Colonization, Cultivation and Visualization of Bacteria on Topographic Surfaces under Zero-Shear Conditions

Laura Sullivan-Green, Charles H. Dowding, Martina Hausner

Abstract*— This research established visualization and growth methods that were initially developed for smooth, nonporous materials for application to rough, porous materials for the purpose of evaluating sparse biofilms. The basic concept of using fluorochromes and confocal laser scanning microscopy (CLSM) to visualize and quantify biota established using smooth surfaces has been extended to topographic surfaces by using additional analyses described herein. The study established that rough, opaque, porous surfaces like mortar colonized with biota can be visualized using CLSM and topographic relief of rough surfaces can be identified by collecting light reflected off the material surface. Volumes of biomass per unit area of surface can be established using optical sectioning to generate image stacks and simple stochastic modeling. Thus, growth accumulation on rough surfaces can be visualized and measured.

***Index Terms*— Confocal Laser Scanning Microscopy, Crack Dating, Forensic Engineering, Infrastructure Health**

I. INTRODUCTION

Microbiological dating of a crack's age depends upon establishing a viable, repeatable protocol for visualizing and quantifying biomass from all types of construction surfaces. Determination of crack age is an important forensic issue. Each year millions of dollars are spent on arbitration and litigation arising from damage claimed to have been produced by some recent adjacent construction activity or some recent natural phenomenon. A protocol such as this can be used to estimate the time crack surfaces have been exposed to the natural environment. With the ability to determine a timeline of cracking, a link between the alleged damage and potential causation may be established. This type of forensic technique can mitigate excessive litigation associated with damage claims due to construction activities, such as blasting, pile driving or use of heavy equipment or natural phenomena, such as earthquakes or hurricanes [1].

Before the biomass accumulation method of dating can be utilized in the field, several principles must be verified in the laboratory, namely the development and cultivation of sparse zero-shear biofilms, verification of visualization of biota on representative building surfaces and verification of ability to quantify one-dimensional growth over time in the laboratory. The work presented herein focuses on colonization and visualization of sparse biofilms on a rough, porous material.

Methods were developed to sparsely colonize surfaces, cultivate these biota under zero-shear feeding conditions and visualize and quantify sparse biomass accumulation under semi-optimal conditions [2]. Semi-optimal conditions are used to bridge the gap between optimal laboratory conditions (frequent, large quantity of fresh nutrients) and oligotrophic conditions (nutrient sources severely limited and/or provided intermittently). The semi-optimum conditions were maintained so that the growth was scaled from long term (months, in many cases) to days in the laboratory. The technique described herein does not precisely mimic natural conditions because of the desire for sustained biofilms and the need to utilize liquid cultures and nutrients to achieve sustainability. The technique does provide a controllable technique to establish sparse biofilms, provide limited nutrition and, ultimately, develop methods to visualize and quantify the sparse biofilms directly on the deposition surfaces of interest.

Confocal laser scanning microscopy (CLSM) was chosen for visualization and quantification of these sparse biofilms because it enables 3-dimensional (3D) visualization of biota directly on an accumulation surface which may be opaque and have an irregular topography. Because confocal microscopy enables examination of optical sections within thick specimens, 3D analysis of biofilms is possible by eliminating the out-of-focus signals. The sparse biofilms generated in this study were thin, often only one microorganism thick (1-2μm), but the surfaces of mortar samples have significant relief on such a scale, requiring the ability to take stacks of images at regular, small intervals to fully characterize the surface and the biofilm deposition on it. Examination of the biota directly on the accumulation surface allows for examination of the in-situ distribution of the biofilm, which can yield additional information about the structure of such dispersed, nutrient-starved biofilms [3]. To visualize biomass using CLSM, acridine orange stain was used. Acridine orange is a nucleic acid stain that interacts with both DNA and RNA. Acridine orange was found to be suitable for this type of work because of its strong, fade-resistant signal and its ability to be detectedover the signal from autofluorescing mortar.

Confocal microscopy can also be used to characterize the surface of a rough, opaque material, such as that of mortar

Manuscript received September 16, 2013.

Laura Sullivan-Green, Department of Civil and Environmental Engineering, San Jose State University, San Jose, CA, USA

Charles H. Dowding, Department of Civil and Environmental Engineering, Northwestern University, Evanston, IL, USA

Martina Hausner, Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada

[4][5]. By collecting reflections of white light (an accumulation of wavelengths from approximately 400 nm to 700 nm) off the examination surface, a topographic map of the surface can be developed by calculating the relief of the stack of images over the analysis area at the same time the fluorescence is collected.

II. MATERIALS AND METHODS

A. *Mortar as a Testing Surface*

Mortar was selected as a testing surface because of its rough surface and porous nature. These properties were felt to be some of the most challenging that could be encountered in a natural accumulation analysis. Thus the methods of visualization and quantification developed for smooth, non-porous surfaces had to be tested and verified as sufficient for these additional material properties [2]. The maximum topographic relief of mortar in this study was determined to be approximately 2 mm, or half of the diameter of the largest grain size of the concrete sand. If the same analogy is applied to concrete, where coarse aggregate is used, the maximum relief for concrete would be in the 12.5 mm range. A comparison of the relative roughness of mortar and concrete with cement paste and glass is shown in Fig. 1. While studies of a material with such a topographic relief of concrete are feasible, they are very time consuming and, in this case, would not provide additional information beyond that of the mortar samples. In the interest of time management, economy, and information yield, mortar was selected as the final testing material.

Use of a cementitious material for these studies raised concern with the ability to colonize an alkaline surface. Cementitious materials are known to have pH levels ranging from 11-14 [6]. To determine if the pH causes a lag in colonization, a pH study was performed on freshly poured cement paste samples. Two cement paste mixes were poured and moist cured for 7 days. After the moist cure, the samples were exposed to the natural environment and the pH was measured every day using phenolphthalein and universal indicator. Phenolphthalein measures pH by a single color change. High pH surfaces turn a bright magenta, while surfaces with pH less than 9 are clear. A pH less than 9 is a critical threshold in the study of concrete and cementitious materials because it indicates that the cement matrix has completely neutralized through a chemical process known as carbonation. Studies have shown that more precise methods of pH measurement in cementitious materials are no more precise than those obtained using phenolphthalein [7].

Universal indicator has color gradations varying from purple to green, where purple is high pH and green is neutral pH (less than 8). The universal indicator was used to evaluate the degree of change over the time and was employed as a means to provide trend data and verify the time of neutralization obtained with phenolphthalein. Results of the pH measurements for both cement mixes are shown in Fig. 2. This fig. shows the surface of the cementitious materials neutralize within days of being freely exposed. The expected accuracy of the biomass accumulation method is on the order of weeks to months, which is far greater than the several days necessary to make the surface an appropriate colonization site.

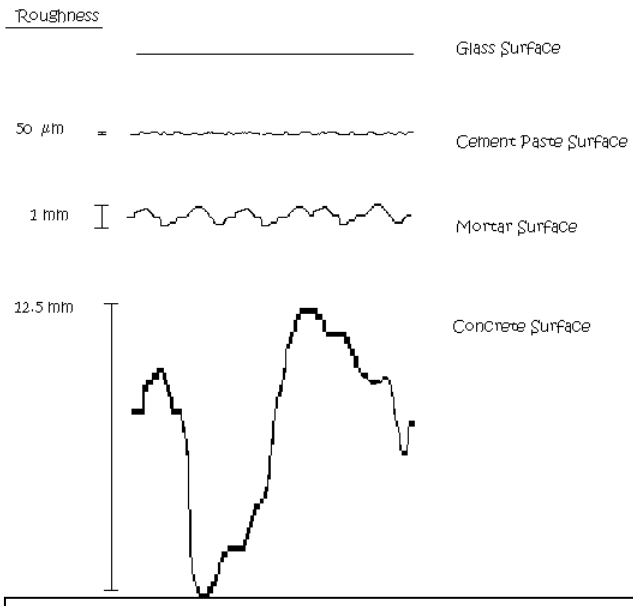

Fig. 1. Illustrative comparison of elevation cross-section for various materials. Roughness was estimated by determining the maximum particle diameter and dividing by 2.

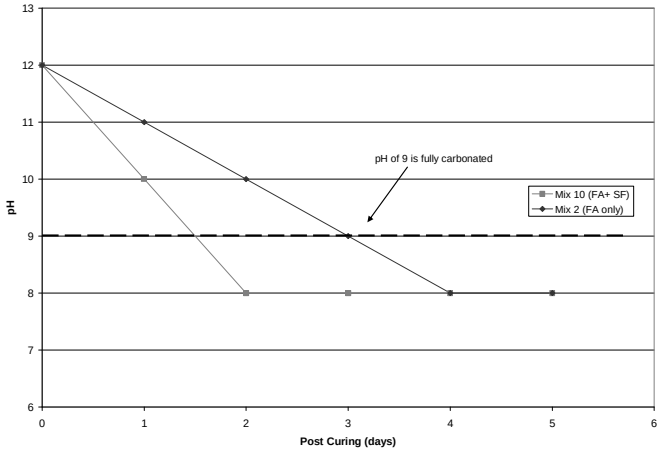

Fig. 2. The mortar surface fully carbonates (a pH less than 9) quickly when exposed to air, thus not inducing a significant lag between exposure and neutrality.

B. *Sparse Biofilm Development*

As discussed in previous publications [1][2], sparse biofilm colonization requires consideration of cell density, coverage consistency and repeatability. As in studies conducted on glass samples, *Pseudomonas putida*, a common soil-inhabiting bacterium, was chosen as the representative microorganism. *P.putida* was ideal for this work because of its relative safety in an aerosol form, its similar behavior to other species in the *Pseudomonas* family and its capability to form biofilms [8]. Because *P.putida* can be found in locations of fluctuating nutrient availability, like soil, laboratory conditions were developed to make the nutrient provisions small relative to its some of the more typical nutrient-rich environments.

Preparation for a *P.putida* liquid culture followed standard microbiological laboratory methods [9] to ensure a pure

culture at the desired cell concentration and consistency. A stock culture was obtained [10] and plated on nutrient agar (Nutrient Broth and Agar 15g/L, Difco Laboratories, Detroit, MI). One colony was removed with an inoculation loop and placed in 100mL of a full concentration nutrient broth solution. The liquid culture was incubated at 100rpm and 28°C for 24 hours. A small sample of the full concentration liquid culture was taken for serial dilution and plate count quantification [11]. The full concentration liquid culture was diluted to 5% of the original concentration. Again, a small sample was taken for serial dilution and plate count quantification to verify the dilution to 5%.

Once the 5% culture was prepared, it was transferred to a calibrated sterile plastic spray bottle. Several bottles were calibrated for consistent spray volume and coverage area [2]. Samples were placed in a container for the spraying process so that surrounding areas wouldn't be contaminated and to reduce the risk of inhaling the aerosolized culture.

Cement mortar (Portland Type I/II cement, concrete sand, w/b = 0.3, 30% cement replacement with fly ash) was chosen to represent porous, rough materials for the purpose of this study. Mortar was mixed and poured into bar molds 0.64 cm x 2.5 cm x 15.2 cm (0.25 in x 1 in x 6 in). After 28 days of curing, the samples were placed in a glass bottle of water to maintain saturation and autoclaved for sterilization. The bars were broken into smaller chips, with a maximum dimension no larger than 1 cm. In order to maintain the biota on the surface for this study during colonization and feeding, capillary forces from the drying process of the mortar needed to be negated. Mortar chips were placed in a water bath so that capillary forces maintained saturation throughout the sample during incubation. Maintaining a saturated sample prevented the liquid culture and nutrient broth from being suctioned into the sample before the culture could colonize the surface. Fig. 3 shows several mortar chips in a water bath. This suctioning force is believed to be the result of the need to use liquid cultures for colonization in the laboratory for the purpose of visualization and quantification. Its potential effects on environmental samples will be addressed in field studies to be conducted.

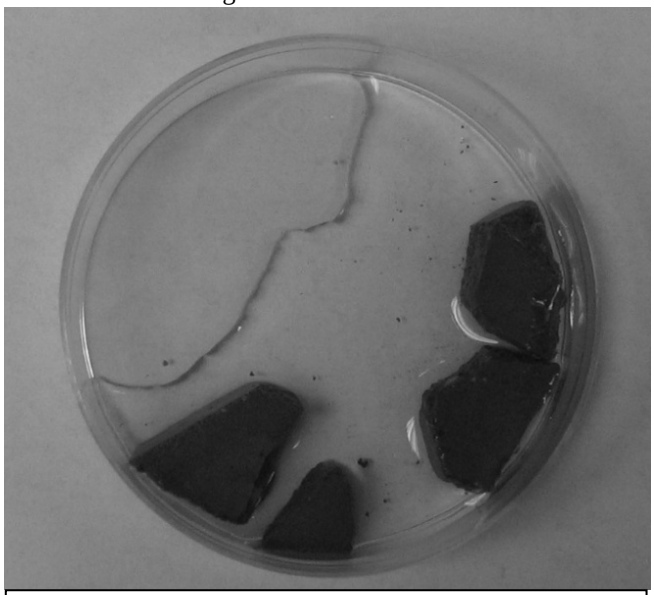

Fig. 3. Mortar chips were incubated in a sterile water bath to maintain saturation of the sample, thus preventing loss of the culture and nutrients through negative capillarity (suction) into the sample.

1) Controllable Acceleration of Growth

Once samples were sprayed with the inoculum, they were prepared for incubation. Samples were first fed with a diluted nutrient broth solution. The nutrient broth was diluted to avoid excessive deposition of concentrated nutrients on the sample surfaces as the fluid evaporated off the surface. A dilution of 50% below the manufacturer's recommendations (7.5 g/L instead of 15 g/L) was sufficient to provide reduced nutrient conditions, simulating those that may be found in a soil environment. The feeding process developed for this research needed to satisfy two criteria: maintain growth and hydration without generating shear forces and provide only limited nutrition to more closely simulate conditions encountered on construction materials. Samples were fed on a daily basis by pipetting diluted broth onto the sample surface with each mortar chip receiving 50 µL per day. This feeding schedule reduced the nutrient quantity within the system by a factor of 24 compared to the nutrients within a flowcell system [2]. More complex comparison of nutrient quantity used by the biofilm within the system is beyond the current scope of this work. After feeding, the samples were sealed in a petri plate to maintain humid conditions and prevent dehydration of the biofilm even if all the broth evaporated off the sample surfaces. Samples were incubated at room temperature (20-22°C), slightly below the optimal temperature of 26°C for *P.putida.*

2) Sample Collection and Preparation for Visualization

Samples were collected at 24-hour intervals to evaluate increasing biomass coverage over time. Samples were collected in 24-hour intervals beginning at the time of incubation. After collection each sample was subjected to a heat-fixation procedure (10 min at 46°C) to aid in attaching the biofilm to the colonization surface and in improving stain receptivity. After heat fixation, samples were subjected to an additional fixation step. Samples were fixed by immersing in a solution of 50% ethanol and 50% Phosphate Buffered Saline (PBS) for one minute to dehydrate the biofilm, and then air dried. Often, structural collapse of biofilms architecture is a concern when dehydrating a biofilm, but it is not a concern for sparse biofilms because growth occurs mostly as a single-cell layer along the material surface, with little growth in the z-direction.

3) Microscopy and Image Processing

After fixation, samples were stored in a sterile water bath in a 4°C refrigerator until analysis with Confocal Laser Scanning Microscopy (CLSM). CLSM was used to determine the spatial density achieved with spray colonization and verify growth over time with the stationary feeding method. A Zeiss ConfoCor3/510 Meta Confocal system provided excellent control of x, y and z positioning and automated tiling of images to analyze large areas without sacrificing high resolution. While the method of image collection is straightforward, an automated process has been developed [2]

To prepare for CLSM analysis, samples were stained with a fluorochrome, acridine orange, to allow visualization of the biota over the autofluorescence of mortar surfaces. Several fluorochromes were compared, but acridine orange (1 mg/mL, Molecular Probes, #A3568) was found to be the most

suited for these types of analyses. Acridine orange proved to be a robust fluorescent stain that provided a strong, fade-resistant signal visible over the autofluorescence of the mortar.

Samples stained with acridine orange were excited by a wavelength of 488 nm. Emissions with wavelengths between 522 nm and 661 nm were collected to visualize the biota. Since mortar samples have significant topographic relief, the location of the surface needed to be identified in relation to the deposited biota to determine if a link exists between colony location and location within the valleys and hills of the surface. The surface and the biota could be scanned simultaneously with two channel images, where the fluorescence was collected with the fluorescence channel and the mortar surface was located by reflecting the same 488 nm laser and collecting all visible wavelengths reflected off the surface. For clarity on images, the signal from the fluorescent biota was given an orange pseudo-color and the signal from material surface was given a white pseudo-color. Images were collected with a 63x magnification to accurately distinguish the biota signal from the autofluorescence signal of the mortar and provide better observation of the surface location. Stack images collected from mortar samples were single location images from the 63x objective, which yielded an analysis area of approximately 20,400 μm^2 (142.9 µm x 142.9 µm). The 3D mortar stacks were generated by collecting 2D images at 1 µm intervals for either 14 or 19 µm (15 or 20 images), as shown in Fig. 4. The stacks yielded analysis volumes of either 285,880 μm^3 or 387,987 μm^3.

Images containing only the fluorescence signal were processed using ImageJ, a public domain Java image processing software (available at http://rsb.info.nih.gov/ij/). A threshold value of 100 was determined to be suitable to distinguish biota based on the 8-bit images collected in this work. Pixels with threshold intensities greater than 100 and less than the maximum 255 (2^8-1= 255) were measured as 'on' and the percentage of the 'on' pixels over all available pixels yielded a percentage of the image area that was occupied by biota. Cell volumes were interpolated using the 2D area covered on each image of a stack and multiplying it by the height of the optical section that the image represents. The optical section thickness of 0.9 µm is approximately equal to the thickness of a single cell of *P.*putida. The general linear equation is:

$$V = h (A_1 + A_2 + A_3 + \ldots A_{n-1} + A_n) \qquad (1)$$

Where V is the interpolated volume
h is the height of the optical section
A_n is the area coverage for each image

The concept of 3D volume approximation is illustrated in Fig. 5. Through this interpolation, the total cell volume is then determined by summing the interpolated volumes for each image pair as measured throughout the entire stack.

III. RESULTS

Quantification of biomass accumulated on a surface can be evaluated qualitatively and quantitatively with the use of microscope images obtained from the CLSM. Qualitative analysis is useful to verify and define growth trends and

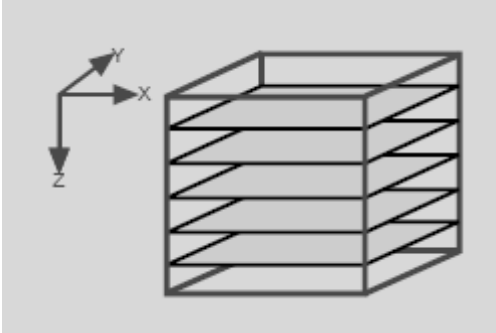

Fig. 4. Stacking enables a 3D view with 2D imaging. Images are taken at specified z-intervals throughout the depth of the sample.

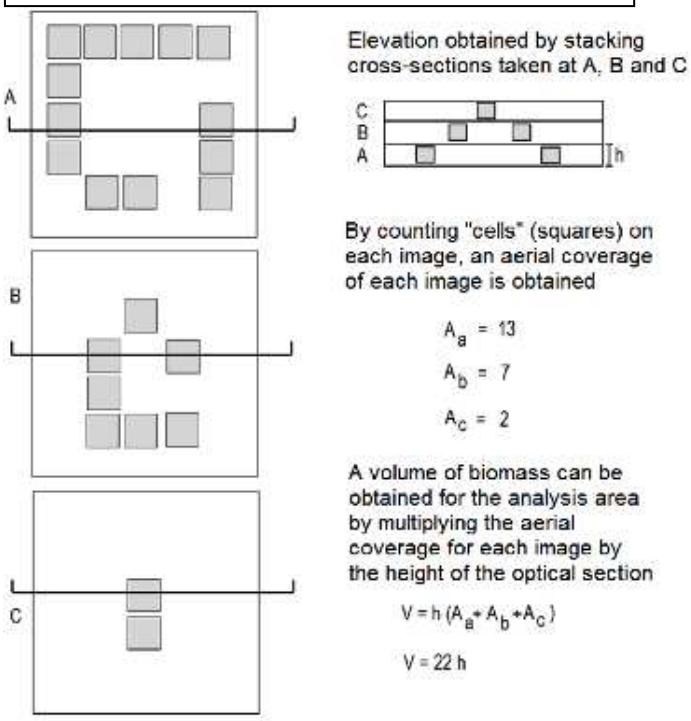

Fig. 5. Interpolation of 3D volume from 2D images. The conical hill of biomass is described by the 2D images shown on the left taken at equal intervals of depth. Taking a cross-section of each image (A-C) and stacking the elevations gives a complete cross-section of the conical hill of biomass at that location. Using this information, trapezoidal interpolation between the images provides a volume measurement of biomass within the analysis area.

colony dispersion [1]. Qualitative analyses of accumulation on mortar required additional imaging than those on smooth surfaces. The relief of the mortar surface required a stack of images be collected to accurately follow the surface, and thus the biota colonizing the surface. Images were collected at 1 µm intervals through the relief in the particular field. Most stacks were either 15 or 20 images thick, i.e. 14 or 19 µm. Fig. 6 presents a stack series for a mortar sample. These images begin at the top elevation in the field and progress down to the lowest elevation in the field, with biota following the elevation of the surface (not shown in grayscale images).

The stack of images can be used to identify the surface location by viewing the 'movement' of the reflected light signal through the stack. Fig. 6 presents both

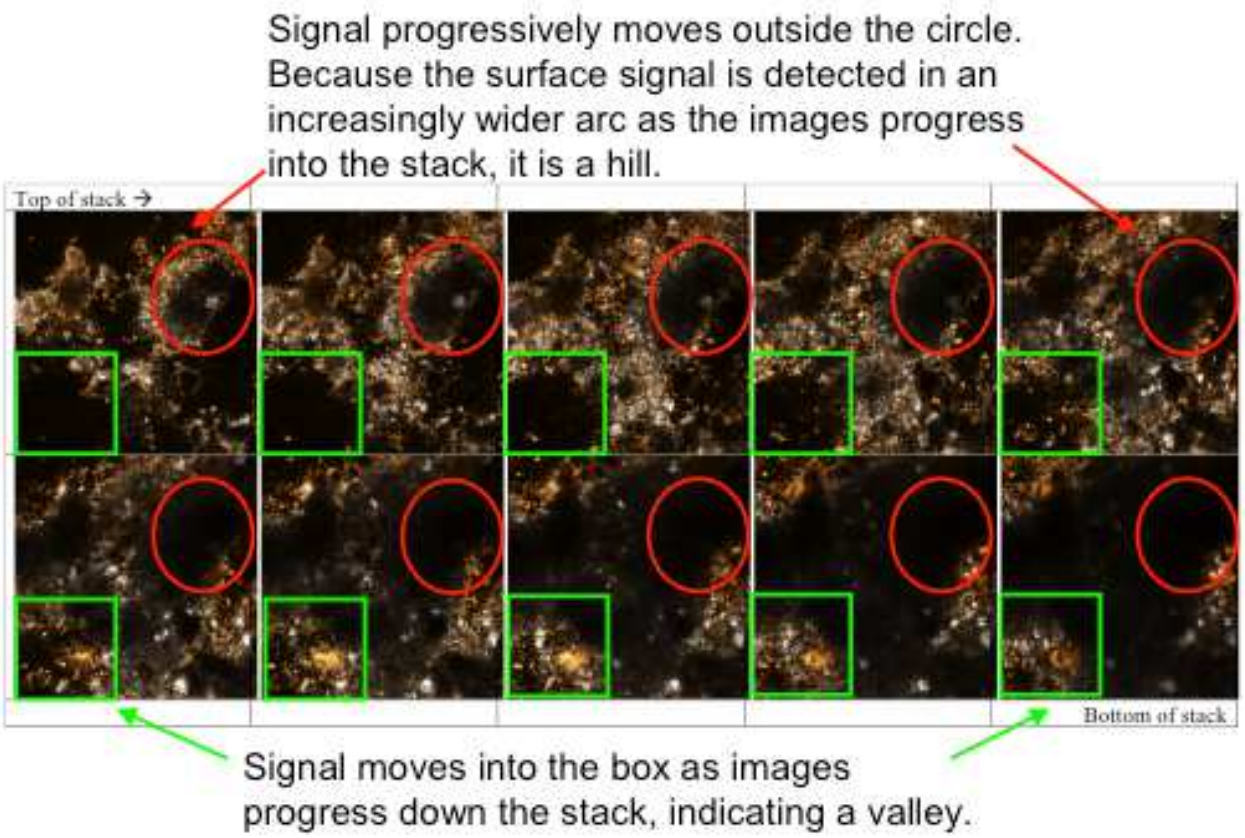

Figure 6. A series of images taken on a rough mortar surface. These composite images show both the white light channel (white) and the fluorescence channel (orange). Red circles show the signal moving in an outward direction through the depth of the stack, indicating a hill, while the signal in the green box moves inward, indicating a valley.

a hill and a valley within the image. The circles are used to identify the location of a hill. Each circle is the same size and placed at the same location in each image. Examination of the signal within the circles demonstrates that the signal progressively moves from the center of the circle outward, indicating the surface location spreads as the stack progresses. The boxes indicate the location of a valley on the surface. Again, all the boxes are the same size and at the same location within the image. This time the signal is absent inside the box at the top of the stack and the signal progressively moves into the center of the box, indicating the surface is converging into the center of the box. This surface convergence indicates that a valley is present.

Defining the material surface topography for rough materials is necessary to define biomass location with respect to the material surface. The reflected light signal is employed to create a topographic map of the area to define the relief on a single 2D image. Topographic maps of several mortar samples are presented in Figs. 7 and 8. These topographic maps utilize color to define the relief of the surface rather than contour lines. Each map uses blue and green color hues to represent areas with little or no relief. Yellows and reds are used to define areas of higher relief. The relief scale for each image is based on the stack thickness. For instance, Fig. 7 has a stack thickness of 9 μm and the color variation is based on that 9 μm, while Fig. 8 has a stack thickness of 39 μm, thus the color variation is based on that thickness. The default scaling of the topography function based on stack thickness within the

Several other features can be identified with these topographic maps. First, the topographic map shown in Fig. 7 is created from the same stack series presented in Fig. 6. The hill defined by the red circles in Fig. 6 is represented on the topographic map by the red color, as identified by the black arrow on Fig. 7. The valley defined in Fig. 6 by the green boxes is represented in the topographic map by the blue and green shades in the lower left portion of Fig. 7, identified by the white arrow. These maps support the visual analysis and interpretation described above to define the topography of the mortar surface using the image stack.

Another feature that can be identified by topographic maps is material edges. Another topographic map shown in Fig. 8 was taken at the edge of a mortar sample. The edge of the sample is marked at the image edges by the two arrows on the top and right boundary of the map. This void is defined as having no relief on the topographic map because it is assigned blue shades. This area could be interpreted as a smooth portion of the sample on the topographic map, so the material location must be verified. The absence of the material is verified by examining the stack of images and confirming a lack of reflected signal in the area.

The topography of a cross-section of an image stack can be developed by utilizing the DepthCod tool in the Zeiss image software. This tool shows elevation views along any selected location parallel to the x- or y-axes [labeled A and B in Fig. 9b]. Fig. 9 demonstrates the effectiveness of elevation views to determine hills and valleys within an image stack. In Fig. 9a the image is

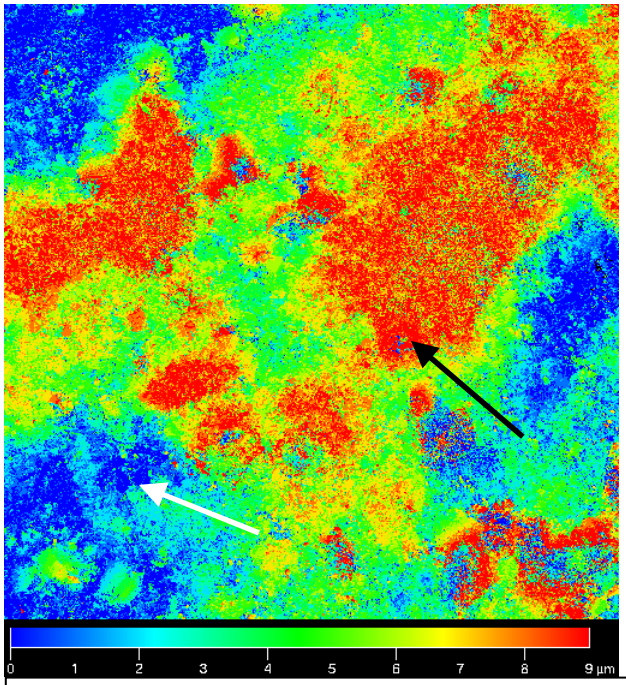

Figure 7. Topographic map for a 3D image stack. The individual images presented in Figure 6 can be used to generate a topographic map to interpret the relief of the material surface. The light arrow identifies the valley and the dark arrow identifies the hill.

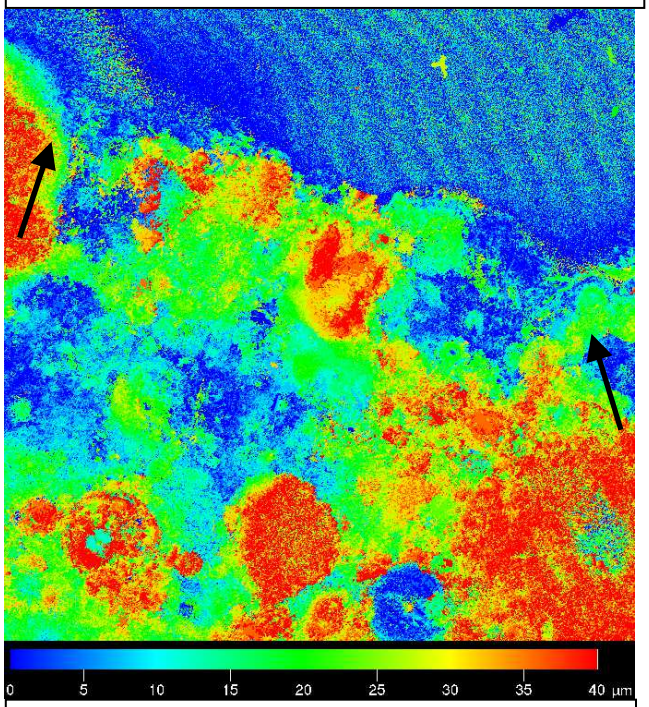

Figure 8. Topographic map taken on an edge of the sample. Void space is characterized as having no relief, as seen in the upper portion of the image, bounded by the arrows.

presented. The black areas of the image are not distinguishable as hills or valleys with one single image of the stack. If elevation views are generated through these black areas [the two rectangles shown in Fig. 9b along the top and right side of the image], it becomes easy to distinguish between hills and valleys. Arrows on each image identify the black areas that are undetermined in Fig. 9a and their corresponding location on the elevation views in Fig. 9b. The topographic map of the image stack [Fig. 9c] is also presented, supporting the identification of certain locations as hills and others as valleys.

Fig. 9 demonstrates results obtained from applying the DepthCod tool. The A-A line parallel to the x-axis correlates to the elevation view at the top of the image. The B-B line parallel to the y-axis thus correlates to the elevation view parallel to that same axis on the right side of the image. Note that the blue line in each elevation view is at the same depth within the elevation, consistent with the image displayed in the main window. Elevation locations were chosen to highlight hill and valley features. The light colored arrows of Fig. 9 identify the hill within the image, as seen in the elevation view at the top of Fig. 9b, and the dark colored arrows of Fig. 9 identify the valley shown in the elevation view on the right side of Fig. 9b.

After the material surface is located, the biota can be located with respect to the surface. Biota location is determined in two ways. First, relative location of the biota to the mortar surface can be seen by examining the progressive images in a stack. Because of the high magnification with the CLSM, the biota tended to be seen slightly above the surface. This 'floating' of biomass is evident in the images by seeing the fluorescence signal (biomass) on one image and the reflected light signal (surface) at the same location on the next image in the stack shown in Fig. 6. Elevation views as described in Fig. 9b show the distribution of the biomass along the entire surface. The biota distribution on these images is identified by noting the position of the fluorescence signal (orange) on the elevation view over the reflected light signal (white). From these elevation views in Fig. 9b and the progression of images in Fig. 6, it can be seen that biota colonizing the rough mortar surfaces did not appear to prefer any particular portion of the surface. Colonization was seen along the ridges and valleys equally, both at the time of colonization and after incubation for several days.

Despite the increased effort required to visualize the topography of the surface and the biota on that surface, quantification methods for volumes of biomass can still be relatively simple. Quantification methods of the biota are consistent with the 2D images collected in a stack than those for a single image. The coverage of each image is determined with image processing software based on pixel intensity and threshold range [2].

To extend these 2D analyses to a 3D volume interpretation requires a volumetric interpolation between each 2D image, as illustrated in Figure 5. An image containing only the fluorescence signal (orange) is analyzed for biomass volume because it represents the biomass present on the mortar surface. The fluorescencesignal images are then processed for aerial coverage based on pixel intensity [2]. Once all images in a stack are processed, the coverage values are input into Equation 1 described above.The interpolated volume between each set of images is calculated and all the interpolated volumes are summed, and then divided by the image area. The result is the volume per unit area with dimensions of$\mu m^3 / \mu m^2$, or simplified as µm.

Though growth was not the primary focus of these mortar investigations, it was a secondary consideration to establishing visualization and quantification methods. Through developing the quantification methods on mortar surfaces, some growth patterns were detected. By applying the above visualization and quantification

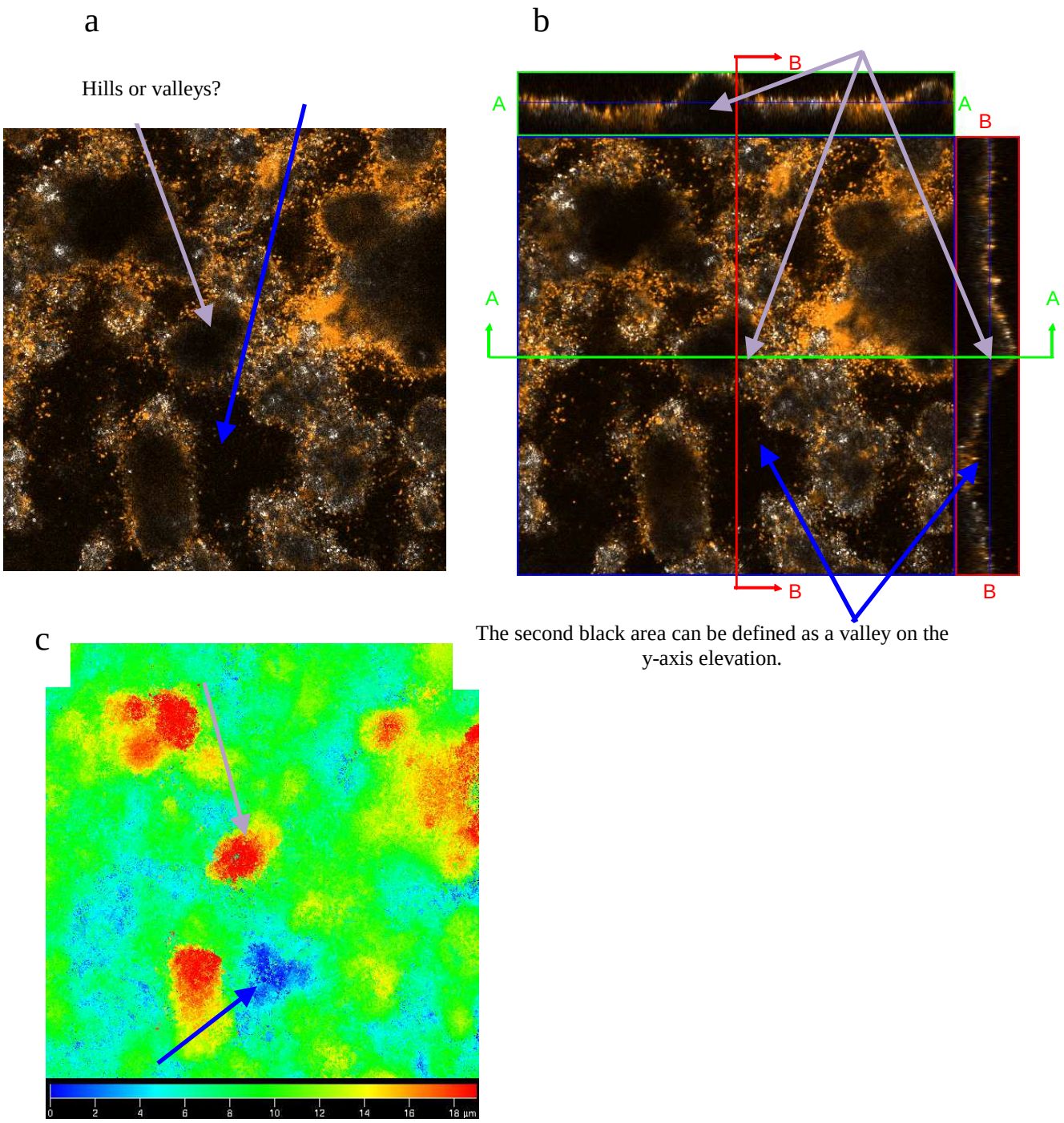

Figure 9. Hills and valleys are indistinguishable on the single image from a stack. Elevation views can be generated through these black areas and each area can be defined as either a hill or a valley. The line A-A parallel to the x-axis correlates to the elevation view at the top of the image. The B-B line parallel to the y-axis thus correlates to the elevation view parallel to that same axis on the right side of the image. The topographic map supports the relief identifications. Light colored arrows identify a hill and the dark colored arrows identify a valley.

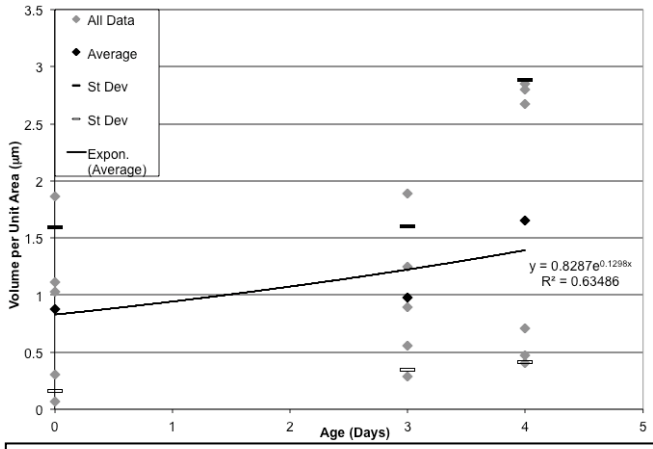

Figure 10. Volume per unit area measurements are effective in measuring biomass quantitatively on rough surfaces. To establish stronger statistical significance, studies of this nature that cover larger areas of the sample need to be conducted.

analyses to a number of samples over a four day period, a growth trend can be verified. Figure 10 shows quantification of biomass volume per unit area over time. The quantification is no longer on a percentage scale like the aerial coverage quantification for 2D analyses. Biomass volumes per unit area are on the order of 1-3 µm over 4 days of growth.

The same exponential best-fit lines applied to 2D analyses on glass [2] were applied to the 3D volumetric data on mortar. Overall quality of data was expected to be lower with the 3D volumetric study than the 2D analyses, for two reasons. First, analysis area for stacks used in these 3D analyses was only 22,350 μm^2 as compared to an analysis area of 2,350,000 μm^2 for a tiled image in 2D [2]. Second, volumes are interpolated from 2D images and, therefore, can have a greater uncertainty associated with each data point. Uncertainty in volumetric measurements can be improved by incorporating tiling with stacked images to increase the analysis area and reducing the z interval at which images are taken to reduce interpolated volume [1].

IV. CONCLUSIONS

This research established visualization and growth methods that were developed for smooth, nonporous materials can be applied to rough, porous materials for the purpose of evaluating sparse biofilms. The basic concept of using fluorochromes and CLSM to visualize and quantify biota established used smooth surfaces, has been extended to topographic surfaces by using additional analyses. The study established that:

- Rough, opaque, porous surfaces like mortar can be colonized with biota. The biota can be cultivated on that surface and be visualized directly on the surface using Confocal Laser Scanning Microscopy (CLSM)
- Topographic relief of rough surfaces can be identified by collecting white light reflected off the material surface. The reflection can be collected at intervals and stacked to obtain a 3-dimensional representation using 2-dimensional images. Cross-sections and topographic maps can be derived from such data.
- Volumes of biomass per unit area of surface can be established with image stacks and simple stochastic modeling.
- Growth accumulation on rough surfaces can be visualized and measured, though additional work needs to be completed to establish statistical significance of the natural growth variation with biofilms.

V. ACKNOWLEDGEMENTS

This research was funded in part by the National Science Foundation through a Small Grant for Exploratory Research. Special thanks to Dr. Bill Russin at the Biological Imaging Facility at Northwestern University for his assistance in image collection and analysis.

VI. REFERENCES.

[1] L. Sullivan-Green (2008) *Quantification of Biomass Accumulation on Building Surfaces through Simulation of Natural Modes of Deposition.* PhD Dissertation. Department of Civil and Environmental Engineering. Northwestern University.

[2] L. Sullivan-Green, C.H. Dowding, M. Haunser and B. Prince (2012) Sparse colonization and cultivation of bacteria on construction surfaces under zero-shear conditions. *International Journal of Applied Science and Technology.* Vol 2, No. 1 January 2012.

[3] H. Daims and M.Wagner (2007) Quantification of uncultured microorganisms by fluorescence microscopy anddigital image analysis. *Applied Microbiology and Biotechnology*, 75, 237-248.

[4] K.E. Kurtis, et al. (2003). Examining cement-based materials by laser scanning confocal microscopy. *Cement and Concrete Composites, 25*, 695-701.

[5] D.A. Lange, H.M. Jennings and S.P. Shah (1993) Analysis of surface roughness using confocal microscopy. *Journal of Material Science, 28*, 3879–84.

[6] S. Mindess and J.F. Young (2002) *Concrete*, 2nd edition, Prentice Hall, Inc., Englewood Cliffs, N.J.

[7] Campbell, D.H. et al. (1991) "Detecting Carbonation", *Concrete Technology Today*, Vol. 12, No. I.

[8] Klausen M. et al. (2006).Dynamics of development and dispersal in sessile microbial communities: examples from Pseudomonas aeruginosa and Pseudomonas putida model biofilms. *FEMS Microbiology Letters, 261*, 1-11.

[9] Madigan, M.T., J.M. Martinko, J.M. and Parker, J. (2003). Brock Biology of Microorganisms. Pearson Education, Inc. Upper Saddle River, NJ.

[10] ATCC.org (2008). "Pseudomonas putida, 12633",. www.ATCC.org (April 26, 2008)

[11] Reasoner, D.J. (2004). Heterotrophic Plate Count Methodology in the Unites States. *International Journal of Food Microbiology, 92*, 307-31.