

Direct estimation of biofilm density on different pipe material coupons using a specific DNA-probe

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Abstract

A variety of approaches to quantify biomass in biofilms 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 (e.g. 4',6-diamino-2-phenylindole, 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, 'concrete' lined cast iron, cast iron, and galvanized steel. 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-PRO™-3 (Molecular Probes, Inc., Eugene, OR, USA) was identified which has an emission intensity maximum at 599 nm (red), with emission intensity 200 times greater when it is bound to DNA. The BO-PRO™-3 has greatly reduced non-specific staining and background problems. In the preliminary experiment, using diluted waste water, a significant exponential relationship was found between stained surface area/total area ratio and fixed biofilm inventory measurements from scraping heterotrophic plate counts (SHPC) on R2A medium. In addition, the biofilm inventory on different pipe material coupons from pilot distribution systems was also correlated to the stained surface area fraction and SHPC.

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1. 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 [1–4]. 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 [5,6]. 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 [5,7–11]. One basic approach is the combination of advanced microscopy with molecular staining [12–15]. Advanced microscopy alone (e.g. Scanning Electron Microscopy) is unable to consistently differentiate cells and inorganic material based on morphology alone [16]. Molecular stains specific to DNA, respiration, or membrane components and other molecules unique to biotic material seem to provide a mechanism to achieve this [17,18]. 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

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material are present [19–21]. In addition some pipe materials cause interfering background fluorescence obscuring biomass when using epifluorescent microscopy.

The new refinement discussed in this article uses fluorescence spectroscopy as well as DNA-staining and confocal laser scanning microscopy (CLSM) coupled with image analysis (thresholding) in order to design an enumeration method for fixed biomass on pipe coupons without disruption of the biofilm. To evaluate this technique, stained surface area fractions were compared to traditional scraping heterotrophic plate counts. This article describes the application of a specific DNA molecular probe to directly quantify biofilm on different pipe material coupons.

2. Materials and methods

2.1. Fluorescent spectroscopic analysis

Fluorescent spectra were acquired on a custom-built 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 10 mW for laser power.

2.2. BO-PRO™-3 staining

Coupons were rinsed once with Phosphate Buffer Saline (PBS, pH7.6 = 7.4; Bio-Rad Laboratories, Hercules, CA, USA) and then put into 250 ml beaker. Paraformaldehyde (4%) diluted 3 × (17 ml in 50 ml 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. Five hundred microliters of BO-PRO™-3 (Molecular Probes, Inc., Eugene, OR, USA) at a final concentration of 10 μM (diluted with PBS), was dropped on the coupon to only cover the upper surface, in order to spare some dye.

2.3. Scraping heterotrophic plate counts for bacteriological enumeration

Coupons colonized by biofilm were sampled and rinsed very carefully with PBS twice. For SHPC, 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 Tearor™, 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, MI, USA) and incubated for 7 days at 25 °C. Each dilution series was plated in duplicate. SHPCs numbers were expressed as CFU per cm² after dividing by the surface area of the coupon.

Total direct count using acridine orange staining after detachment of the cells from the supporting material has been attempted on the coupons, but corrosion clumps and



Photograph 1. Sliding coupon holder with biofilm coupons

inorganic matter made microscopic observations really difficult and lead to irrelevant results. Therefore, heterotrophic plate count has been performed on the coupons after detachment of the cells. Past work showed similar difficulties. Servais et al. [22] observed that direct epifluorescence microscopic observations of bacteria fixed on cast iron is impossible and their experiment showed that it was quite impossible to obtain a total detachment of the fixed bacteria by sonication without breaking some of them. Moreover, sonication of cast iron coupons resulted in the production of a lot of small metallic particles, which caused difficulties for microscopic enumeration of bacteria. Below are the references of these articles.

2.4. Confocal laser scanning microscopy

Coupons with intact BO-PRO™-3 stained biofilm were affixed to microscopic slides using Silly Putty™ (Binney and Smith, Inc., Easton, PA, USA) and placed on the stage of an inverted confocal laser scanning microscope on which the biofilm was observed. Using a helium/Neon laser tuned to 543 nm and a long-pass emission filter at 580 nm, serial optical sections through the entire thickness of biofilm at three randomly chosen areas of series were scanned. Data were 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.

2.5. 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 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

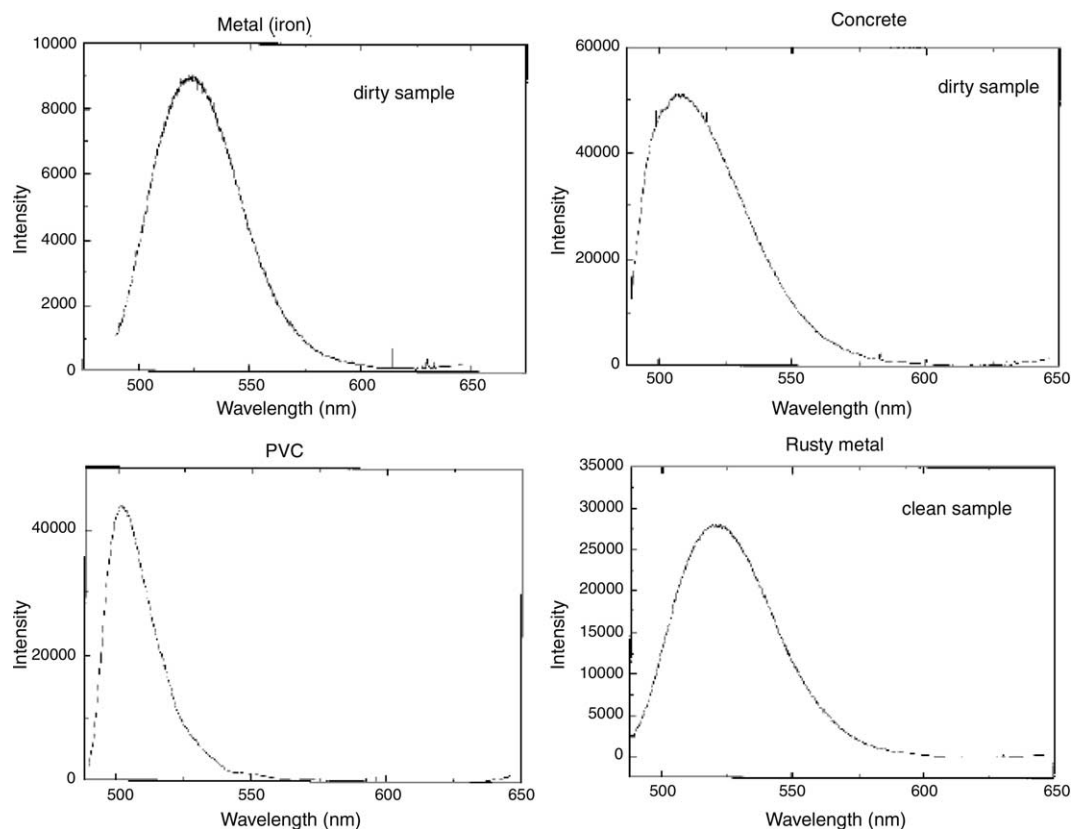


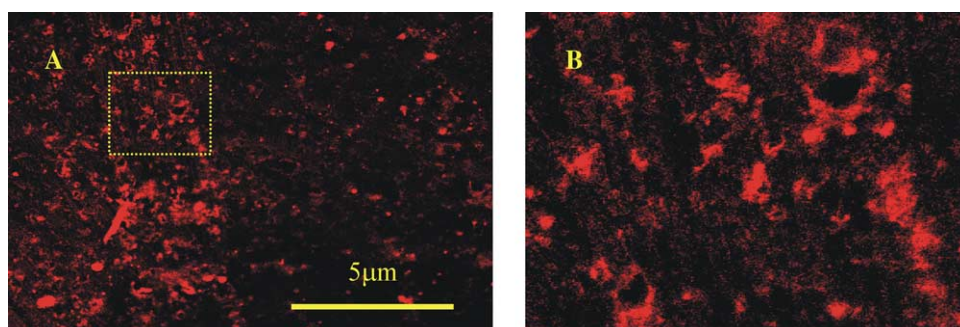
Fig. 1. Spectra of coupon materials.

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, which 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 displayed a histogram of the image with a single sliding marker that was

moved to separate the brightness values, which were displayed as black and white images. The area of the image measurement region selected was reported using the Filter → IP*Measure → Global function.

2.6. Experimental set-up for biological coupons in pilot distribution systems cradles

The University of Central Florida is conducting an American Water Works Research Foundation 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



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 (2x) of biofilm (dotted line in A.).

Table 1
Comparison of SHPC with threshold results—galvanized steel coupons

Sources	Ratio stained area/total area (%)	SHPC ($\times 10^3$ cfu/cm ²)
DI	0.1 (0.06)	13 ^a
Tap water	2.4 (1.14)	24
Waste water (25%)	11.2 (2.45)	125
Waste water (50%)	31.0 (9.93)	933
Waste water (100%)	37.0 (9.26)	1544

The values in parentheses represent the standard deviations for duplicates coupons.

^a Mean value of duplicates.

softening (G3), Blended NF (G4), CSF-O₃-BAC (S1), IMS (CSF-NF or S2) and high pressure RO are blended and distributed to 18 different pilot distribution systems (PDS). The preceding acronyms are defined accordingly:

G: ground water; S: surface water; NF: nanofiltration; CSF: coagulation–sedimentation–filtration; BAC: biological 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, Polk County, FL, 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, Hillsborough County, FL, 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 four 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) \times 3 (biofilm assays) \times 2 (aged and pristine) \times 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 were placed upstream and aged pipe coupons downstream so that corrosion products from the aged material were not transported onto the pristine coupons. Each PDS had a cradle consisting of 4 in. diameter PVC pipe housing and a sliding 3 in. diameter half PVC pipe sliding coupon holder. The length of the cradles was approximately 12 ft for the single material pipe distribution systems. The old and pristine pipe coupons were attached to sliding pipe coupon holders as presented in Photograph 1. These pipe coupon holders were 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.

Some of the experiments were conducted on pure PDS coupons. In addition some experiments were conducted with coupons which 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.

3. Results and discussion

The stain selected for direct cell counts for intact biofilms was BO-PRO™-3 from Molecular Probes, Inc. (Eugene, OR, USA). The probe was selected by first conducting Fluorescence spectroscopy on coupons of each material to determine the wavelength for background fluorescence (Fig. 1).

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-PRO™-3, was identified with emission intensity maximum at 599 nm (red). This probe had an emission intensity that was 200 times greater when it was bound to DNA (Photograph 2). The BO-PRO™-3 probe had very low emission intensity unless it was bound to DNA. So far, this seems to have greatly reduced non-specific staining and background problems.

The stained coupon was immobilized on a glass slide using Silly Putty™ (Binney and Smith, Inc., Easton, PA, USA). Under the CLSM, the stain allowed the biological

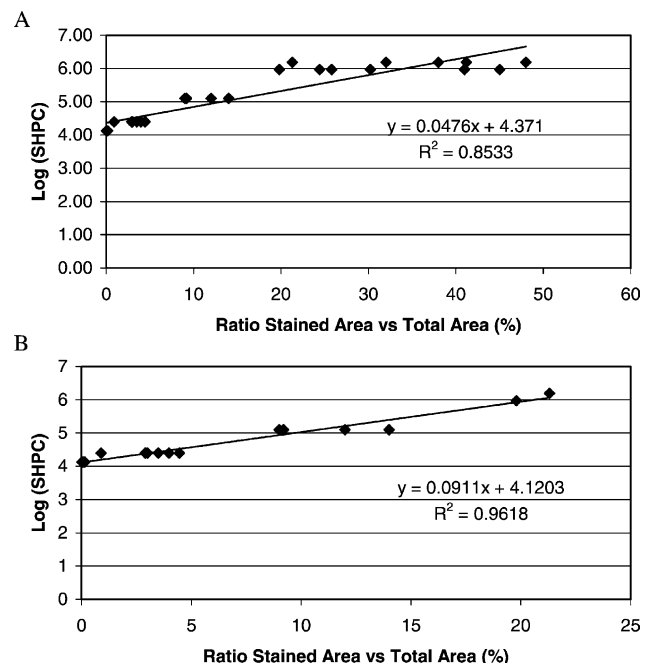


Fig. 2. (A) Correlation log SHPC vs stained area fraction—galvanized steel coupons. (B) Correlation log SHPC vs stained area fraction—for results below 20% only.

Table 2
Comparison of SHPC with threshold results—pilot system coupons

Coupons	Sampling date	Ratio stained area/ total area (%)	SHPC ($\times 10^3$ cfu/cm ²)
p-lined cast iron	6/06/02	1.72 (0.95)	69 ^a
p-PVC	6/06/02	0.47 (0.22)	3.1
p-galvanized steel	6/06/02	10.00 (1.71)	124
Aged-lined cast iron	6/06/02	6.23 (2.09)	122
Aged-PVC	6/06/02	0.48 (0.21)	8.7
Aged-galvanized steel	6/06/02	10.93 (3.52)	131
p-cast iron	8/22/02	36.43 (13.84)	3100
p-lined cast iron	8/22/02	14.51 (6.19)	676
p-PVC	8/22/02	1.23 (0.51)	29.3
p-galvanized steel	8/22/02	18.44 (3.22)	230
Aged-cast iron	8/22/02	19.65 (4.45)	552
Aged-lined cast iron	8/22/02	26.98 (11.51)	3700
Aged-PVC	8/22/02	1.76 (0.23)	73.8
Aged-galvanized steel	8/22/02	25.16 (4.72)	2500

p: pristine; The values in parentheses represent the standard deviations for duplicates coupons.

^a Mean value of duplicates.

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 (10%, w/v), which was added to a final concentration of 125 mg/l. Then BO-PRO™-3 staining was performed with pristine galvanized coupons (no biofilm) and incubated pristine galvanized coupons (with biofilm). Control pristine galvanized coupons did not 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, SHPC was used for bacteriological enumeration and assessed for correlations with BO-PRO™-3 enumeration combined with the threshold technique (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 at 100, 50, 25%). After observation under CLSM, three representative pictures of the attached biomass were taken. The stained

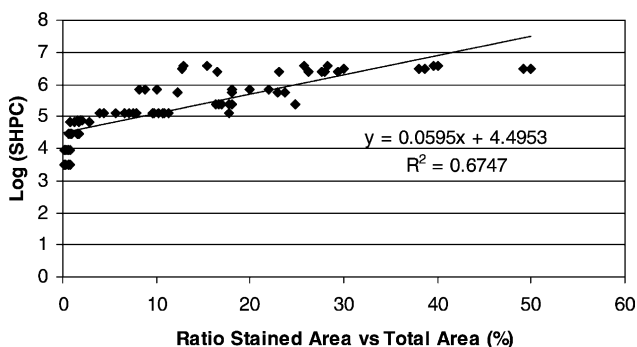


Fig. 3. Correlation log SHPC vs stained area fraction—PDSs coupons.

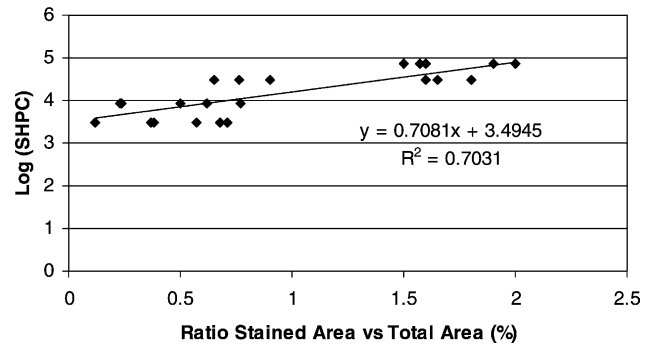


Fig. 4. Correlation log SHPC vs stained area fraction—PDSs PVC coupons.

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 and SHPC increased in proportion to the biofilm volume (Table 1). A significant exponential relationship was found between stained surface area/total area ratio and fixed biofilm inventory measurements from scraping heterotrophic plate counts (SHPC) on R2A medium with $R^2 = 0.85$ (Fig. 2(A)). The method gives strong correlations when SHPC is below 1.5×10^6 cfu/ml with $R^2 = 0.98$ (Fig. 2(B)).

Coupons cut from the actual pipe distribution system (lined-cast iron, PVC, cast iron and galvanized steel) of Tampa Bay Water network (Photograph 1) were used to see if the trends were similar to the results of the preliminary bench-scale study (Table 1). These coupons were exposed to drinking water. However, cast iron coupons results are not included in much of the analysis in this article due to the fact that only a few data points have been collected for that material, which did not permit meaningful comparisons.

Two sets of coupons were incubated in the pure PDSs from 3/14/02 to 6/06/02 (first set) and from 6/14/02 to 8/22/02 (second set) and then were harvested to measure biofilm on their surface (Table 2). PVC had a lower biomass inventory than the other materials. It is interesting to notice that this trend was similar for both pristine and aged materials (Table 2).

SHPC (analyzed on duplicate coupons) was compared to the stained surface fraction (Table 2) to confirm the trends

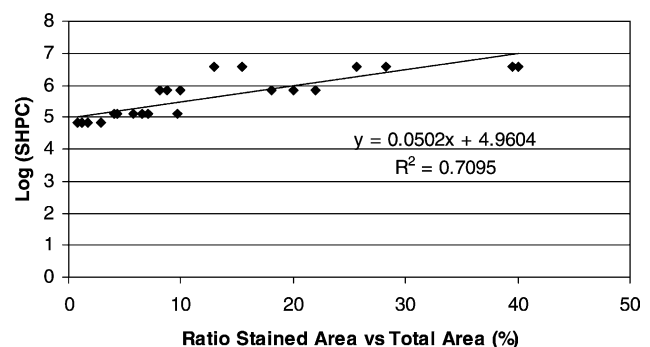


Fig. 5. Correlation log SHPC vs stained area fraction—PDSs lined cast iron coupons.

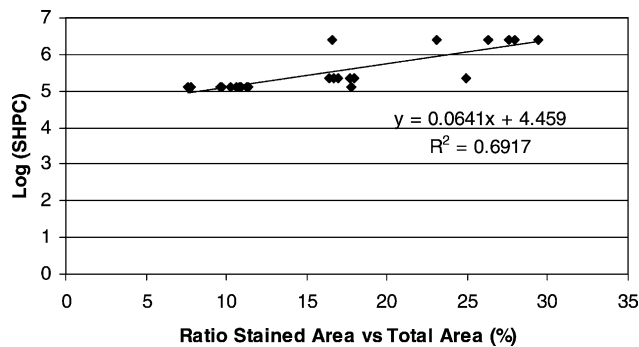


Fig. 6. Correlation log SHPC vs stained area fraction—PDSs galvanized steel coupons.

observed in the preliminary study (Table 1). For BO-PRO™ - 3 staining, two coupons of each material were analyzed. For image analysis, thresholds for three representative images of the stained surface from each of the duplicate coupons were determined. The stained area and SHPC increased in proportion to the biofilm density (Table 2). In addition, the results show important linear correlations between Log SHPC and stained surface area/total area ratio. A fair correlation ($R^2 = 0.67$) is obtained from the results combining all the materials, i.e. PVC, lined cast iron and galvanized steel (Fig. 3). Figs. 4–6 present results for single materials, i.e. PVC, lined cast iron and galvanized steel coupons, respectively. The correlations obtained from these results are significant ($R^2 = 0.70, 0.71$ and 0.69 , respectively). However, the difference between the slopes obtained for each of those correlations ($0.71, 0.05$ and 0.06 , respectively), implies that the supporting materials might have an effect on the validity of the technique. Previous observations have already shown the impact of the pipe materials on biofilm density [2,23,24]. The results for the pilot experiment are not correlated as well as the batch experiment. One of the causes of this may be radically different experimental condition between the two experiments. Indeed, the longer incubation time (3 months opposed to 1 week) and different hydraulic conditions in the pilot system lead to radical changes in the structure and distribution of the biofilm on the coupons. Furthermore, it can be noted that standard deviations were high for all the materials under study. This high variability can be explained by the choice that has been made to take only three representative pictures of the biofilm and by the discontinuity and the heterogeneity of the biofilm structure that leads to a non-uniform dispersion at the surface of the materials in contact with water [25]. Taking more pictures would decrease the variability but it would be contradictory with one of the main purposes of this technique, which is to be an easy and rapid method for quantification of fixed biomass.

4. Conclusions

The proposed method has shown utility in relative comparisons between coupons yielding trends comparable

to the traditional SHPC method. The method gives strong correlations when SHPC is below 1.5×10^6 cfu/ml. This technique is really promising since it is a rapid, inexpensive (when a confocal laser scanning microscope is already set-up in the facility) way to quantify fixed biomass without disrupting the biofilm. This method can also be used when inhibitory compounds or compounds that attenuate bacterial ability to be cultivated are present.

At this time, this technique is being adjusted to epifluorescence microscopy instead of CLSM in order to make this technique inexpensive by lowering the capital cost of the equipment and to allow its use directly on site.

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