area of the image measurement region selected is reported using the Filter -> IP*Measure -> Global function.

Experimental Set-up for Biological Coupons in PDS Cradles

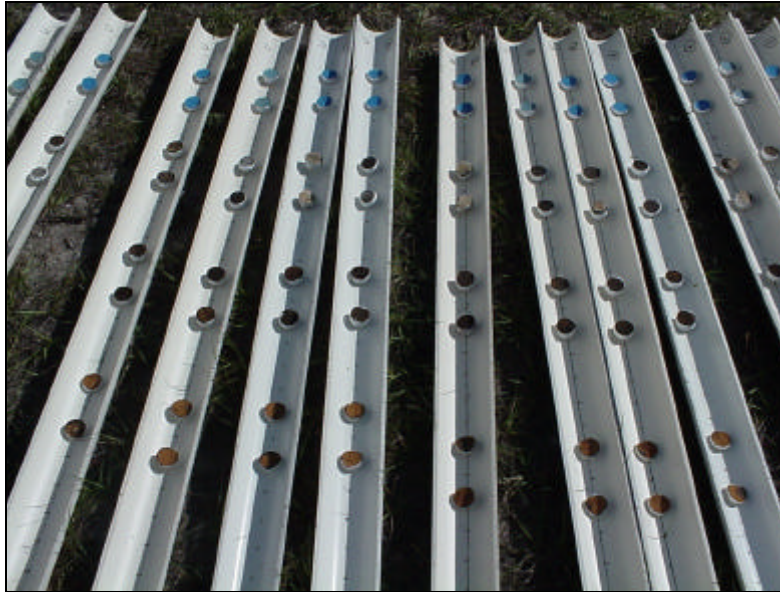
UCF is conducting an AWWARF and Tampa Bay Water tailored collaboration project to determine the effect of blending different water qualities on distribution system water quality. Waters produced from seven different treatment systems (aeration (G1), softening (G2), Blended softening (G3), NF (G4), CSF-O₃-GAC (S1), IMS (CSF-NF or S2) and high pressure RO are blended and distributed to 18 different pilot distribution systems (PDS).

G: ground water; S: surface water; NF; nanofiltration; CSF: coagulation-sedimentation-filtration; GAC: granulated activated carbon; IMS: integrated membrane system; RO: reverse osmosis

G1, G2, G3, G4 and RO finished waters are produced from the same groundwater (Cypress Creek Water Treatment Plant, Florida, USA). Salts are added in RO permeate to simulate typical finished water from a desalination process. The S1 and S2 finished waters are produced from the same surface water (Hillsborough River Water Treatment Plant, Florida, USA). The PDSs consist of combined PVC, galvanized, lined ductile iron and cast iron pipes taken from actual distribution systems and have a 5-day HRT. The last 4 PDSs (i.e. numbers 15-18) are made of a single pipe material and are referred to as the "Pure" PDSs. The pure PDSs were receiving the same blend during the study (G1(60%), S1(30%), RO(10%)). The number of coupons incubating in the Pure PDSs during the experiment was determined as follows:

2 (duplicates) * 3 (biofilm assays) * 2 (aged and pristine) * 1 (material) = 12 coupons per cradle (i.e. 6 aged + 6 pristine, all of the pipe material the Pure PDS consists of).

In the Pure PDSs, the Pristine coupons are placed upstream and aged pipe coupons downstream so that corrosion products from the aged material will not be transported onto the pristine coupons. Each PDS has cradle consisting of 4 in. diameter PVC pipe housing and a 3 in. diameter half PVC pipe sliding coupon holder. The length of the cradles is approximately 12 feet for the single material pipe distribution systems. The old and pristine pipe coupons are attached to sliding pipe coupon holders as presented in Photograph 1. These pipe coupon holders can be easily removed from the cradle and replaced after each experimental phase for biofilm analysis. The cradles were transported in a 4 in. diameter PVC pipe, with the coupons still in place. Placing a wet sponge inside elevated the humidity in the pipe. Upon arrival at the labs the coupons were harvested for biofilm studies.



Photograph 1. Sliding coupon holder with biofilm coupons

Coupons were incubated in varying dilutions of activated sludge effluent. This effluent was obtained from two lab scale sequencing batch reactors (SBRs) treating a septic domestic wastewater spiked with acetic and/or propionic acid. The SBRs were receiving a nitrification inhibitor also.

RESULTS AND DISCUSSION

The stain selected for direct cell counts for intact biofilms was BO-PRO3 from Molecular Probes (Eugene, OR, USA). The probe was selected by first conducting Raman spectroscopy on coupons of each material to determine the wavelength for background fluorescence (Figure 1).

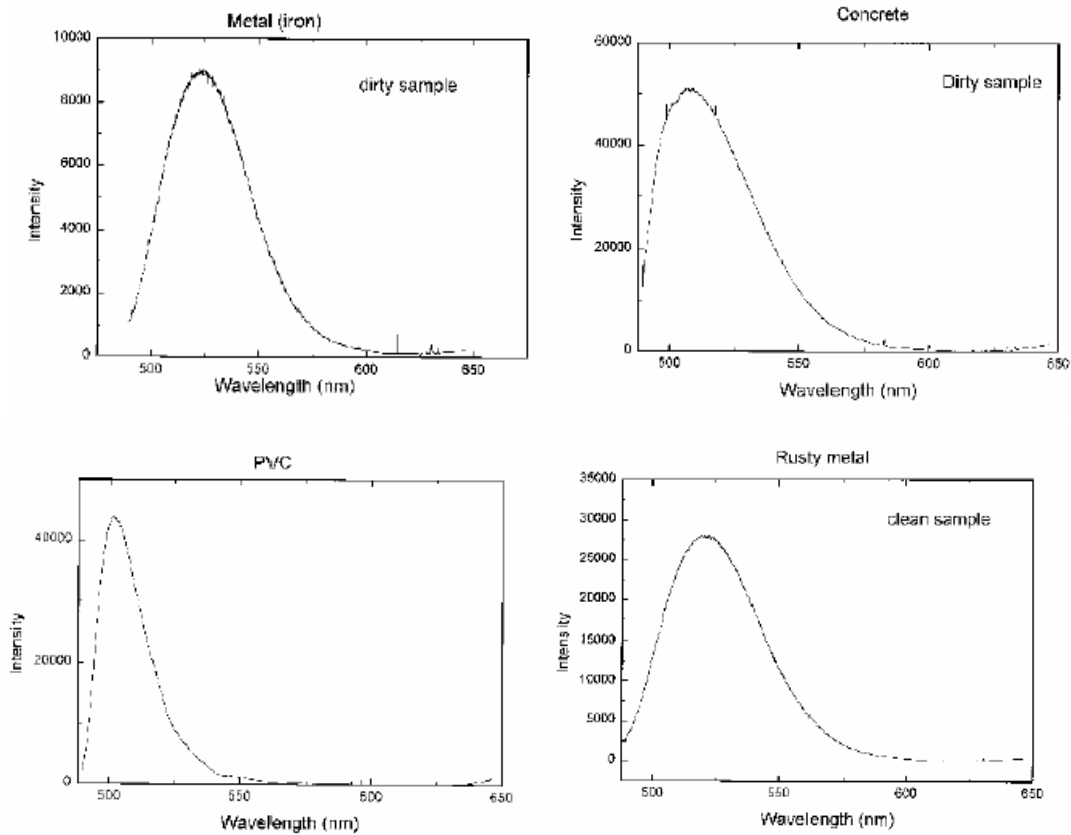
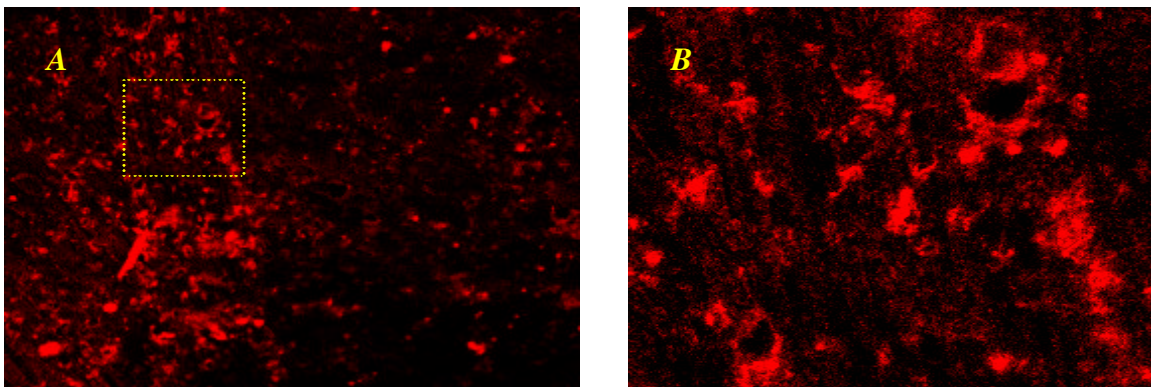


Figure 1. Spectra of coupon materials.

The emission maximum for all four materials was between 500 and 550 nm, but emissions radically decreased around 575-600 nm. A molecular probe, BO-PRO3, was identified with an emission intensity maximum at 599nm (far red). This probe had an emission intensity that was 200 times greater when it was bound to DNA (Photograph 2). The BO-PRO3 probe had very low emission intensity unless it is bound to DNA.



Photograph 2. A. image of biofilm using a confocal laser scanning microscope (CLSM), showing red stained areas of biomass within the biofilm. B. Magnified area of biofilm (dotted line in A.).

The stained coupon was immobilized on a glass slide using Silly Putty™ (Binne&Smith, Inc., Easton PA, USA). Under the CLSM, the stain allowed the biological surface material (and often to a considerable depth within the film) to be differentiated from the non-biological material. For the control experiment, pristine galvanized coupons were incubated for 7 days at room temperature in tap water. These samples were dechlorinated using sodium thiosulfate (125 mg/L), which was added to a final concentration of 125 mg/L. Then BO-PRO3 staining was performed with pristine galvanized coupons (no biofilm) and incubated pristine galvanized coupons (with biofilm). Control pristine galvanized coupons didn't display any red color on their surface while incubated galvanized coupons (i.e. with biofilm attached to it) displayed red color on their surface (data not shown).

In preliminary experiments, methods for bacteriological enumeration (HPC and TDC) were used and assessed for correlations between BO-PRO3 direct cell count methods combined with the threshold techniques and corresponding bacteriological enumeration results (Table 1). In order to grow biofilm, pristine galvanized coupons were incubated for 7 days at 25°C in different source waters (deionized water, University of Central Florida tap water, SBR effluent (100, 50, 25%). After observation under CLSM, three representative pictures of the attached biomass were taken. The stained biofilm image was then transferred into a computer and analyzed, using Image Processing Tool Kit 4.0, to calculate the stained surface fraction of the coupon. The stained areas, HPC and TDC increased in proportion to the biomass volume (Table 1). A significant linear relationship was found between stained surface area/total area ratio and fixed biomass measurements from heterotrophic plate counts (HPC) on R2A medium and total direct count (TDC) (Figures 2 and 3).

Table 1. Comparison of HPC, TDC with threshold results – galvanized steel coupons.

Sources	Ratio Stained Area/Total Area (%)	HPC (x10 ³ cfu/cm ²)	TDC (x10 ³ cells/cm ²)
DI	0.1 (0.05) ^a	13 ^b	5 ^b
Tap water	2.6 (1.32)	24	155
SBR Effluent (25%)	11.2 (3.11)	125	226
SBR Effluent (50%)	27.1 (9.52)	933	424
SBR Effluent (100%)	35.7 (9.04)	1544	1131

^aMean value are provided with 95% confidence intervals in parentheses.

^bMean value of duplicates.

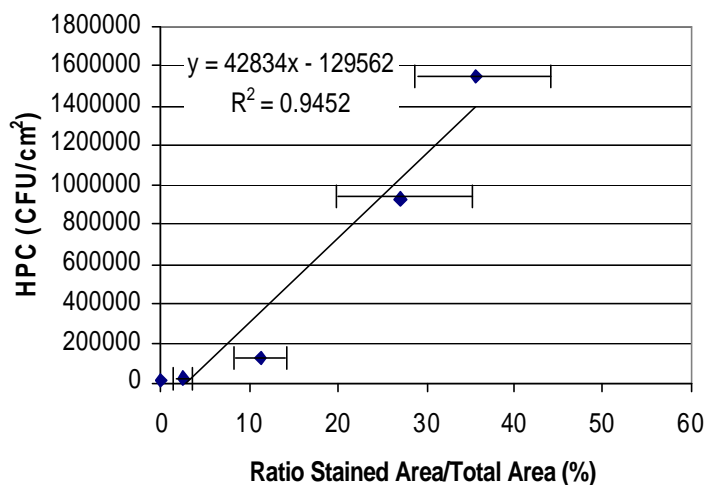


Figure 2. Correlation HPC vs stained area fraction results – galvanized steel coupons.

Horizontal error bars are based on the 95% confidence limits for the stained area fraction data.

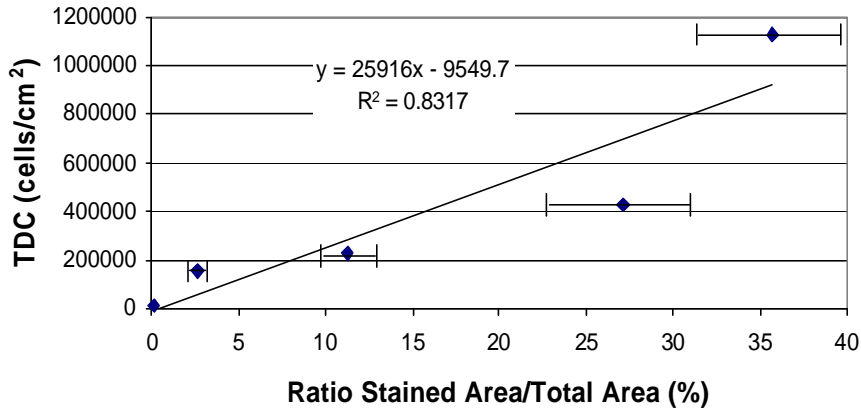


Figure 3. Correlation TDC vs stained area fraction results – galvanized steel coupons.

Horizontal error bars are based on the 95% confidence limits for the stained area fraction data.

Coupons cut from the actual pipe distribution system (lined-cast iron, PVC and galvanized) of Tampa Bay Water network have been used to confirm the results of the preliminary study (Table 2). These coupons were exposed to drinking water.

Table 2. Comparison of HPC with threshold results - pilot system coupons.

Coupons	Ratio Stained Area/Total Area (%)	HPC (x10 ³ cfu/cm ²)
p-lined cast iron	1.29 (0.72)*	69
p-PVC	0.47 (0.18)	3.1
p-galvanized steel	10.00 (1.37)	124
aged-lined cast iron	5.74 (2.33)	122
aged-PVC	0.48 (0.17)	8.7
aged-galvanized steel	9.06 (4.44)	131

p: pristine *Mean value are provided with 95% confidence intervals.

Coupons incubated in the pure PDSs from 3/14/02 to 6/14/02 were harvested to measure biomass on the surface (Table 2). Galvanized steel supported the greatest biofilm inventory, followed by lined cast iron, and finally PVC. However, it can be noted, that there is a significant difference between PVC and the other two materials; PVC being the lowest in terms of amount of fixed biomass. It is interesting to notice that this trend is similar for both pristine and aged materials.

HPC was compared to the stained surface fraction to confirm the results of the preliminary study (Table 2). For BO-PRO3 direct cell count, two coupons of each material were analyzed. For image analysis, thresholds for three representative images of the stained surface were determined. The stained area and HPC increased in proportion to

the biofilm biomass (Table 2). In addition, the correlation between stained area and HPC was relatively significant ($R^2 = 0.81$) (Figure 4). Aged PVC and concrete lined coupons showed higher biomass than pristine coupons, but galvanized coupons did not show any difference.

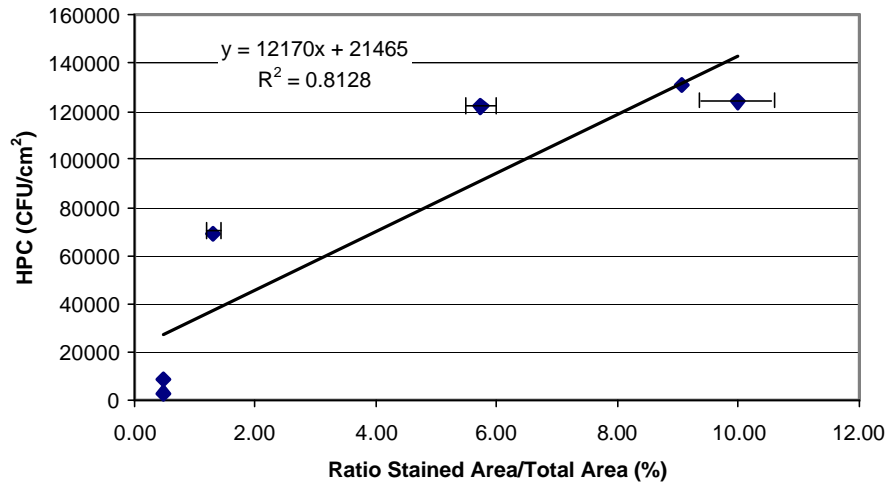


Figure 4. Correlation HPC vs stained area fraction – PDSs coupons

The fact that there is more fixed biomass on galvanized steel than on lined cast iron and PVC can be explained by the fact that the galvanized steel corroded extensively and relatively rapidly compared to lined cast iron (PVC is considered to be practically non-corrodible material). The galvanized layer provides a sacrificial anode for the steel and therefore is preferentially corroded. This corrosion provides microorganisms with crevices (in which they are protected from disinfectant stresses) and more surface area to attach and settle on the coupon. The roughness and porosity of the lining for the lined cast iron may provide more surface available for microorganisms to colonize on than smooth PVC, explaining the higher biofilm inventory.

Finally, this technique presents several advantages:

- Even though corrosion products have sorbed on the surface of the coupons, it was still possible to obtain significant correlations.
- The dye used and described in this paper allows for specific staining of fixed biomass without detaching it, reducing the variability of the results thanks to a low contribution of fluorescence from the support in terms of emission intensity, i.e. less background interferences.

This technique is promising, however, additional investigations are still on-going to confirm the results presented in this paper. The intact stained area provides a simple technique for relative biofilm comparisons.

ACKNOWLEDGMENTS

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