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# Color measurement as a means of quantifying surface biofouling

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**Abstract**

Laboratory reactors fitted with removable ceramic porcelain growth surfaces were inoculated with a consortium of biofilm forming environmental isolates. A Minolta colorimeter CR-200 (Minolta Camera Co., Ltd, Ramsey, NJ) was used in conjunction with a specially designed adapter to evaluate the reflective color of the porcelain disks as biofilm accumulated on them. Areal viable cell counts were monitored over a period of eleven days in two separate experiments and direct color measurements of the untreated, microbially fouled test surfaces were collected. This colorimetric assay was both non-destructive and immediate. A strong linear relationship between log cell density and log color change was observed. The Pearson product moment correlation coefficient for all 45 observations combined was $r = 0.95$. Separate regression lines for each experiment were not significantly different ($P = 0.19$). When adjusted for time, the (partial) correlation coefficient between log cell density and log color change was $r = 0.87$, which suggests that the relationship between the two measures can not be explained by their mutual dependence on time. Reflective color measurement provided a rapid, non-destructive and quantitative measure of biofilm accumulation. 

*Keywords:* Biofilm; Colorimeter; Color

## 1. Introduction

Currently accepted methods of monitoring surface biofouling are often labor-intensive and require invasive manipulation of the attached microbial community with either dyes or stains or destructive sample preparation. Viable counts in particular usually rely on lengthy sample processing and incubation times while integrity of the surface-attached structure is generally compromised. The present study investigated the use of a novel colorimetric assay to quantify viable biofilm bacteria. We know of no other published reports of direct colorimetric measurement of biofilm accumulation on a surface.

## 2. Theory

The color of an object can be defined by specifying its two chromaticity coordinates, $a^*$ and $b^*$, and a lightness factor, $L^*$ (Fig. 1). The $L^*a^*b^*$ system is

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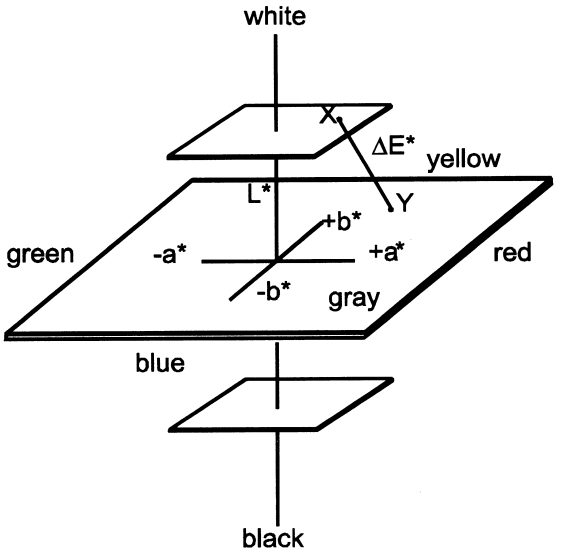



Fig. 1. A graphical representation of the $L^*a^*b^*$ color space. The baseline color is represented by X and the specimen color by Y. The difference in color between X and Y is given by $\Delta E^*$.

one of many color systems determined by the CIE (Commission Internationale de l'Eclairage, 1976) that can be used for measurement (Berger-Schunn, 1994). The $L^*a^*b^*$ system was chosen for this study because it best quantifies color as perceived by the human eye (Billmeyer and Saltzman, 1966). Total color difference $\Delta E^*$ is given by:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2},$$

where $\Delta L^*$, $\Delta a^*$and $\Delta b^*$ represent the difference between the measured values of the baseline and specimen coordinates. In our application, the measured color of a clean white disk served as the baseline color; color measurements of that same disk as bacterial biofilm accumulated on it were specimens.

## 3. Materials and methods

### *3.1. Growth surfaces and reactor conditions*

This study was part of a larger effort to characterize toilet bowl biofilm, therefore bacterial growth conditions were chosen so as to simulate the toilet bowl environment in laboratory reactors.

Biofilm growth reactors consisted of 1-liter beakers fitted with drain spouts. At the bottom of each vessel an unattached rotor base was seated. The rotor was constructed from a star-head magnetic stir bar to which a teflon and silicone rubber disk was attached. Six removable porcelain ceramic growth surfaces were inserted into each rotor. Growth surfaces (Tyler Research Instruments Corp., Edmonton, Alberta) were disks 1/2 inch in diameter and 3/16 inches thick and mildly bead blasted to promote bacterial adhesion. Reactor vessels were set on magnetic stirrers, and when these were turned on the entire rotor mechanism was free to spin. The rotational speed of vessel bases was set at 500 rpm with a tachometer.

Prior to use, disks were scrubbed with a commercially available cleanser, soaked in chlorine bleach, then rinsed thoroughly and autoclaved. Growth reactors were inoculated with a group of biofilm-forming bacteria isolated from toilet bowl biofilms which included *Pseudomonas vesicularis* and *Pseudomonas fluorescens*. Reactors were operated open to the air under cyclic fill-and-draw conditions. Once per hour, sterile tryptic soy broth (TSB, Difco Inc., Detroit, MI) was added to the 400 ml working volume to produce a final nutrient concentration of 1 g/l. After 5 min, the liquid volume was simultaneously stirred and drained. When the vessel was empty, a fresh supply of refill tap water was delivered to 400 ml. Thus, the organisms were exposed to 55 min of tap water conditions and 5 min of TSB per hour. This cycle, controlled by a programmable timing module (Chrontrol Corp., San Diego CA) was repeated once an hour for the duration of the experiment.

Two separate but otherwise similar experiments were completed, one nine days long (Experiment 1) and the other eleven days long (Experiment 2). A total of 30 observations were made in Experiment 1 and 15 in Experiment 2.

### *3.2. Colorimeter*

The Minolta CR-200 colorimeter uses a xenon arc lamp to produce an outgoing light pulse. This pulse illuminates an 8mm diameter area of the surface to be measured. Light reflected perpendicularly off the surface is collected by the measuring head and converted to a current, which is then digitized and

fed to the instrument's microcomputer. There, digital signals are transformed to a set of coordinates in the color space chosen. Data was output from the colorimeter directly to a personal computer where it was then analyzed.

The Minolta CR-200 colorimeter is a handheld device consisting of a data processor and keyboard which are attached by a cable to the measuring head. The head is designed to rest flush against the surface being evaluated to minimize introduction of ambient light into the optical system. The measuring head has a 7/16 inch diameter opening through which light exits and enters, and this is surrounded by a flange which must be placed flat against the surface to be measured.The flange ensures that stray light does not interfere with a measurement. Because placing the flange in contact with the biofilm surface would ordinarily disrupt the structural integrity of the biofilm, we designed an adapter that would protect the biofilm. The adapter allowed us to apply culture techniques after performing color measurements.

The adapter consisted of a 3 inch square of nylon cut from a 1/4 inch sheet, with a 1/2 inch diameter hole cut in the center. The nylon square and hole were nearly bisected through by a single cut - one small uncut section served as a hinge. The adapter was designed specifically for use with the ceramic disks, and could easily be fit around a disk and pinched closed without disturbing the surface. The colorimeter measuring head was placed flat against the adapter face during use. Marks on the adapter face served as placement guides for the measuring head (Fig. 2).

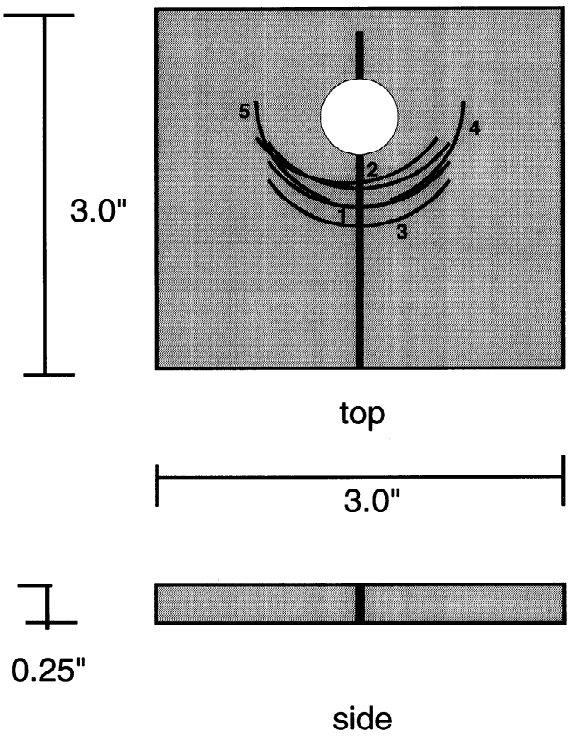



Fig. 2. Colorimeter adapter with placement lines. In the top view, the light area represents the ceramic disk. The arcs show the five positions where the edge of the colorimeter flange was aligned to take five readings. 1=Center, 2=North, 3=South, 4=East and 5=West. Note that measured areas included only the disk face and did not overlap onto the adapter.

### *3.3. Color measurement and viable cell counts*

Baseline color measurements were taken of clean, sterile disks. To take into account variations in color or reflectivity over the surface of a disk as well as patchiness of biofilm, five color readings were taken of each disk and averaged to give a single value (Fig. 2).

After baseline color readings were collected, disks were inserted into reactor vessels and bacterial growth conditions were implemented. The disks were subsequently removed from reactors over the course of each experiment and assayed for color change and viable areal cell density. Color measurement proceeded as above: disks were pried from reactor bases with a dissecting probe, and removed fully with tongs. Each disk was then set on a sterile work surface and the adapter was fit around it with the edges held tightly together. Five color readings were taken.

The change in color $\Delta E^*$ was computed using the average baseline and specimen components ($L^*$, $a^*$ and $b^*$) for each disk. Plots of $L^*$, $a^*$, and $b^*$ and of $\Delta L^*$, $\Delta a^*$ and $\Delta b^*$ for our data suggested that the direction of color change could be described by a line in three-dimensional space. The quantity $\Delta E^*$ reliably measured distances along this line, allowing us to replace the three-dimensional measure of color change with a one-dimensional measure. Statistical analysis was done on the scale $\log_{10}(\Delta E^*)$.

The disk was set into a 1/8 inch well in the work surface and assayed for viable bacterial counts. The

surface was scraped until dry with sterile wooden applicator sticks. Sticks were then stirred vigorously for 30 s in 5 ml of sterile phosphate buffered water (PBW) to dislodge bacterial cells. Five ml volumes of PBW and scraped material were disaggregated using a tissue homogenizer (Tissuemizer, Type SDT 1810, Tekmar Company, Cincinnati OH) for 30 s. Appropriate dilutions were spread plated on R2A medium (Difco, Detroit MI) in triplicate and incubated at room temperature for 6 days. All samples were processed within 5 h of collection. Triplicate plate counts were standardized to colony-forming units per square centimeter (cfu/cm$^2$), transformed to a $\log_{10}$ scale, then averaged to obtain a measure of log areal viable cell density.

### *3.4. Statistical methods*

The $\log_{10}$(cfu/cm$^2$) versus $\log_{10}(\Delta E^*)$ points were plotted, each point corresponding to a single disk. A different plotting symbol was used for each experiment. The plot showed the correlation between viable cell counts and color change. From that plot, a calibration line was calculated by fitting a least squares regression line. Prior to combining data from the two experiments, the regression calculations were performed separately for each experiment, and the null hypothesis of equal regression lines was tested using the $F$-test described in Seber (1977).

Let $W$ denote either $\log_{10}(\Delta E^*)$ or $\log_{10}$(cfu/cm$^2$), depending on the context, and let $t$ denote the time in days at which $W$ was observed. The observed $(t,W)$ points were plotted to show biofilm accumulation as a function of time. A smooth curve was drawn through the points based on the least squares estimates of the coefficients $\alpha$, $\beta$, and $\gamma$ of the accumulation curve defined in Eq. (1):

$$W = \alpha + \beta \cdot e^{-\gamma \cdot t}. \quad (1)$$

Eq. (1) was fit separately to each of four data sets - the color change measurements and the viable cell counts in each of the two experiments.

The partial correlation between $\log_{10}$(cfu/cm$^2$) and $\log_{10}(\Delta E^*)$, removing the effect of time, was calculated. The partial correlation coefficient, which is described in Draper and Smith (1981), was calculated to see whether the association between viable cell counts and color change was only a statistical artifact, attributable mainly to the causal effect of time on both biofilm accumulation variables.

## 4. Results

Fig. 3 shows that there was a strong linear relationship between log cell density and log color change. The Pearson product moment correlation coefficient, $r$, for all 45 observations combined was $r = 0.95$. Separate regression lines were fit for each experiment. Let $y = \log_{10}$(cfu/cm$^2$) and $x = \log_{10}(\Delta E^*)$. The fitted regression lines and correlation coefficients were $y = 7.28 + 1.29x$ and $r = 0.96$ for Experiment 1 and $y = 7.19 + 1.15x$ and $r = 0.93$ for Experiment 2. The two regression lines were not statistically significantly different ($F$-test, $P$-value = 0.19). These results provide evidence of the repeatability of the linear relationship between log cell density and log color change from experiment to experiment.

Figs. 4 and 5 show that, for each experiment, the color change curve imitated the viable cell count curve. The color change measurements, which were

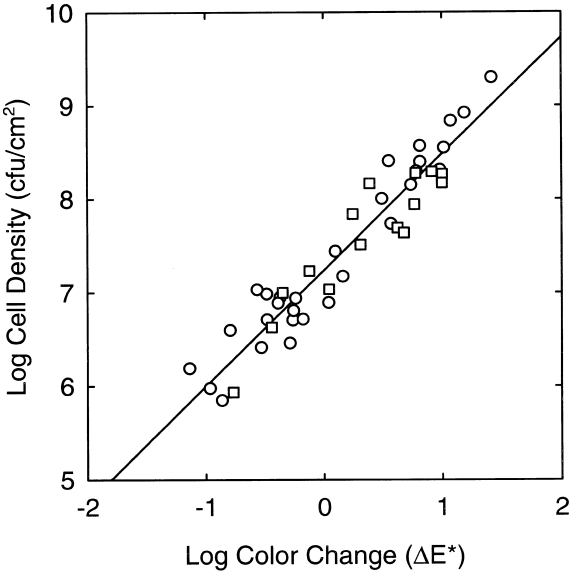
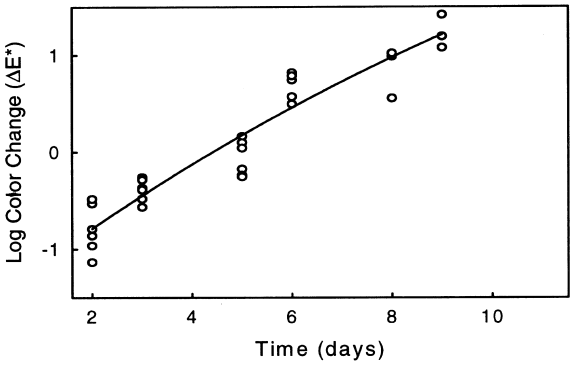
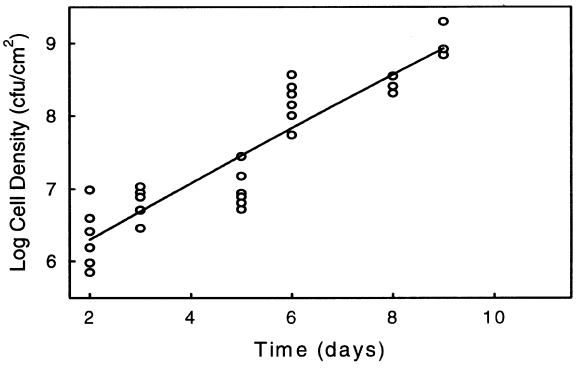



Fig. 3. $\text{Log}_{10}$ areal cell density, $\log_{10}$(cfu/cm$^2$), versus $\log_{10}$ color change, $\log_{10}(\Delta E^*)$, for Experiment 1 (○) and Experiment 2 (□). For the combined data, $r = 0.95$.

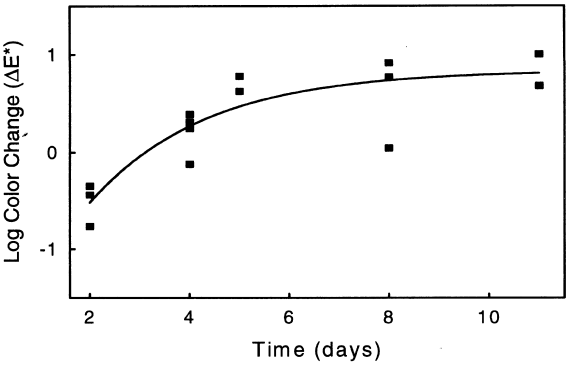



(a)

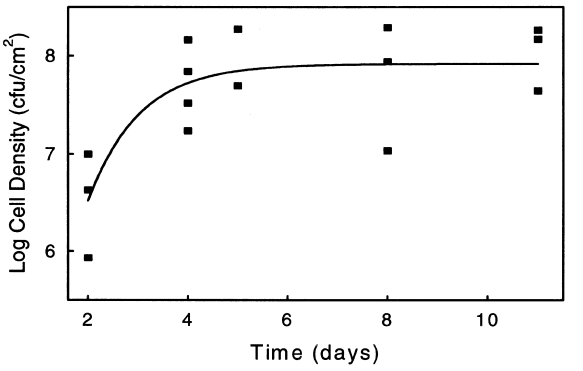



(b)

Fig. 4. Biofilm accumulation as a function of time for Experiment 1. $Log_{10}$ color change over time (a) and $log_{10}$ areal viable cell density over time (b).




(a)




(b)

Fig. 5. Biofilm accumulation as a function of time for Experiment 2. $Log_{10}$ color change over time (a) and $log_{10}$ areal viable cell density over time (b).

immediately available, accurately indicated biofilm growth dynamics as gauged by viable cell counts, which were available only after the six day incubation period. In Experiment 1 (Fig. 4), both the color change curve and the viable cell count curve increased steadily over the nine days of observation. The estimated coefficients in Eq. (1) were $\alpha = 5.0$, $\beta = 6.5$, and $\gamma = 0.061$ for $W = \log_{10}(\Delta E^*)$, and $\alpha = 35.0$, $\beta = 29.5$, and $\gamma = 0.014$ for $W = \log_{10}(\text{cfu}/\text{cm}^2)$. It is not evident that the biofilm reached steady state conditions during Experiment 1.

In Experiment 2 (Fig. 5), both the color change curve and the viable cell count curve leveled off after a few days, indicating that the biofilm had reached steady state. The estimated coefficients in Eq. (1) were $\alpha = 0.83$, $\beta = 3.3$, and $\gamma = 0.44$ for $W = \log_{10}(\Delta E^*)$, and $\alpha = 7.9$, $\beta = 9.9$, and $\gamma = 0.98$ for $W = \log_{10}(\text{cfu}/\text{cm}^2)$.

Because both $\Delta E^*$ and viable cell counts increase in time, one would expect that they would be correlated, even if they were only weakly associated. It may be that color would change as any material - viable bacteria or abiotic matter - accumulated on a surface over time. It is possible to remove the effect of time before doing the correlation calculations by performing separate least squares fits of Eq. (1), and then computing the correlation coefficient of the residuals of the regressions. At each observation time point, the residual for color change, which is the difference between the observed and predicted

$\log_{10}(\Delta E^*)$ values, is paired with the corresponding residual for viable cell counts, which is the difference between observed and predicted $\log_{10}(\text{cfu}/\text{cm}^2)$. The correlation coefficient for these paired residuals - called a partial correlation coefficient - describes the linear relationship between log cell density and log color change after removing the effect of time. The partial correlation coefficients were 0.77 for Experiment 1 and 0.90 for Experiment 2. The overall partial correlation coefficient, found by combining the Experiment 1 and Experiment 2 residuals, was 0.87. These high partial correlation coefficients indicate that the linear relationship between log cell density and log color change cannot be attributed to their mutual dependence on time. We conclude there is an intrinsic relationship between viable cell density and color change. Although abiotic material is undoubtedly a component of biofilms such as those studied here, the color change of our biofilms appears to be due to the accumulation of bacterial cells.

### *4.1. Summary and conclusions*

The results obtained in this study indicate that the colorimeter has potential use as a quick and easy means of estimating viable bacterial cell numbers attached to a surface. The basis of such an assay has been investigated here. Most importantly, the link between color and viable cell numbers associated with a surface has been established. The data collected in this study show that it is not simply the length of time spent in a fouling environment that determines the color of these biofilm-covered surfaces, but the number of viable bacteria per unit area.

Examination of Figs. 4 and 5 suggest that reflective color techniques could supplant plate counting for biofilm bacterial quantification for the same reasons that light scattering methods (also referred to as absorbance, optical density or turbidimetry) are often chosen to monitor bacterial growth in planktonic cultures. Both techniques offer immediate and nondestructive measurement. As is often the case with bacterial turbidimetry, reflective color measurement could be used to monitor growth until a desired level is reached, at which point treatments might be applied or other quantification techniques implemented.

As with bacterial turbidimetry, reflective color measurement must be carefully calibrated and applied. Optical density measurements for example, are highly dependent on the size of the bacteria, the color of the suspension and especially dependent on the degree of dilution of the suspension culture (Gerhardt, 1994). Similarly, prior to any application colorimeter measurements would need to be calibrated to the particular system - which would include surfaces, bacteria and disinfecting agents used - to ensure that a linear relationship exist between log $(\Delta E^*)$ and log $(\text{cfu}/\text{cm}^2)$. Our experience indicates that surface colorimetry is not useful for monitoring the early stages of biofilm formation when areal cell densities are less than $10^5$ $\text{cfu}/\text{cm}^2$. In contrast, at steady state we did not reach an upper limit, beyond which the linear relationship between log $(\Delta E^*)$ and log $(\text{cfu}/\text{cm}^2)$ did not hold.

Preliminary work not reported here using the colorimeter and antimicrobial agents indicated that the potential for interference by a biocidal agent is high. Interference might be due to bleaching of accumulated cell color by a treatment such as sodium hypochlorite or it might be due to addition of color by effects such as chelating. It is possible that a treatment would allow the extra-cellular polysaccharide matrix to become stickier, so that it actually collects more material and color. Also, as is the case with some quaternary ammonium compounds, the treatment itself may be colored and may add a background that dominates more subtle biofilm colors. For these reasons, it may not always be useful to use $\Delta E^*$ measurements before and after biocide treatment to estimate the log reduction in viable bacteria.

At present, most methods employed for assessing either biofilm bacterial growth or the effect of a treatment on an established biofilm are laborious and non-standardized. Reflective color measurement offers a simple and immediate alternative that may be as appropriate to biofilm study as optical density techniques are to monitoring of planktonic cell cultures.

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