

## **Sparse colonization and cultivation of bacteria on construction surfaces under zero-shear conditions**

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### **Abstract**

*This paper describes a method to evaluate microbial growth on solid surfaces under stationary feeding conditions and establish visualization and quantification methods on representative construction materials to provide a foundation for biological age dating of crack surfaces in construction materials that are sparsely colonized after exposure to the environment. Cultivating bacterial growth under stationary conditions, coupled to confocal laser scanning microscope techniques which were optimized to visualize accumulation directly on the colonization surface, assists in verifying foundational hypotheses for the expansion of a biomass quantification method for use in determining the age of a crack in construction materials, termed crack dating. Dating cracks can establish a timeline that may allow forensic engineers to link the crack to any atypical events occurring at the same time.*

**Keywords:** Crack Dating, Infrastructure Health, Forensic Engineering, Microbiology, Biofilms, Confocal Laser Scanning Microscopy.

### **1. 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. A protocol such as this can be used to estimate the time crack surfaces have been exposed to the natural environment. Establishing a timeline of exposure can lead to links between the crack and its cause. 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. 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. With the ability to determine a timeline of cracking, a link between the alleged damage and potential causation may be established.

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 development and cultivation of sparse biofilms on two representative building materials: glass and mortar.

Development of techniques for sparse colonization and zero-shear feeding is a necessary first step in the development of biological methods to quantitatively determine the age of exposed construction surfaces. Biological age dating is based upon the hypothesis that the amount of biomass on a given crack surface is an indication of the amount of time that has passed since the surface was exposed and verification of the applicability of basic microbiological methods must be established.

Methods were developed to visualize and quantify sparse biomass accumulation under semi-optimal conditions 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). This research established methods to develop sparse, 1-dimensional biofilms in the laboratory, cultivate organisms on surfaces in zero shear conditions, and visualize and quantify the biomass directly on the surface of deposition. These conditions of sparse nutrients require special experimental approaches to faithfully reproduce the growth environment, yet at the same time it is necessary to accelerate the growth in order to reduce the period of observation from natural months or years to laboratory days and also provide a pattern of growth to verify the visualization and quantification methods for the materials.

Competition results from two competing goals, replication of the nutrient-poor, zero-shear growth environment and acceleration of a patterned growth, were overcome through controlled provision of hydration and nutrients under stationary conditions. This approach departs from the traditional use of flow cells for incubation, which also provides large quantities of nutrients, continuous hydration and induces shear stresses on the accumulated biomass. Because of the limited growth at solid-air interfaces and the lack of shear stresses on construction surfaces, it was necessary to modify typical laboratory methods to mimic the zero shear conditions while still providing sufficient nutrients to maintain a healthy biofilm for the purpose of establishing visualization and quantification methods for sparse biofilms. 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 patterned growth; however, it 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 (1-2 $\mu$ m), but the surface of most construction materials is irregular and has 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 (Daims and Wagner, 2007). 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 visibility over the signal from autofluorescing materials.

Confocal microscopy can also be used to characterize the surface of a rough, opaque material, such as that of cementitious materials (Kurtis, 2005; Lange, 1993). 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.

## 2. MATERIALS AND METHODS

### 2.1 Sparse Biofilm Development

Sparse biofilm colonization requires a method of cell deposition that controls the cell density of the selected microbe. An ideal deposition method is one that results in repeatable and consistent initial coverage of the surface to be colonized, independent of the microbial strain and the culture media. Culture concentration and media can be optimized for the selected microbial strain to determine the culture concentration and resulting initial coverage area. Concentration of the liquid media can be selected based on the degree of nutrition desired and the acceptable amount of residue left on the surface after evaporation of the culture liquid.

*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 (Klausen et al., 2006). Because *P.putida* has preferences for nutrient-rich locations, like soil, laboratory conditions were developed around making the nutrient provisions small relative to its typical environment and providing a pattern of growth, not necessarily the more severe conditions expected on a construction surface.

Preparation for a *P.putida* liquid culture followed standard microbiological laboratory methods (Madigan, 2003) to ensure a pure culture at the desired concentration and consistency. A stock culture was obtained (American Type Culture Collection, #12633) 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 100 mL of a full concentration nutrient broth solution. The liquid culture was incubated at 100 rpm and 28°C for 24 hours. A small sample of the full concentration liquid culture was taken for serial dilution and plate count quantification (Reasoner, 2004). 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 culture was prepared, it was transferred to a calibrated sterile plastic spray bottle. Ordinary spray bottles available at any local convenience store provide a simple, economic method to aerosolize the liquid culture for colonization. Spray bottles can be selected for their spray characteristics (geometry, quantity, consistency, etc), depending on the desired result. For an even, consistent coverage it was determined that a mist spray setting was ideal to disperse the culture evenly over the sample surface. Bottles were sterilized in a 5% bleach solution and reused for repeatability and economy.

Several bottles were calibrated for consistent spray volume and coverage area. Calibration was performed using two parameters: average volume per spray and coverage area at a 20.3 cm (8 in) spray height. First, the bottles were filled with water tinted with ordinary food coloring available in any grocery store and primed to remove all air from the spray mechanism. Once the bottle was primed, it was held 20.3 cm (8 in) above a piece of plain white paper and sprayed 2 times. Figure 1 presents a schematic of the spray calibration method. The tinted water provides easy viewing of the approximately round spray area on the white paper. Dimensions of the sprayed area were measured through the midpoint of the spray area along the two main axes. The spray was adjusted so that the spray area dimensions were between 19 cm and 20.3 cm (7.5 in and 8 in). After the bottles were calibrated for area, they were evaluated for spray volume. Each bottle was sprayed into a graduated cylinder ten times. The total volume was recorded and the average volume per spray calculated. The process was repeated several times for each bottle. Table 1 presents the calibration data for several bottles. Bottles were selected based on their consistency and correlation to other bottles in the group. Bottles 1, 3 and 6 were chosen for the experimentation because they generated similar spray volume over a consistent spray area.

Two materials were selected for colonization: glass and cement mortar (Portland Type I/II cement, concrete sand, w/b = 0.3, 30% cement replacement with fly ash). These surfaces were selected to represent a range of construction surfaces. Glass was chosen to represent smooth, non-porous surfaces and mortar was chosen to represent porous, rough materials. For glass samples, 10-well slides (SPI Supplies, #02286-AB) were selected because of the possibility to utilize 10 independent analysis areas on one 75 mm x 25 mm slide. 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.

Mortar chips were placed in a small amount of water so that capillary forces maintained saturation throughout the sample during the colonization and incubation process. Maintaining a saturated sample prevented the liquid culture and nutrient broth from being suctioned into the sample before the culture could colonize the surface. Figure 2 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 and its potential effects on environmental samples will be addressed in field studies to be conducted.

Colonization experiments were performed in a container to prevent contamination of surrounding surfaces with the aerosolized culture. Each sample or slide was placed in a tray, which was contained in a plastic bag. The spray bottle was held 8 inches above the surface and pumped twice. The colonization setup is shown in Figure 3. Mortar chips were immediately transferred to the water bath to prevent suction of the culture fluid into the pores. Sample surfaces were allowed to air dry to encourage deposition of the *P.putida* on the sample surface.

### 2.1.1 Controllable Acceleration of Growth

Once samples were sprayed with the inoculum and air-dried, 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 limited nutrition and reduce nutrient residue. 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 oligotrophic conditions encountered on construction materials. Samples were fed on a daily basis by pipetting diluted broth onto the sample surface with each well on the glass slide receiving 5  $\mu$ L per day, while each mortar chip received 50  $\mu$ L per day. This feeding schedule significantly limited the nutrients within the system, compared to the nutrients within a flowcell system. Figure 4 presents a graphical, cumulative comparison of the quantity of nutrition passed within the system in a slow flowcell (flow rate of 5  $\mu$ L/hour) and the quantity of nutrition within the system with stationary feeding over a 4 day period. Nutrient quantity within the system (not necessarily that which is used by the biota) is reduced by a factor of 24 over 5 days with the stationary feeding method, while still maintaining hydration of the biofilm. 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.

After feeding, samples were incubated at two temperatures: room temperature (20-22°C) and oven temperature (26°C). The oven temperature of 26°C is considered optimal for *P.putida*, but led to high growth rates and saturation of the 2D colonization surface (defined by percent coverage of the surface area) after only 2-3 days. The temperature was then dropped to room temperature, in an attempt to reduce growth rates. Growth, as measured by the percentage of the analysis area that is covered by biota, at the two incubation temperatures is compared in Figure 5. Though the growth rate was reduced by incubating at room temperature, the surface still saturated after 3-5 days. Additional investigations are ongoing to determine appropriate laboratory conditions for slower growth than that described while maintaining a suitable pattern of growth for visualization and quantification technique verification and relating laboratory growth to field conditions.

### 2.1.2 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 and air-drying of any remaining broth, 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.

### 2.1.3 Microscopy and Image Processing

After fixation, samples were stored in a 4°C refrigerator until analysis with Confocal Laser Scanning Microscopy (CLSM). CLSM was used to verify the spatial density at colonization and the 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 straight-forward, development of a methodology to automate the process required some effort to perfect.

To prepare for CLSM analysis, samples were stained with a fluorochrome to allow visualization on the glass surfaces and to allow visualization 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 frequencies between 522 nm and 661 nm were collected with the detector for the fluorescence channel for both the glass and mortar samples to visualize the biota. The mortar samples were two channel images, with the same 488 nm laser being reflected off the surface and all visible wavelengths being collected to identify the surface location in relation to the deposited biota. Images were collected with a 20x objective for the glass samples, but a higher magnification, 63x, was needed to accurately distinguish the biota signal from the autofluorescence signal of the mortar and provide better observation of the surface location. Single-layer images from glass samples were collected in a 4 x 4 tile pattern with 20% overlap between the images. The tile pattern yielded an analysis area of approximately 2.35 mm<sup>2</sup> (1530 μm x 1530 μm). Stack images collected from mortar samples were single location images from the 63x objective, which yielded an analysis area of approximately 20,420 μm<sup>2</sup> (142.9 μm x 142.9 μm). The mortar stacks were generated by collecting images at 1 μm intervals for either 14 or 19 μm (15 or 20 images). The stacks yielded analysis volumes of either 285,880 μm<sup>3</sup> or 387,987 μm<sup>3</sup>.

Images containing the fluorescence channel 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 area that was covered by biota. This percent area coverage was used to verify growth over time. Further discussion of the quantitative growth analyses in 3D will be presented in later publications.

### 3. 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 quantification is useful to verify and define growth trends and colony dispersion (Sullivan-Green, 2009). Figures 6-8 present sets of images collected during growth cycles on glass. Initial concentration of the inoculum culture varied between trials, with samples in Figures 6 and 8 having concentrations of 10<sup>4</sup> colony forming units (cfu) per mL and samples in Figure 7 having a concentration of 10<sup>7</sup> cfu per mL. Figure 6 and 7 samples were incubated in an oven and Figure 8 samples were incubated at room temperature.

In order to evaluate growth rates, images were first assessed visually. Qualitative analyses of growth show progression of coverage without enumeration. A visible change in the coverage of biota over several days verifies that, indeed, growth has occurred. It can be seen that the signal from biota covers a larger portion of area as time passes. This simplistic growth verification ensured that more rigorous analyses would be useful and provide quantifiable data for growth rates with stationary feeding. Figure 9 presents quantitative growth curves for trial samples in Figures 6-8. These figures verify the expected increasing growth and growth rates over time. These figures also demonstrate the ability to overcome inherent variability in these types of natural systems by multiple point analyses. Growth rates were determined using coefficients from the equation for the best-fit curve.

Qualitative analyses of accumulation on mortar required additional imaging than those on glass. The relief of the 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. Figure 10 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 the grayscale images).

#### 4. DISCUSSION

Qualitative analyses of 2D images provide a quick, simple way to determine success of a growth trial. These visual analyses also allowed assessment of the inherent variability associated with growth of microorganisms. Day 0 samples in Figure 6 demonstrate the variability of colonization with the spray deposition method, while Day 1 samples demonstrate the inherent variability of growth. The second image in the Day 1 series shows considerably more growth than other images taken on Day 1. This variability in growth can be accounted for by increasing the analysis area or increasing the number of analysis points for an investigation, both which were undertaken in this work. Averaging a number of analysis points (tile images) provides an average growth rate that is sufficient to quantify growth.

While qualitative analyses are helpful for describing and ordering growth, it not sufficient to quantify growth statistically. Growth must be quantified for full characterization. Quantification of a biofilm volume in two dimensions is possible if thickness is similar to the thickness of the image slice, thus images used to establish growth qualitatively can then be used to quantify growth quantitatively, as well. Progressive coverage was calculated for each set of images presented in Figure 6-8. Coverage is characterized by the intensity of the pixel at each location and when the intensity of the pixel was greater than the threshold, the pixel was counted as 'on' and, thus, correlated to biomass coverage. Pixels that are 'on' are then summed and coverage is calculated as a percentage of the total number of pixels in the image.

Once each tile image is processed and the coverage is calculated, the average coverage for each time step is determined. These average coverage values are represented by the black diamonds on the growth curves. Averaging of all data points for a time step aids in accounting for the variability in growth over a large surface, as discussed in the qualitative analysis. Exponential best-fit curves are fit through the average coverage values to determine the overall average growth rate. The equations of each growth curve and the square of the correlation coefficient ( $R^2$ ), which relates to the fit of the curve through the data points, are presented for each curve. Typically, bacterial growth is described as exponential (Madigan 2003), but other forms of the relationship have been employed such as the power function (Nan, 1995; Matis, 2007). Exponential functions were selected for this work to correlate with the most common equation format for bacterial growth. Exponential functions utilize a coefficient and a power value to describe the relationship. A basic exponential function is in the form:

$$y = ae^{xb} \quad (\text{Equation 4.1})$$

where  $a$  is the intercept of the equation at  $x = 0$  and  $b$  is the exponent that defines the shape of the curve, i.e. the growth rate. In this research, the variable  $x$  would be the age of the sample, in days, and  $y$  would be the percent coverage of biomass. The exponential function parameters would describe the percent coverage at initial coverage ( $a$ ) and the growth rate constant ( $b$ ) for the trial. Exponential curves were fitted through the average data points and the two parameters,  $a$  and  $b$ , were defined for each trial's data set.

##### 4.1 Image Segmentation and Analysis Area

Determination of the minimal analysis area is necessary for verification of statistically significant results. Korber's (1992) study showed that the minimum required analysis area was the area that the mean cell density no longer changed with increasing area size. The tapering range indicates that the variability is decreasing as the analysis area increases. The sufficient analysis area is interpreted as the point where the range variation is suitable for the desired results. Image segmentation analysis was performed to determine the minimum analysis area necessary for consistent coverage analyses. Figure 11 presents a plot of the number of segmented images versus the coverage for a single tiled image. Plots like this were generated for each image in the analysis. The mean coverage does not vary with increased image segmentation, indicating that the 4x4 tile with a 20x objective and image 20% overlap is more than sufficient to define these laboratory-generated biofilms.

Analysis of biomass coverage on rough surfaces required significantly greater effort than on smooth surfaces. The topography of the surface required that 2D images be collected at constant elevation intervals and stacked to represent entire volume the material surface inhabits. These 2D image stacks allow interpolation of volumes of biomass between images, yielding quantification of bio-volume, a more accurate representation of biomass quantity than 2D coverage would provide on 3D surfaces. Examination of the mortar focused on overcoming autofluorescence and ensuring sufficient stack depth to cover the relief of the surface. Acridine orange enabled visualization of biota over the material signal. The signal emitted from the stained biota was sufficiently strong and distinct to distinguish it from that of the material.

The material signal was filtered out by the confocal pinhole, allowing only the biota signal to be collected. Images collected for mortar are single field images and not tiles as used in glass analysis, since visualization and imaging were the focus, not quantification. Further investigations are exploring tiling with stack images and volume quantification.

#### **4.2 Threshold Comparison**

Threshold intensities are a critical component of image processing. The threshold is a function of the A/D converter that subdivides light intensity. In this case the A/D converted 8-bit intensity is subdivided into 256 ( $2^8 = 256$ ) steps. Threshold selection can vary from image set to image set, but should be fairly consistent within a sample if conditions are the same. The threshold value controls the pixel intensity that is counted as an occupied pixel, thus controls the coverage that is biomass. The most critical aspect of threshold selection is consistency from image to image and trial to trial. Manual threshold selection provides the most accurate correlation between visual biomass coverage and intensity values associated with occupation of a pixel. Manual selection was used in this analysis. Images in Figures 6 and 7 were analyzed using three threshold values: 100, 150, and 200. Figures 12 and 13 graphically compare growth rates determined with the three threshold values. Comparing results with the varying threshold values emphasize two factors: growth rate,  $b$ , and coverage. All three threshold values return an exponential form curve and growth rates are within a reasonable range of 1.56 to 1.9 for both trials. These observations indicate all reasonable threshold values demonstrate growth. The key to these types of analyses is to be consistent in the method so data obtained is comparable.

The second factor to note when comparing threshold selection is that by varying the threshold, the overall measured quantity of biomass changes. As the threshold is increased, the returned coverage and, thus, biomass quantity is reduced. Again, this comparison emphasizes the importance of remaining consistent in the selected analysis parameters over the importance of the actual parameter itself.

#### **5. CONCLUSIONS**

This research validates several hypotheses that form the foundation for the concept of dating surfaces by measurement of accumulated biomass. This method of dating surfaces can establish a timeline of cracking, an important link between connecting the crack to potential causation due to an atypical natural or man-made event, which can mitigate excessive litigation associated with damage claims due to construction activities. First, this work has established the ability to visualize and quantify both qualitatively and quantitatively, sparse biofilms generated under specialized laboratory conditions that simulate the sparse colonization that occurs on exposed construction surfaces. Performing controlled growth experiments of sparse biofilms allows for greater understanding of the limited growth that occurs on construction surfaces. It was shown that sparse biofilms could be established on smooth and rough surfaces by a spray deposition method. These sparse biofilms were nourished and hydrated by a stationary feeding method that maintains the no-shear conditions anticipated on most crack surfaces. Repeated hydration, controlled nutrient supply and optimum incubation temperature sufficiently accelerated growth to permit quantification. Finally, growth of the sparse biofilms was verified using CLSM technology. Analyzing colonized surfaces with Confocal Laser Scanning Microscopy allows for visualization directly on the accumulation surface and produces digital images that can be manipulated using image processing software to statistically quantify the amount of biomass present. Verification of visualization and quantification methods for such biofilms is a critical first step in utilizing microbiological accumulation as a means to determine the exposure time of the analysis surface, namely crack surfaces within the material. CLSM software enabled tiling of images, increasing the analysis area without sacrificing high resolution and stacking of images, which allowed a 3D interpolation of rough surfaces with 2D images.

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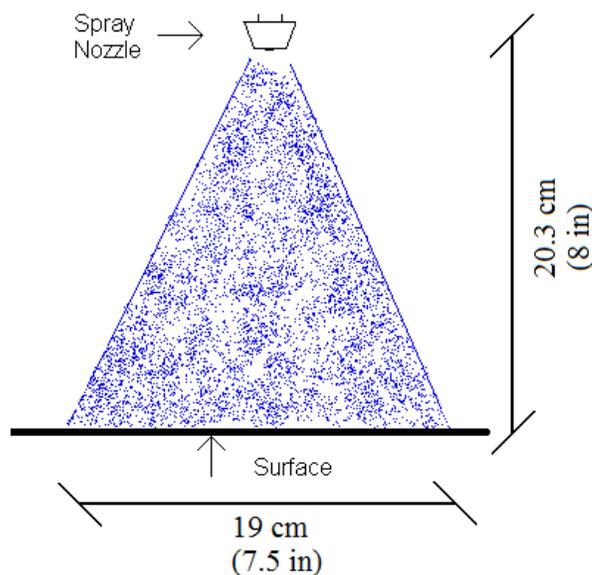


Figure 1: Spray bottles were calibrated for their coverage area when held 20.3 cm (8 in) above the surface to be colonized. Spray nozzle was adjusted so the volume of water sprayed and coverage area were consistent from bottle to bottle.

Table 1: Calibration of Plastic Spray Bottles. Bottles 1, 3 and 6 were chosen because of their similarity and consistency in spray volume and pattern as demonstrated in Figure 1.

Number	mL per 10 sprays										Avg mL/spray	$\sigma$ per 10 sprays	$\sigma$ per spray	
1	9.4	9.8	10	9	9	10	9.2	9.8	9.6	9.8	9.58	0.958	0.42	0.04
2	Leaks													
3	10	9.2	10	9.2	9.8	9.8	10	10	10	9.6	9.82	0.982	0.39	0.04
4	Leaks													
5	8.4	8.6	8.6	9	8.8	8.6	8.8	9.2	8.8	9	8.78	0.878	0.24	0.02
6	9.6	9.2	9.8	9.8	9	10	10	9.8	10	9.6	9.68	0.968	0.34	0.03
7	Poor Spray													
											0.9465	0.35	0.03	

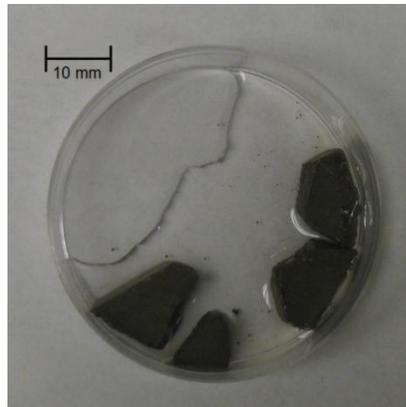


Figure 2: Mortar chips in a water bath. Mortar represents rough, porous materials for the purpose of visualizing sparse biota on construction materials.

Figure 3. Colonization of surfaces in a confined space prevents contamination of surrounding objects. The tray provides a flat area to place the samples to be colonized.

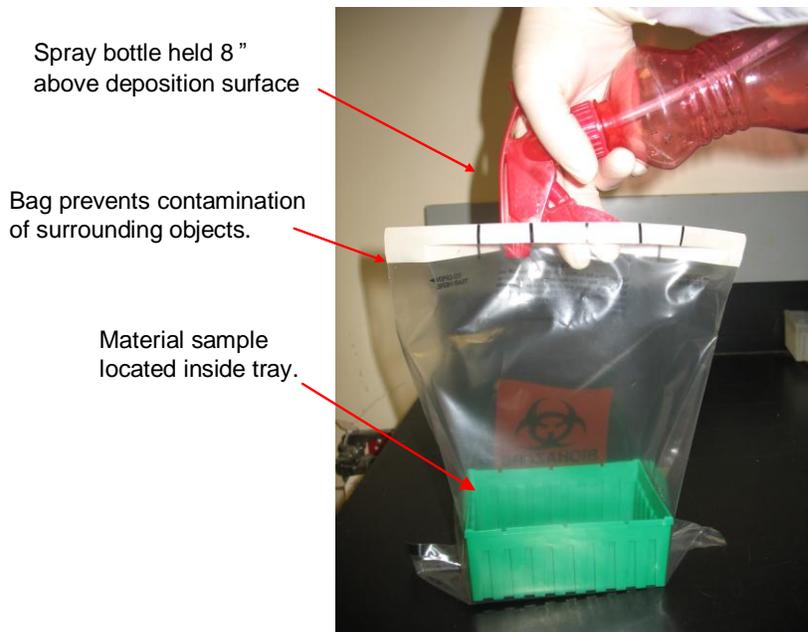


Figure 4. Cumulative nutrition passed through the system is greatly decreased over a 5 day period with stationary feeding over that in a flowcell. Hydration is maintained by feeding daily and by incubating the sample in a sealed petri plate.

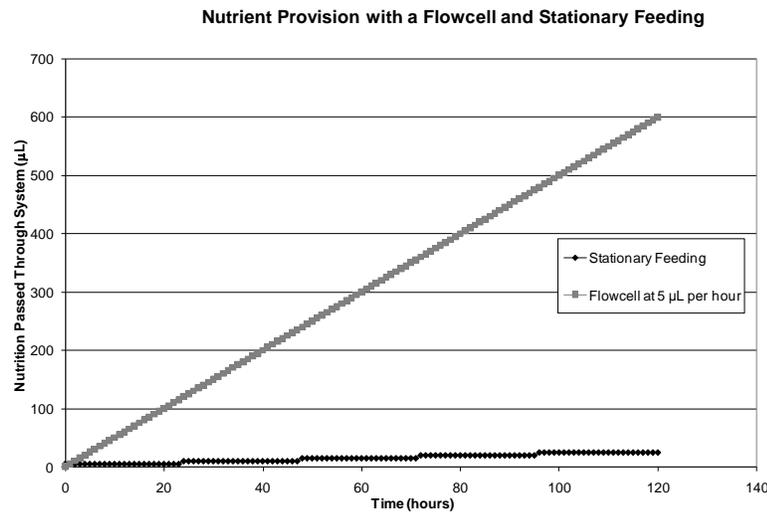
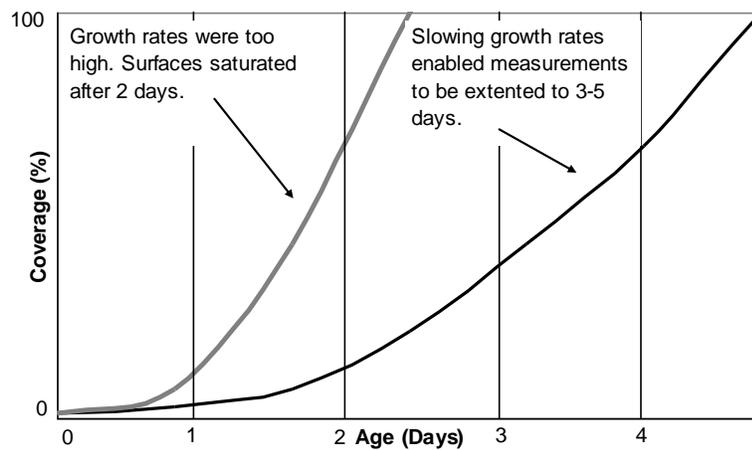


Figure 5. Growth rates (as measured by the percentage of the analysis area that is colonized) were extended from 2-3 days to 3-5 days when the incubation temperature was reduced from 28°C to 22°C. Further efforts to reduce the growth rate are being continued to replicate oligotrophic environments while maintaining a repeatable growth pattern for the remaining verification studies.



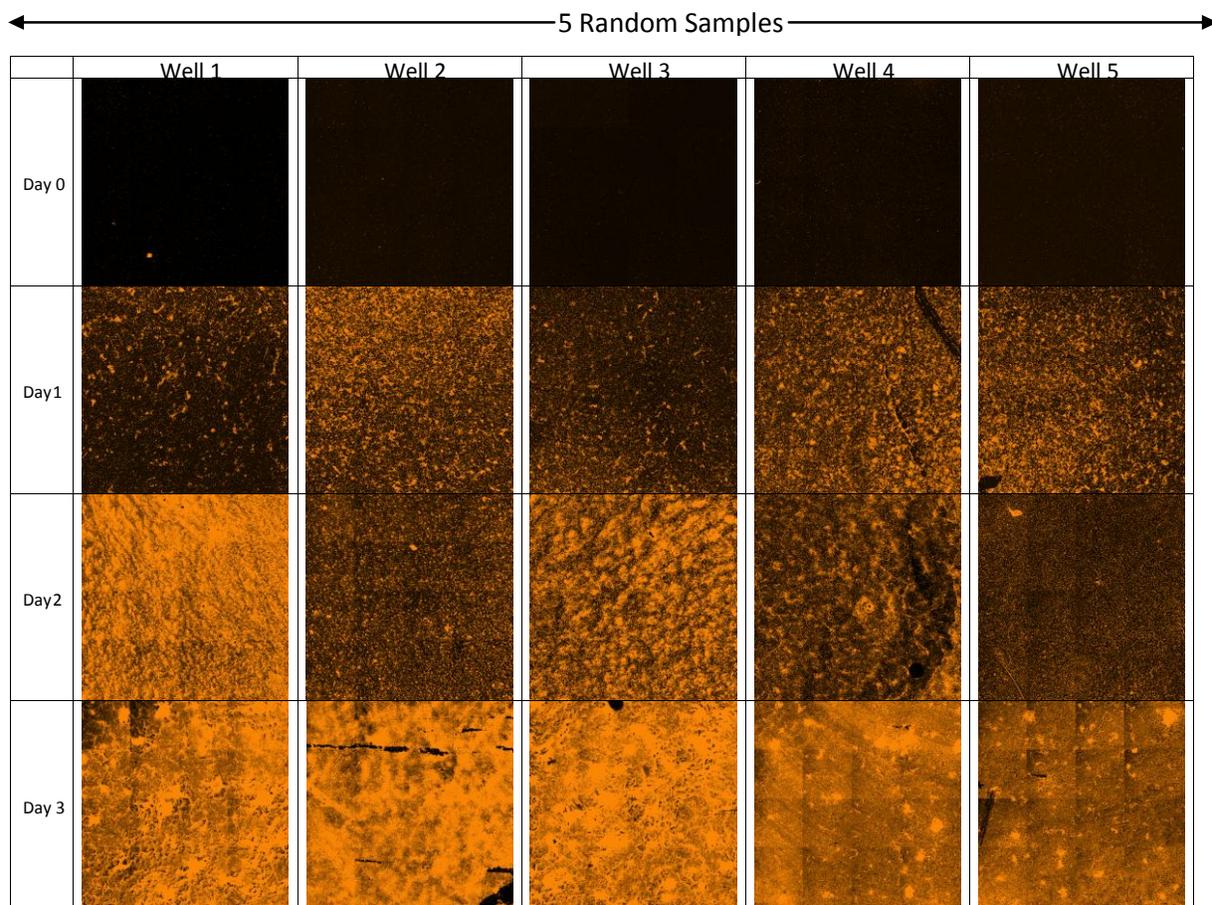


Figure 6. Growth (orange area) quickly covered the surface after providing optimum hydration and nutrition as shown by these five random samples from successive days of collection. The increase in signal (orange area coverage and intensity) represents increase in biomass quantity. The initial colonization of this experiment was low ( $10^4$  cfu/mL).

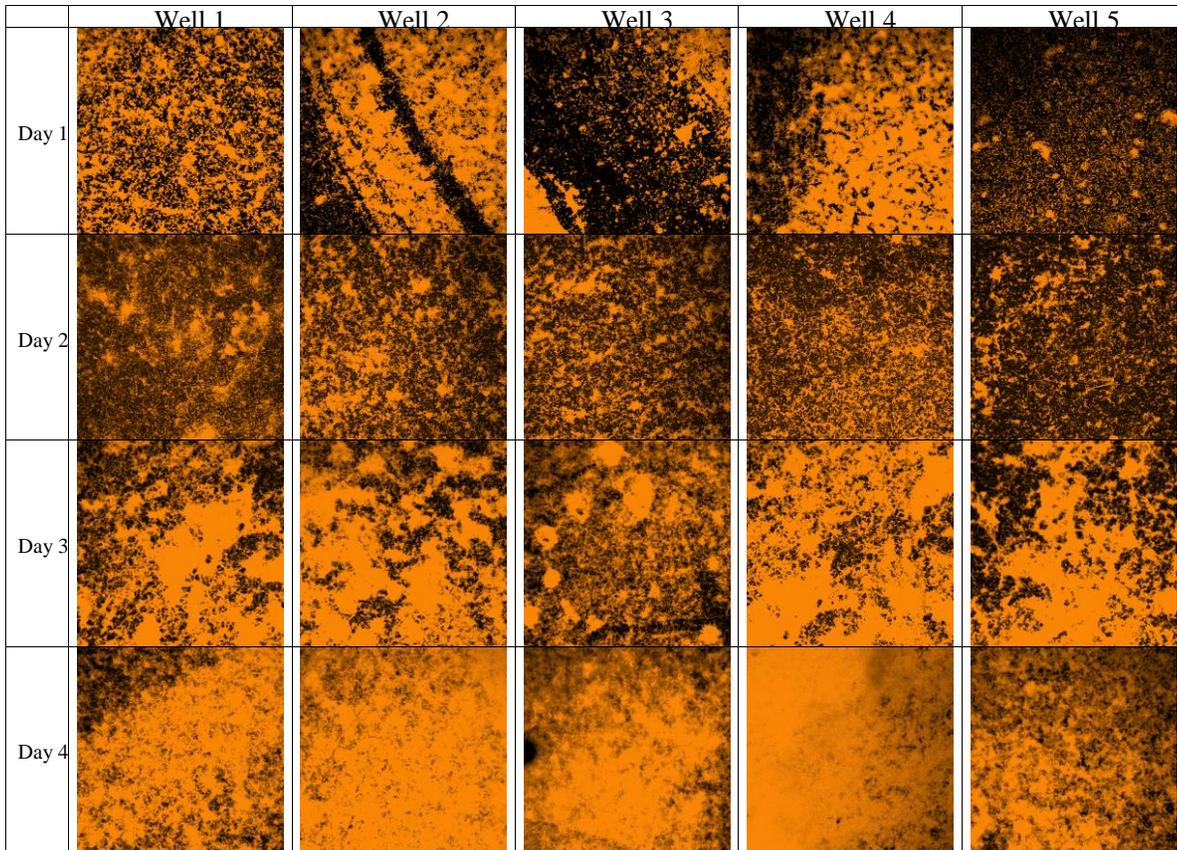


Figure 7. Growth in this experiment began at a higher concentration ( $10^7$  cfu) than other experiments, but growth rate was slower, potentially due to an unmeasured difference in temperature or humidity in the oven as compared to other experiments. Growth was still quantifiable and statistically differentiable over the course of the experiment, as shown in Figure 9.

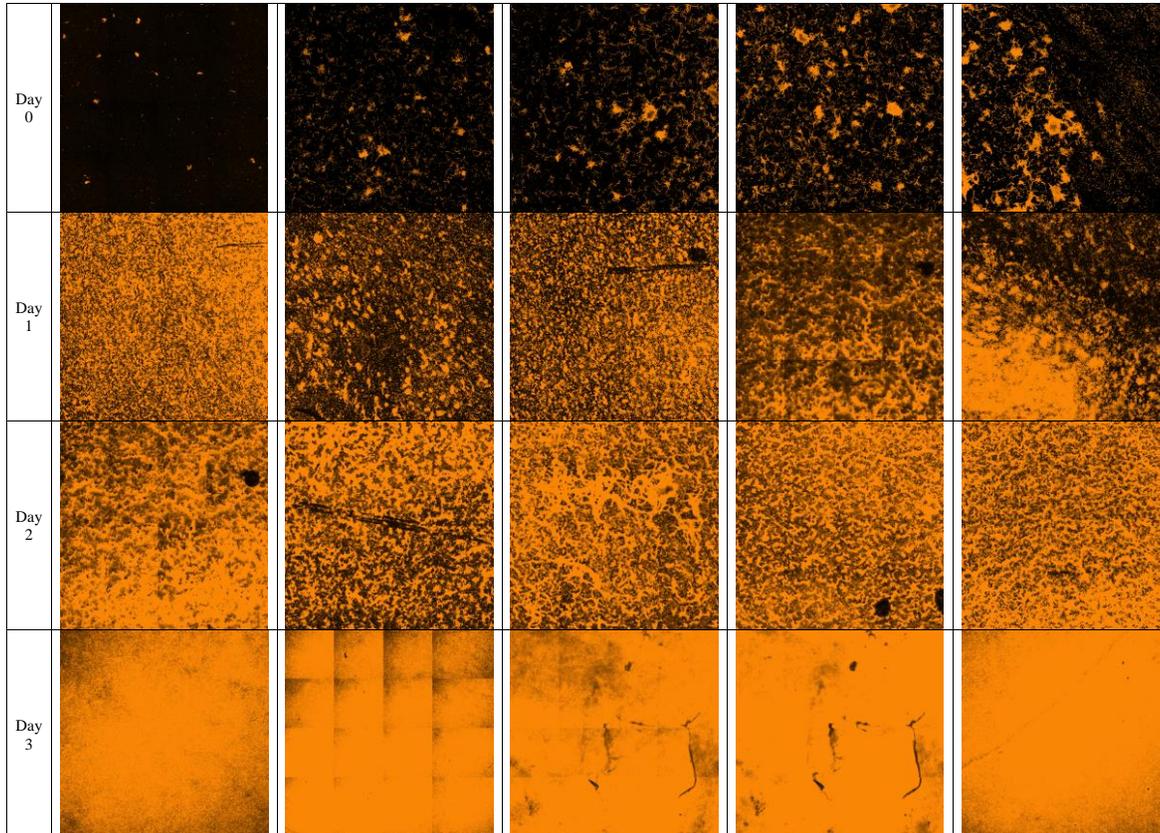
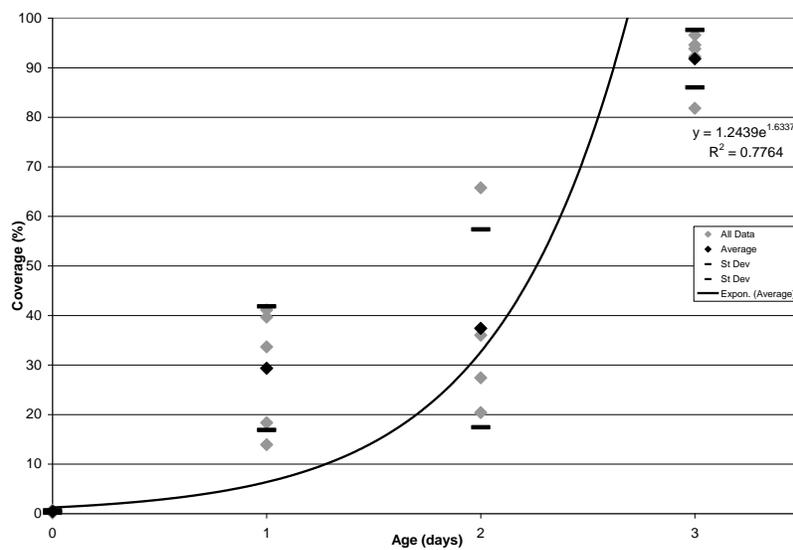


Figure 8. Room temperature incubation did not significantly reduce the growth rate for the biofilms in this experiment. The experiment started at a similar concentration as the experiment shown in Figure 6 and had a very similar growth pattern.



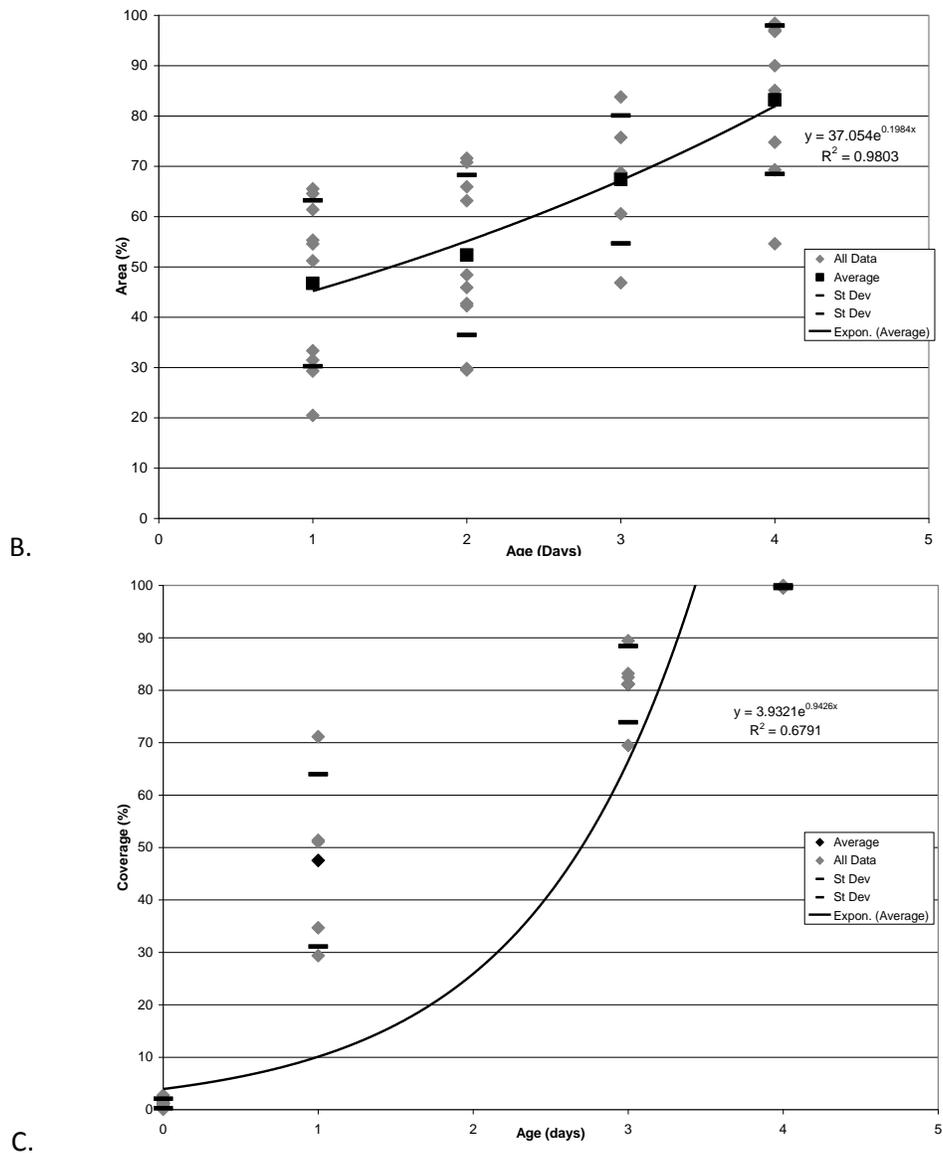


Figure 9. The images in figures 6-8 were quantified by a pixel on/off count and represented as a percent coverage of the total area analyzed. Growth patterns are consistent with trials shown in Figures 6 and 8, but differ in the trial presented in Figure 7.

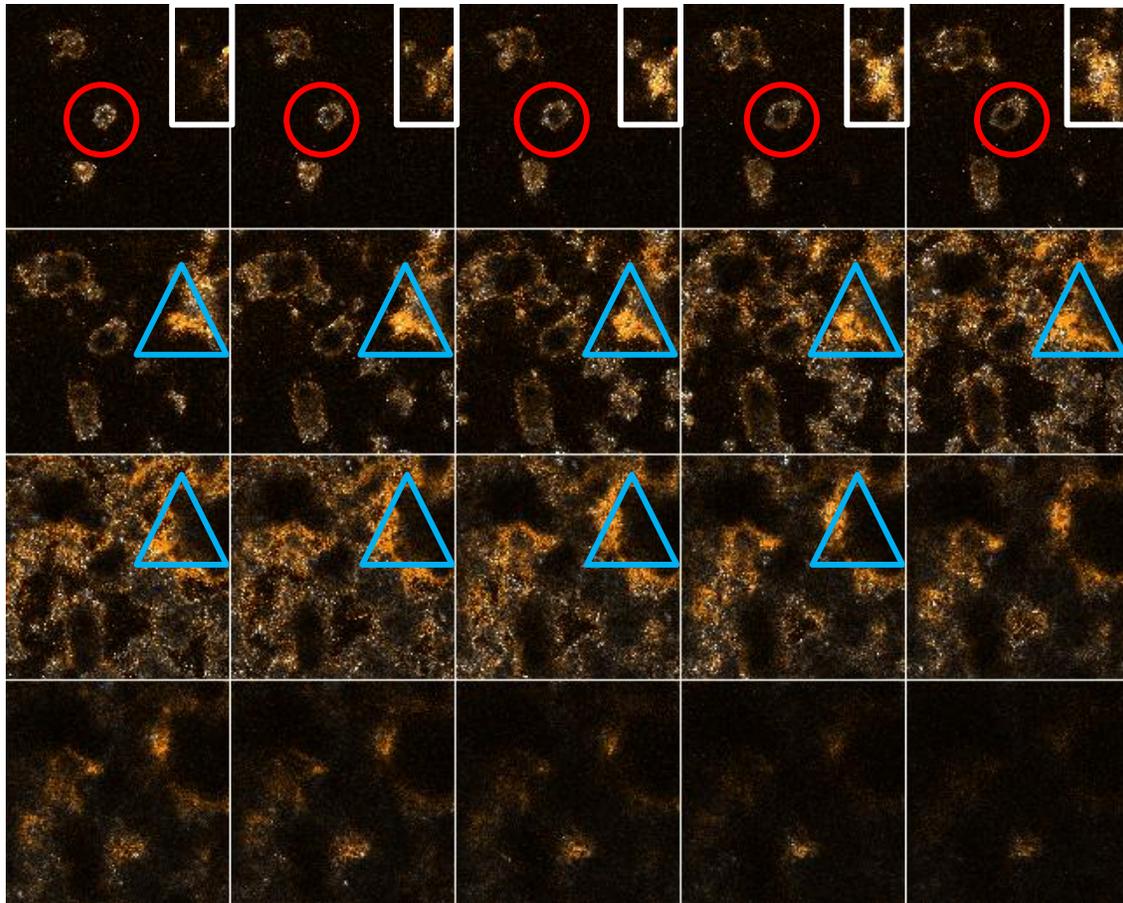


Figure 10. This single-location image shows the biota (orange) resting on the surface of the mortar (white), following the topography of the surface. The shape of the surface can be determined by following the progression of the signal from the top of the stack (top left image) to the bottom of the stack (lower right image) image. The circle shape identifies a peak in the topography, demonstrated by the spreading of the signal outward towards the circle itself as the images progress. The box identifies a downward, left-striking slope in the topography (signal moves from right to left), while the triangle identifies a valley where the slope of the peak and the left-striking slope meet at a common low point in the topography (signal is moving towards a common point). The black area on the right side of the triangles in the deeper images shows an absence of signal, which indicates the section is within the mortar sample.

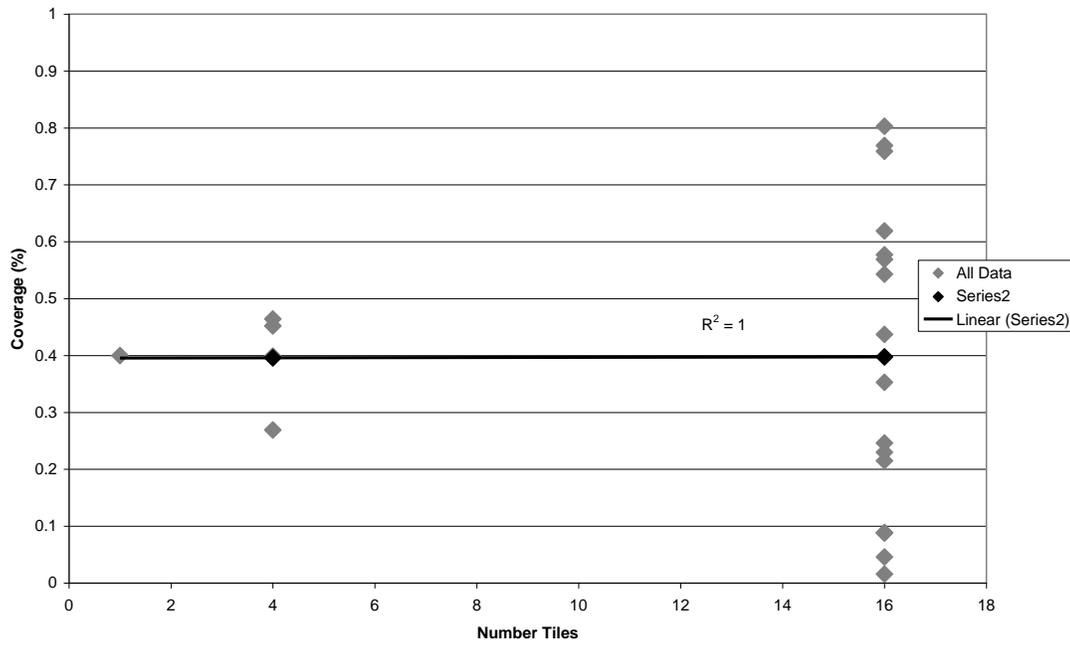


Figure 11. Image segmentation assists in determining if the analysis area is sufficient to statistically characterize the biomass coverage and if a method of averaging a number of images to represent a growth trend is acceptable, which is shown by the linear best fit line’s accuracy.

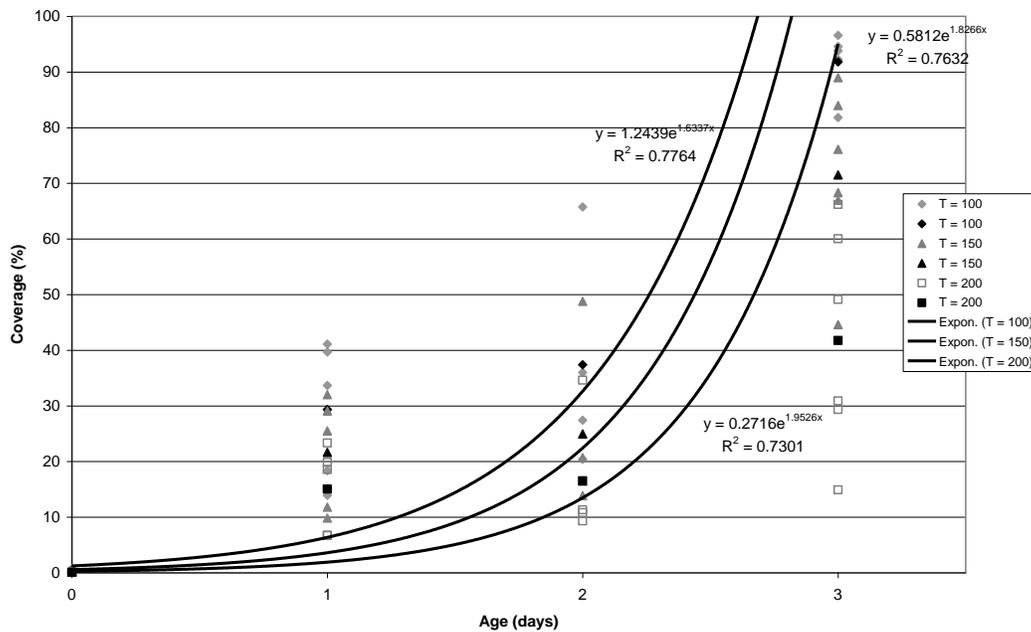


Figure 12. The threshold chosen for image processing can affect how many pixels are considered “on” or “off” for the purpose of quantification. This threshold analysis for the experiment shown in figure 8 indicates that curve shape and fit is consistent, no matter which threshold value is used, but consistency is key to comparing across experiments.