

## Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces

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Received 23 September 2002; received in revised form 7 January 2004; accepted 8 January 2004

Available online 27 February 2004

### Abstract

Biofilms of a wild type *Escherichia coli* were grown on 316 stainless steel slides in a nutrient starved medium. The stainless steel surfaces were either polished to a smooth finish or scribed. The scribes consisted of lines and crosses. Biofilm samples were taken after 3, 6, 12, 24 and 48 h of growth. After sampling, the slides were soaked in deionized water or 50 or 200 ppm free chlorine prior to vital staining. Images were captured and the areas of viable and total biofilm were estimated. The individual biofilm patches, circularities, total percentage coverage, and viability percentage coverage were analyzed. The biofilms tended to increase in size between 6 and 24 h. A 3–6 h old biofilm on a polished stainless steel surface detached when 200 ppm sodium hypochlorite was applied. When grown in scribes, the circularity decreased up to 24 h, but thereafter increased. As the film grew older, it detached with or without a sodium hypochlorite treatment from the part of the surface that was polished, but remained in the neighborhood of the scribe. Based on the results, we recommend sanitizing at intervals of less than 12 h for this and similar strains of bacteria and protection of stainless steel surfaces to minimize scratching.

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**Keywords:** Chlorine; Biofilms; Surface morphology; 316 stainless steel; Image analysis; *Escherichia coli*

### 1. Introduction

It is well known that bacteria can attach and grow on various surfaces utilized in the food processing industry (Mafu et al., 1990). Microbial biofilms can form on virtually any surface that is in contact with water (Stewart et al., 1996). The cells that are associated with the biofilms have growth and survival advantages over planktonic cells. According to James et al. (1995), this advantage is due to the formation of an exopolysaccharide (EPS) matrix that surrounds the biofilm, protects it from attack by sanitizers, and supplies it with nutrients. Another explanation for the sanitizer resistance is that the bacteria in the biofilms are no longer actively growing (Mattila-Sandholm and Wirtanen, 1992). When active growth stops, an EPS matrix is

formed, which further helps to anchor the bacteria cells to the surface (Kumar and Anand, 1998). According to Nichols (1991), the increased resistance a biofilm shows to a sanitizer is not due to an impermeable EPS matrix, but rather due to a mass-transfer rate limitation in the gel-like EPS, in concert with detoxifying enzymes expressed from the bacteria cells.

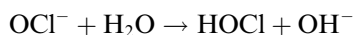
A major issue in the investigation of biofilms in the food processing industry is the development of methods to break up and remove the EPS matrix (Zottola and Sasahara, 1994). If the matrix is not completely removed when sanitizing a surface, pathogens will more readily reattach to the surface and a biofilm will form again, even if the previous pathogens were killed (Gibson et al., 1999). The use of chlorine as a means to remove the EPS has been discussed by Samrakandi et al. (1997). Further, Kumar and Anand (1998) list chlorine as one of the chemicals that depolymerizes the EPS.

Sodium hypochlorite is the oldest and most widely used of the chlorine compounds employed in chemical

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disinfection. Upon dissolution in water (such as in bleach), ionization takes place, and the hypochlorite ion establishes equilibrium with HOCl (Bloomfield, 1996):



It has been shown experimentally that the bactericidal action of chlorine releasing agents results from an oxidative interaction with the sulfhydryl groups on certain enzymes in the cell membrane or protoplasts. Due to chlorine's high oxidizing reactivity, the activity of cellular proteins is destroyed. In addition, it is believed that chlorine induces irreversible decarboxylation reactions. Care must be taken to make sure that sufficient free chlorine is available in the sanitizing solution, since chlorine reacts competitively with organic material to reduce the concentration of sanitizer will reach the bacteria (Kotula et al., 1997; Bloomfield, 1996).

Stainless steel has been reported as the ideal material for food processing since it is chemically and physiologically stable at various food processing temperatures, easy to clean, and has a high resistance to corrosion (Zottola and Sasahara, 1994). Scribing a polished stainless steel surface is one example of a deformation process where tensile and compression forces are introduced (Allen and Thomas, 1999). Tensile and compressive forces may cause general corrosion (Beddoes and Parr, 1999). Although no corrosion due to scribing was introduced in this research, it should be noted that the corrosion may create a different environment for the bacteria than that of the un-corroded stainless steel.

There have been several evaluations of a biofilms' strength of attachment and growth rate on different surfaces (Eginton et al., 1995; Frank and Koffi, 1990; Hyde et al., 1997; Jansen and Kohnen, 1995; Ronner and Wong, 1993). Hyde et al. (1997), found that biofilms consisting of *Escherichia coli* grew better on polypropylene than stainless steel, while they did not grow well on glass. Biofilms are not continuous sheets of EPS and bacteria. Instead they consist of a multitude of small patches of bacteria. In addition, biofilm has been shown to preferentially attach and grow in the crevices of a surface (van Haecke et al., 1990). Because of this, we anticipated that biofilms grown on polished then scribed billet of stainless steel would exhibit differences in overall surface coverage, biofilm patch area, and biofilm patch circularity when compared to those grown on undamaged, polished stainless steel. Circularity is a metric comparing the perimeter of the biofilm patch to that of a circle having the same the same area. The effect of preferential biofilm growth near a scribe was expected to evidence itself as an increased area of the biofilm patches and as a longer, narrower shape of those patches. The shape was evaluated as the biofilm patch cir-

cularity. Furthermore, the effect of treatment with free chlorine on the biofilm was considered.

To accomplish these goals, we examined the following null hypotheses regarding the effects of deionized water, 50 or 200 ppm free chlorine on biofilms grown on polished stainless steel and those around surface defects created in the stainless steel. First, there are no differences between individual patches of biofilm areas or circularities depending on the time the biofilms have been allowed to grow (3, 6, 12, 24 or 48 h). Second, there are no differences between patch areas or circularity depending on the surface morphologies that the biofilms were allowed to adhere and grow on (polished stainless steel, scribed, or specifically grown on a scribed line or a scribed cross). Finally, there are no differences in patch area or circularity depending on treatment of the biofilms (immersion in deionized water, 50 or 200 ppm free chlorine).

## 2. Methods

### 2.1. Choice of material and surface morphology

A common material in the food processing industry is 316 stainless steel (Mattila-Sandholm and Wirtanen, 1992). This type of stainless steel has a higher strength at elevated temperatures than other stainless steel types (Jastrzebski, 1976). van Haecke et al. (1990) has shown that surfaces with an increased roughness are less resistant to bacterial adhesion than surfaces with a polished surface. However, in practice, highly polished surfaces are subject to scratching.

The biofilms were grown on 25 × 75 mm billets prepared from a single sheet of 316 stainless steel. The billets were buffed to a bright finish prior to each experiment. On some of the polished stainless steel billets, lines and crosses were scribed using an industrial diamond. The width of the scribe was 14 μm. The depth of the scribe was estimated to be 2.1 μm. The scribes were located 1 cm apart in the horizontal and vertical direction of the slide surface. After preparation, the billets were washed in acetone to remove residual oils and buffing compound, and then rinsed in deionized water.

### 2.2. Bacterial and biofilm culture

The wild type *E. coli* was recently isolated from fresh strawberries obtained by the Food and Drug Administration in a metropolitan Washington, DC supermarket. It has been identified as attaching well to solid matrices. Since the strain was found in a natural habitat, there is a smaller risk of, by sub-culturing techniques in the lab, selecting for a fast growing mutant that lacks the mucoid extracellular polymer (Characklis, 1984). The wild type *E. coli* was transferred to plates containing tryptic soy broth (TSB) and agar (both VWR, West Chester,

NJ). The culture was maintained by plating onto fresh TSB agar at regular intervals. The bacterial cultures were maintained at 30 °C.

The use of a circulating culture medium for the development of biofilms has been reported by others (Camper et al., 1996; Sternberg et al., 1999; Tolker-Nielsen et al., 2000). The culture vessels were manufactured from 13.6-l polyethylene containers (VWR, West Chester, NJ) as previously described (Lomander et al., 2002). Circulation of the medium was maintained by a peristaltic pump (Masterflex®, Cole-Parmer Instrument Co., Vernon Hill, IL) at a flow rate of 200–220 ml/min to minimize localized nutrient depletion. The stainless steel slides were randomly inserted with their short side into channels with a 2 cm spacing. The assembled apparatus was covered with aluminum foil and autoclaved prior to use.

A limiting carbon medium was chosen for this research, since it mimics the limited nutrient concentrations found in whole fruit processing operations. The medium was prepared from TSB (3%) in deionized water. In addition, yeast extract (YE) (Difco Laboratories, Detroit, MI) was added to a concentration of 0.6%. The medium was then diluted three times with 0.9% sodium chloride (yielding  $1/3 \times \text{TSBYE}$ ) and autoclaved (Khambathy and Shah, 2002). Sodium chloride in the culture medium reduces the susceptibility of Gram-negative bacteria to lysis (Beuchat et al., 1998).

Bacteria were cultured on five TSB agar plates overnight. The resulting mat of bacteria on each plate was then transferred to a 500-ml culture flask, containing approximately 230 ml of sterile TSB. The flask with its contents was incubated on an Orbitron II rotator (Boekel Industries, Inc.) overnight at 30 °C. The absorbance of the culture in the flask was then adjusted with  $1/3 \times \text{TSBYE}$  to an absorbance of 0.7 at 610 nm. When the starter culture was first added to the culture vessel, a dilution of  $1500 \times$  with additional  $1/3 \times \text{TSBYE}$  was performed yielding a final volume of 5.4 l. The dilution ensured that the cell density was low, and that the cells would multiply as they attached and formed biofilms (Khambathy and Shah, 2002).

### 2.3. Preparation of sanitizer

The 50 and 200 ppm chlorine solutions were prepared from sodium hypochlorite (household bleach, Wonder Chemical Corp., Fairless, PA) diluted with deionized water. The concentration of free chlorine in the sodium hypochlorite solution was determined using a standard method (Chlorine Institute, Rosslyn, VA, 2000). The chlorine concentration was determined each time a chlorine solution was prepared. The chlorine solutions were stored in sealed bottles and prepared fresh every 24 h. To confirm the sodium hypochlorite solution's short-term stability, the concentration of free chlorine in an

open bottle was also determined at 12 h intervals during three days. The concentration of free chlorine did not change within that time frame.

### 2.4. Sampling of biofilms and application of sanitizer

At times 3, 6, 12, 24 and 48 h after the growth of the biofilms was initiated, slides were removed from the culture vessel using sterile gloves in order to prevent contamination of the medium and slides that remained. One polished stainless slide, and one stainless steel slide with a scribed surface was immersed in sterile deionized water for 2 min in order to remove loose debris. The slides were subsequently immersed in the chlorine solution for 5 min, and finally immersed in a sodium thiosulphate solution for 2 min in order to stop the chlorines' reaction with the biofilm. Since a 0.1 M sodium thiosulphate solution was used to titrate a tenfold dilution of the original manufacturers bleach, this molarity was adjusted to the 200 and 50 ppm bleach solution that was used for the sanitation of the slides. The slides were then immersed in two baths of sterilized deionized water for 2 min each time in order to rinse off excess sodium thiosulphate and chlorine compounds. Each study of a sanitizers' impact on biofilm on the different surface morphologies was repeated four times.

### 2.5. Viability staining

One polished and one scribed slide were evaluated at each of the sampling times from the tank. To evaluate a slide, it was stained using propidium iodide (PI) and SYTO-16 (both Molecular Probes, Eugene, OR). In this study, the staining solution was a 1:5 ratio of 1 mM SYTO-16 in DMSO with 1 mg/ml PI in deionized water. The stain was pipetted onto the billet in three to four aliquots of 3  $\mu$ l each. The stains were then allowed to incubate in darkness for a few minutes. While SYTO-16 stained all bacteria green by binding to the nucleic acids, propidium iodide only affected bacteria with damaged cell membranes, such as dying or dead cells. These cells were stained red. Using a FITC filter (which passes light with a wavelength  $>490$  nm), both SYTO-16 and PI stained cells could be visualized. When changing the filter to a Rhodamine filter (which passes light with a wavelength  $>545$  nm), only PI stained cells were seen. Hence, an estimation of total biofilm coverage and viable biofilm coverage (obtained by image subtraction) could be made.

### 2.6. Image acquisition and analysis

Images were generated using a black and white GBC CCD (charge coupled device) camera (GE Interlogix, Corvallis, OR) and acquired and stored on a computer (Macintosh® model 8600/250, Apple Computer, Inc.

Cupertino, CA) for further analysis. The images were acquired at a gray scale resolution of 8 bits and an image size of 640×480 pixels. Biofilm patches smaller than 5 pixels were omitted from our analysis, since this was the maximum size of dark noise and pixel defects for our CCD camera. On the polished stainless steel surface (SP), four images were randomly captured. On the scribed stainless steel surface, images were captured in three different ways: four image pairs were captured randomly (RA) over the surface, sometimes on a scribed line, and sometimes on a smooth surface; four image pairs were specifically captured on the scribed lines (SL), and four image pairs were specifically captured where the scribed lines crossed each other, forming a cross (SQ). On each slide, images were captured on four random locations. For each spot, one image was captured using the FITC filter, and one image was captured using the Rhodamine filter.

The image analysis techniques used in this research have been described in detail elsewhere (Lomander et al., 2002). We have determined the ratio of total biofilm to total slide area on a polished or scribed polished surface, the ratio of viable biofilm to total biofilm on a slide, the areas of the individual biofilm patches, and their shape factors for every image pair. To proceed with this analysis, every image was rank filtered and contrast enhanced. After the image taken through a Rhodamine filter had been subtracted from the corresponding image taken through a FITC filter, the resulting image, showing viable biofilm was contrast enhanced. The gray levels in this image were then manually thresholded (converted to a binary image) and the pixel counts that were generated were imported into Excel® (2000, Microsoft, Inc., Redmond WA). The FITC filtered image was also manually thresholded and the corresponding pixel counts, showing the total biofilm pixel counts, were imported to Excel®. The pixel count of the total biofilm was calculated as a mean of the pixel counts for the four images on each slide. This value was then divided by the total slide area in one image, 480×640 pixels. This value was finally converted to a percentage. The percentage of viable biofilm was calculated in a similar way. The mean coverage of viable biofilm was subsequently divided with the corresponding average value of total biofilm coverage for the same slide and the percentage computed. When analyzing the individual biofilm patches, all FITC filtered images were also rank filtered, contrast enhanced, manually thresholded, and the number of biofilm patches was counted, but this time, biofilm patches that were touching the edge of the images were omitted. This was done because the shape factor would be incorrect if not accounting for an entire patch. In this analysis, the results that were imported into Excel® consisted of areas and perimeters (in pixels) of the individual biofilm patches on every slide. The actual perimeters and areas were computed

from the pixel counts using conversion factors obtained by calibration using a stage micrometer. The shape factor, circularity has been defined (Sieracki and Viles, 1998; Lomander et al., 2002) as  $C = \frac{P}{2\sqrt{\pi A}}$  where  $P$  is the perimeter and  $A$  is the area. The shape factor, circularity, is the ratio of the perimeter of a biofilm to the perimeter of a circle with the same area as the biofilm. Hence, the circularity is “1” for a circle and larger for more irregularly shaped objects. Since background studies indicated that all biofilms were at least five pixels in size, thresholded images with a size less than five pixels were ignored.

### 2.7. Statistical analysis

The experiment was treated as a Randomized Complete Block Design with individual tanks as blocks. An analysis of variance (ANOVA) was performed on the data using SAS (version 8, SAS Institute, Inc. Cary, NC). In this study, ANOVA was performed on areas and circularities of the biofilm patches, the percentage of viable biofilm coverage, and percentage of total biofilm coverage. When analyzing the individual areas and circularities, ANOVA was performed on the medians, 10th and 90th percentiles. The percentage of viable and total biofilm was analyzed on means generated over time, material, treatment, and replication. To increase the homogeneity of variances, all values were logarithm transformed prior to using ANOVA. The statistical level of significance was 0.05 throughout the study. When comparing means of percentage coverage, patch area or circularity, the  $F$ -statistics of the main effects were generated (time, treatment, morphology, and their interactions). Non-significant main effects were thereafter removed, and the statistical program was run again on a reduced model. Least square means were compared, using adjusted  $p$ -values generated according to Sidak (Kuehl, 1994).

## 3. Results and discussion

### 3.1. Biofilm culture medium

As previously noted, limiting carbon medium was chosen for this research. Reduction of the available carbon has been shown to have a variety of impacts on the development of biofilms. The production of the polysaccharides is often a result of excess levels of simple sugars in the medium that is used for the growth of the biofilm. At the same time, a limitation of nutrients seems to enhance biofilm formation (Groat et al., 1986; Hood and Zottola, 1997; Jenkins et al., 1991). Dewanti and Wong (1995) found that biofilms from *E. coli* O157:H7 developed faster, and that a higher number of cells were recovered when grown on stainless steel in a low nut-

rient medium. Dodds et al. (2000) reported on the hypothesis that some fractions of the cell coverage in the biofilm inhabit zones of nutrient depletion and therefore exhibit a slow growing or starvation-like state and assemble into a biofilm. In addition, it has been shown that a low level of nutrients induces an increase in cell surface hydrophobicity, which in turn leads to a greater number of irreversibly bound cells in some bacteria (Zottola and Sasahara, 1994).

### 3.2. Overall trends

In this study, we examined the effectiveness of chlorine as a killing and/or biofilm removal agent on stainless steel surfaces of three different morphologies. Overall, the growth trends of a biofilm on a polished stainless steel surface followed those on a scribed surface. This was due to the very small area of scribes on the stainless steel surfaces. When randomly capturing images (RA) over the scribed surface, not many images were captured showing biofilm growth on the scribes. Images were also captured when the microscope was specifically focused on the scribed lines (SL) and where the scribed lines crossed each other (SQ). These images showed distinctive differences from the images captured on the smooth or randomly scribed surfaces (SP and RA).

Statistically, there were differences found over time ( $p < 0.001$ ), surface morphology ( $p < 0.001$ ), and treatment ( $p = 0.013$ ). There were also interactions between time and morphology of the surface ( $p = 0.008$ ), and time and treatment ( $p < 0.001$ ). When capturing images, it was noticed that the total biofilm coverage was significantly reduced when using 200 ppm free chlorine in comparison with 50 ppm free chlorine. The biofilms did not show a large coverage of the surface until 12 h of age. Very large biofilms could be seen at 12–24 h, while the biofilm coverage had decreased at 48 h.

Visually, no major differences were attributed to surface morphology. A comparison of the means of the total percent of biofilm coverage on the different morphologies when subjected to immersion in deionized water, 50 or 200 ppm sodium free chlorine is shown in Fig. 1.

### 3.3. Percentage of viable and total biofilm

Apparently, the individual biofilm patches did not start to actively increase in size until after 6 h. Before 6 h, the bacterial cells were attaching, initiating formation of biofilms, but they were also detaching, and there was no net increase in size. After about 6 h, active growth takes place until the biofilm is about 24 h old. Thereafter, the biofilms tended to reach a steady state where

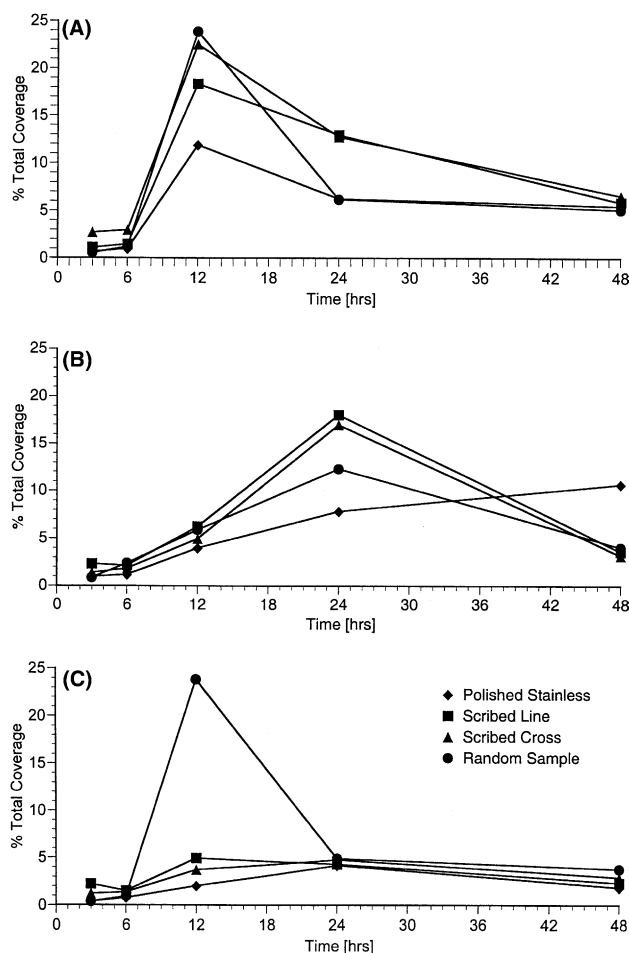


Fig. 1. Percent of a stainless steel surface covered by biofilm after treatment with (A) deionized water (control), (B) 50 ppm sodium hypochlorite, and (C), 200 ppm free chlorine. Each line represents a stainless steel morphology.

the sloughing rate equals the growth rate. This growth trend is indicated in Fig. 1.

In addition, there was a detachment of biofilm after 12–24 h. Thereafter, the biofilms tended to reach a steady state where the sloughing rate equaled the growth rate. The detachment rate has been reported to be due to a weakening in the biofilm matrix. Characklis (1984) reported that the detachment rate increased with an increase in biofilm mass. In addition, the weakening depended on an increase in the fluid shear stress, which increased with the increase in biofilm thickness. It was also assumed that weakness was caused by limitations of oxygen and nutrients in the deep portion of the biofilm. Characklis and Cooksey (1983) also suggested upsweeps of the flowing fluid and detached pieces of biofilm as means of causing cell detachment. Upsweep causes particles to move away from the surface as a result of turbulent bursts. The particles will therefore move away from the surface. A lift force that is normal to the surface is generated and thus, detachment is influenced.

There seemed to be a larger detachment from a polished stainless steel surface compared to a scribed surface. It was not clear whether 50 ppm sodium hypochlorite contributed to a detachment from a surface, but as indicated in Fig. 1(C) shows, a large detachment took place when treating the biofilms with 200 ppm free chlorine from sodium hypochlorite. It appeared that the biofilms attached to the same extent on a scribed line as compared to a scribed cross.

### 3.4. Individual area patches and circularities

The total biofilm coverage varied with the morphology on which it was growing. Biofilms on scribed surfaces mainly consisted of a few large, irregular patches centered in and around the scribes, while biofilms on the polished surfaces consisted of many smaller biofilms. As the biofilm became older (>12 h), the individual biofilm patches increased in size. The sizes appeared smaller when treated with sodium hypochlorite, especially on polished surfaces, which is shown in Fig. 2. In order to compare biofilms on different materials, the 50th, and 90th percentiles were selected as a reflection of a mid-sized biofilm and a large biofilm. Biofilms patches in the 10th percentile of areas were below 10 pixels, many in the 5–7 pixel range. Since, the cut-off level for particles analyzed by NIH Image was set to 5 pixels due to instrumentation limitations, the 10th percentile patches were not considered for further analyses.

The trends in patch size over time and treatment on (A) a polished stainless steel surface, (B) a polished stainless steel surface with scribed lines, and (C) a polished stainless steel surface with scribed crosses are shown in Fig. 2. The trends in circularities on (A) a polished stainless steel surface, (B) a polished stainless steel surface with scribed lines, and (C) a polished stainless steel surface with scribed crosses are shown in Fig. 3.

### 3.5. Medians and 90th percentile biofilm patch areas on polished stainless steel surfaces

The trends in patch sizes in the 90th and 50th percentiles corresponded well. Sodium hypochlorite treatments decreased the biofilm patch areas more than a water treatment on polished surfaces up to 12 h. A 200 ppm sodium hypochlorite solution did not decrease the area patches more than a 50 ppm sodium hypochlorite solution when applied to a young biofilm (6 h). Since the analysis of the total percent biofilm coverage showed that 200 ppm was more efficient in detaching biofilm than 50 ppm, it was believed that a few unusually large biofilm patches were present when randomly capturing biofilms treated with 50 ppm sodium hypochlorite. At 12–24 h, the biofilm patches decreased in area for all treatments (Fig. 2(A)). A theory is that the area patches

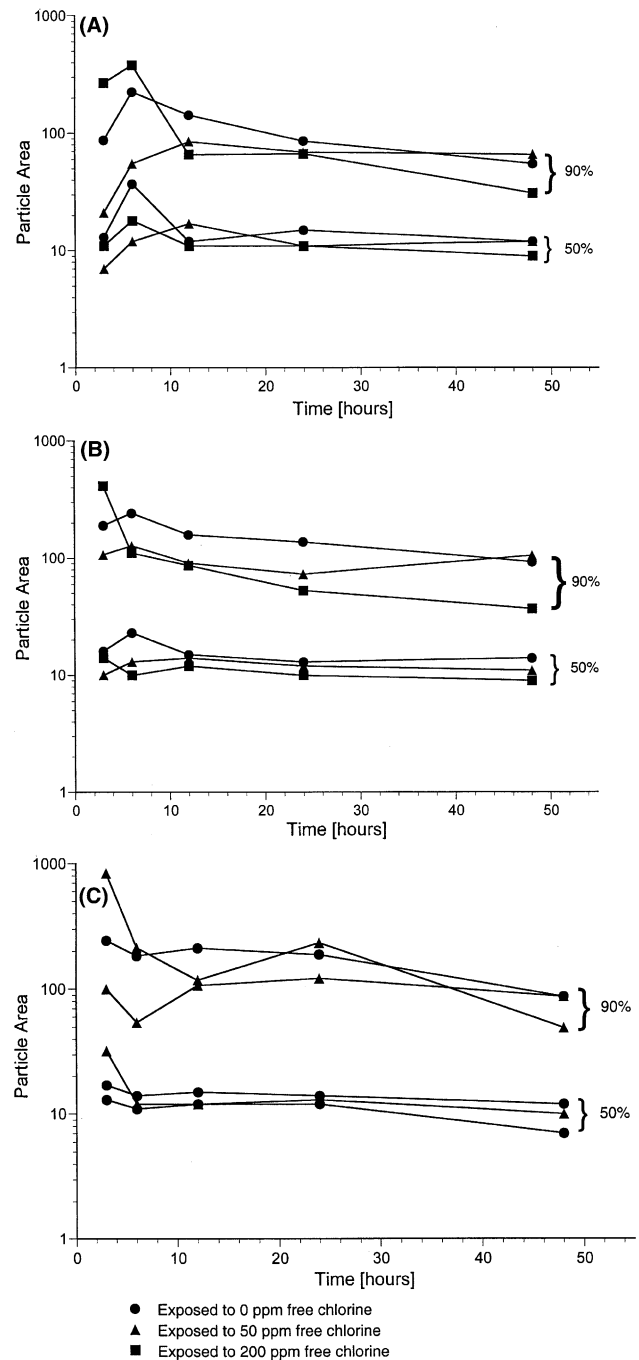


Fig. 2. The sizes of individual patches of biofilm on (A) a polished stainless steel surface, (B) a polished stainless steel surface with scribed lines, and (C) a polished stainless steel surface with scribed crosses after treatment with deionized water, 50 or 200 ppm free chlorine. Each line represents a size and a treatment. The 50% and 90% refer to the biofilm patch sizes in the 50th or 90th percentiles, respectively.

decreased due to nutrient limitation and age. The differences over time and treatment were more apparent for biofilm patches in the 90th percentile. Area patches in the 50th and 90th percentile showed significant interactions over time and treatment ( $p < 0.001$ ). Sodium hypochlorite changed the shape of the biofilm

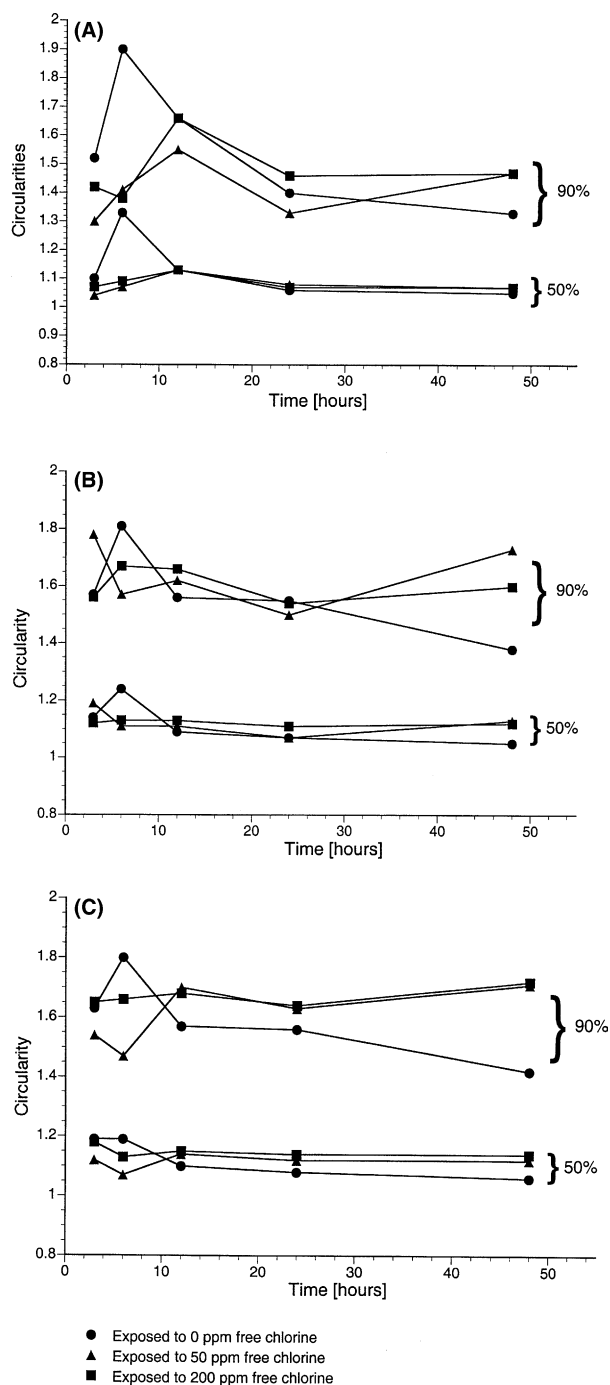


Fig. 3. The circularities of individual patches of biofilm on (A) a polished stainless steel surface, (B) a polished stainless steel surface with scribed lines, and (C) a polished stainless steel surface with scribed crosses after treatment with deionized water, 50 or 200 ppm free chlorine. Each line represents a circularity and a treatment. The 50% and 90% refer to the biofilm circularities in the 50th or 90th percentiles, respectively.

patches, especially in the 90th percentile of the circularities, causing young biofilms to become more circular compared to a water rinse (Fig. 3). As the biofilms grew older, the circularity remained fairly constant. The

graphical visualizations were supported by statistics since there were differences over time ( $p < 0.001$ ).

### 3.6. Medians and 90th percentile biofilm patch areas on scribed stainless steel surfaces

One theory in this research was that biofilms on scribed surfaces would remain protected from the sodium hypochlorite solution. It can be seen that biofilms on scribed surfaces showed smaller reduction in patch size when treated with sodium hypochlorite than the biofilms on polished surfaces, but only for young biofilms (Fig. 2(B) and (C)). However, the patch areas remained fairly constant after 12 h. The interaction between time and surface morphology was significant in the 90th percentile ( $p = 0.010$ ). This supports the theory that biofilms on scribed surfaces were protected over time, although an initial reduction in size did take place. This theory was also supported by an investigation of the circularities of the biofilm patches. Biofilms growing on scribed surfaces became fairly circular in shape compared to a water rinse as long as the biofilm was young. As the biofilm aged, the circularity did not decrease. Instead it increased, which was clearly demonstrated in Fig. 3(B) and (C) and statistically confirmed. There were differences due to morphology ( $p < 0.001$ ), and morphology and treatment ( $p = 0.007$  in the 50th percentile, and  $p = 0.032$  in the 90th percentile).

## 4. Conclusions

In order to demonstrate trends in growth on different surface morphologies over time, the biofilms had to be at least 6 h old. Since the biofilms were viable on the surfaces prior to this time, it was believed that the irreversible adhesion took place during the first 6 h. The largest biofilm coverage was found 12–24 h after growth was initiated, which suggests that cleaning of a stainless steel surface should be performed at least every 6–12 h for organisms like the *E. coli* strain used here. From the analysis of the percent of coverage, it was concluded that a treatment with chlorine did kill biofilm. It was however not necessary to use 200 ppm to reduce the viable biofilm. A 50 ppm sodium hypochlorite solution killed the biofilm to a significant extent compared to rinsing in water. Also, 50 ppm sodium hypochlorite did not detach the biofilm to a larger extent compared to water.

A young biofilm on a polished stainless steel surface detached when 200 ppm sodium hypochlorite was applied. As the biofilm grew older, it detached even if no sanitizer was applied. When grown in scribes, the biofilm was affected differently. The shape factor decreased up to 24 h, but thereafter, the circularity increased. One conclusion from this finding was that biofilm patches, as

the film grew older, detached with or without a sodium hypochlorite treatment from the part of the surface that was polished. The part of the biofilm that remained attached to the surface would be the film that grew in the very neighborhood of the scribe, along the lining of the scribe, or in some cases covering the scribe. Therefore, the contribution to the irregular shape would come from the shape of the scribe.

This study suggests that further research using sodium hypochlorite on stainless steel surfaces with a higher roughness than polished steel should be undertaken. In addition, biofilm attachment surfaces other than stainless steel, along with sanitizers other than sodium hypochlorite, should be tested.

### Acknowledgements

We thank the Center of Food Safety and Applied Nutrition (CFSAN), FDA, Washington, DC for donation of the bacteria strain. This study was funded by JIFSAN (Joint Institute for Food Safety and Applied Nutrition), University of Maryland, College Park, MD.

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