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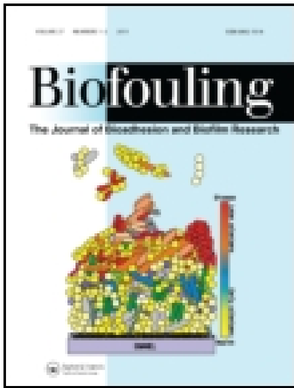

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Bacterial characterization of toilet bowl biofilm

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BACTERIAL CHARACTERIZATION OF TOILET BOWL BIOFILM

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Methods have been developed and applied for sampling, characterizing and quantifying naturally occurring toilet bowl biofilms. Ceramic porcelain disks mounted in neoprene rubber strips were sealed in place in toilet bowls in three residences in Bozeman, Montana. In each bowl, duplicate strips were placed above, at and below the water level. In 7 consecutive weeks, duplicate disks from each zone in each bowl were removed. Surface biofouling was measured by viable cell areal density. Specific fouling rates were calculated and variability among toilet bowls and water levels was assessed. Specific fouling rates ranged from 0.0 to $0.46\,d^{-1}$. Average areal cell densities at the end of 7 weeks ranged from 10^3 to $10^7\,cfu\,cm^{-2}$. The extent of fouling was highest below the water line. Neutralization of the chlorine residual (typically $0.9\,mg\,l^{-1}$) in one toilet did not increase the extent of fouling compared to the controls. Biofilm areal viable cell densities and bowl water viable counts were positively correlated ($r = 0.78$). The visual threshold for detection of toilet bowl biofilm by the naked eye was approximately $10^5\,cfu\,cm^{-2}$. In a heavily fouled toilet bowl, the biofilm was up to $20\,\mu m$ thick. Microorganisms were isolated from the biofilm and identified. Of the 32 organisms that were further characterized, 10 were identified as *Pseudomonas*, *Sphingomonas* or *Chryseomonas* species.

Keywords: biofilm; biofouling; toilet; method; ecology; speciation

INTRODUCTION

The many systems in which biofouling has been studied have not included the toilet bowl. Toilets contain continuously and intermittently wetted surfaces that experience periodic additions of concentrated organic mixtures capable

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of serving as microbial growth substrates and this provides an environment susceptible to biofilm formation. Though toilet biofilms have not been studied, other microbiological issues of toilet use and operation have been addressed (Newsom, 1972; Gerba *et al.*, 1975; Mendes & Lynch, 1976; Scott & Bloomfield, 1985; Yahya *et al.*, 1992; Roach, 1994). Despite the lack of published research, biofilm accumulation in toilet bowls presents obvious cosmetic and hygienic concerns. The goal of this investigation was to characterize basic features of biofilm accumulation in toilet bowls including the net rate and extent of accumulation, variation of fouling with water level, microbial speciation, and the relationship between biofilm and bulk water microbial densities.

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MATERIALS AND METHODS

Experimental System

Toilets in three different Bozeman, Montana households all receiving water from the same municipal drinking water treatment plant were selected for experimental use. The household occupants numbered from two to four persons and were all in good health. One toilet in each residence was fitted with 48 3/16 inch thick, 1/2 inch diameter removable porcelain disks (Tyler Research Instruments Corporation, Edmonton, Alberta) that were held in place by 3/16 inch thick neoprene rubber strips. Disk surfaces were mildly abraded by bead-blasting to promote bacterial adhesion. Disks were scrubbed, soaked in chlorine bleach and autoclaved before insertion into the toilets. The rubber strips were sealed to the bowl surfaces with silicone sealant. Each strip measured 4 1/2 × 1 inches and held four ceramic porcelain disks (Figures 1 and 2). The strips were affixed horizontally at three different levels in each bowl, *viz.* high (H, above the water line), middle (M, at the air-water interface) and low (L, below the water line). These strips were duplicated on the left and right sides of the bowl. The toilets used will henceforth be referred to as A, B, and C.

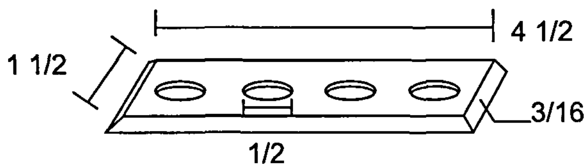

FIGURE 1 Dimensions of rubber disk-holder (inches).

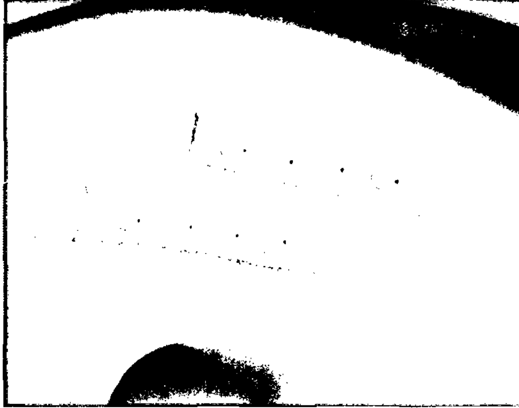

FIGURE 2 Photograph of disk-holders in place in a toilet.

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Sampling Procedure

Biofilm was collected from both disk and bowl surfaces in all three experimental toilets approximately once a week for 7 weeks. At time zero, each toilet was cleaned thoroughly using commercial bowl cleaning products and scrub pads, and flushed repeatedly to rinse. Sterile disks were installed in the toilets and the toilets were flushed immediately prior to sampling. To collect samples for microbiological analysis, the bowl surfaces were scraped with sterile wooden applicator sticks at positions L, M and H, in left-right duplication, using a 1 cm^2 template to demarcate the sampling area. For both L and M scrape samples, the toilets were first drained to expose the bowl surface. Two applicator sticks were used per area, for 30 s each, then deposited into a single vial containing 5 ml of sterile phosphate buffered saline (PBS). The sticks were stirred vigorously for 30 s to dislodge biofilm then disposed of. Bowl temperature and pH were monitored continuously during the sampling period, and recorded approximately every 30 min. Residual chlorine in the bowl water was measured at the start of the sampling session, immediately after flushing and approximately 30 min after flushing. Residual chlorine was determined in the field by the DPD colorimetric method for total chlorine and absorbance measurements were immediately taken with a portable spectrophotometer. Chlorine was neutralized in one toilet (C) by addition of 14 $mg\,l^{-1}$ (final concentration) sodium thiosulfate through a flush-actuated dispenser installed in the toilet tank.

Subsequent weekly sampling included collection of biofilm scraped from the disks. Each week, 6 disks were removed per toilet from high, middle and

low on the left and right side of the bowl. Removal of disks below and at the waterline was accomplished while the disks were submerged. Disks were selected for removal on the basis of a sampling pattern randomized with respect to the specific location in the rubber strip.

At each sampling period, assigned disks were removed from the rubber holders, quickly immersed in bowl water to wet them and then immobilized in a specially designed holder. Disks were scraped until dry. Bowl scrapes, grab samples of bowl water and other water quality data were collected following the procedure described above for time zero, with water samples being taken immediately after flushing. Samples destined for microbiological analysis were returned to the laboratory for processing. Water samples collected from each site were examined for selected metals which were determined by atomic absorption with direct aspiration.

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Microbial Enumeration

Five ml volumes of PBS 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 (Difco, Detroit MI) medium and incubated at room temperature for 6 d. All samples were processed within 5 h of collection.

Microbial Identification

Biofilm bacteria were collected and isolated by restreaking. Isolates were picked from R2A spread plates at countable dilutions and subcultured 1–3 times prior to further characterization. Due to the inherent difficulty in imposing a truly random selection process on the choice of colony types to be examined, a "present or absent" system was adopted as the basis for speciation. Colony types were selected for further examination based upon visual estimation (by colony size, color, morphology) of either presence in great numbers or constant presence. A group of over 50 toilet bowl isolates was amassed, with roughly equal numbers originating from biofilm grown below, at and above toilet bowl water lines, as well as from samples of the toilet bowl water.

The classification process followed one of two paths. Group screening was done by plating sample homogenates directly onto various selective and differential media, including MacConkey's (for an indication of coliform presence or absence) and MacConkey's with 100 mg l^{-1} 4-methyl-umbelliferyl-beta-D-glucuronide (MUG) added (for *E. coli* specifically). Isolates

were also subcultured on various selective and differential media, and further grouped by Gram stain reaction. If growth rates permitted, each isolate was then identified using an appropriate API biochemical test kit. Positive and negative control organisms were included with each group of isolates examined.

Microscopy

Cross-sections of frozen biofilm were prepared by cryoembedding (Yu *et al.*, 1994; Murga *et al.*, 1995). Five µm-thick sections were stained with the DNA stain 4',6-diamino-2-phenylindole (DAPI) and examined using an Olympus BH-2 microscope with epifluorescent illumination. The filter combination used included an exciter filter at 490 nm, dichroic mirror at 500 nm and a barrier filter at 515 nm.

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Data Analysis

A linear statistical model was fitted to the data. Specifically, an analysis of the variance (ANOVA) was performed in S-Plus with $\log_{10}$ transformed cell densities treated as response, toilet treated as a random factor and time and water level treated as fixed factors (Montgomery, 1991). The log transformation of cell densities was necessary to stabilize their variances over time, toilet and water level, which the assumptions for ANOVA require.

The model allowed comparison of the variation in the responses between toilets to the variation within toilets, and to detect other effects, such as responses that differ across water levels, that may merit further study. The components of variation were estimated using the method of restricted maximum likelihood (REML).

Pearson product moment correlations, denoted by r, provided measures of the strength of linear relationship between $\log_{10}$ cell densities on porcelain disks and on toilet surfaces, and between $\log_{10}$ cell densities on porcelain disks and in the bowl water (Montgomery, 1991).

It was expected that during the early part of the study biofilm would accumulate at an exponential rate according to the model $y = ae^{bt}$, where y is cfu cm^{-2}, t is time in days, a is cell density at time 0, and b is a rate parameter. Parameter b is called the specific fouling rate and has units $days^{-1}$. The specific fouling rate was estimated by linearizing the equation $y = ae^{bt}$ and fitting a least squares line. Time units of days were used in this calculation to allow for comparison with specific growth and fouling rates reported in other studies.

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RESULTS AND DISCUSSION

Biofilm Accumulation

A strong correlation ($r = 0.85$) was observed between areal cell densities on porcelain sampling disks and cell densities on toilet bowl surfaces (Figure 3). This relationship suggests that biofilm accumulation as measured using the removable-disk method is representative of natural accumulation on a toilet bowl surface.

Microbial biofilm accumulated on ceramic sampling disks in all toilets over the observation period of 6 weeks (Figure 4). Specific biofilm fouling rates, calculated from the first three weeks of cell count data, ranged from 0.0 to 0.46 d^{-1} (Table I). The specific fouling rate averaged over all toilets and all water levels was 0.23 d^{-1} for the first 3 weeks. This average rate is well below the specific growth rate that is attained in most laboratory cultures, but is typical of the relatively slow growth that is encountered in nature (Brock, 1971). In addition, this study was conducted in Bozeman, Montana, where year-round tap water temperatures are relatively low and chlorine residuals relatively high (1 mg l^{-1}). It is possible that in a different geographic location, significantly different growth rates would be observed.

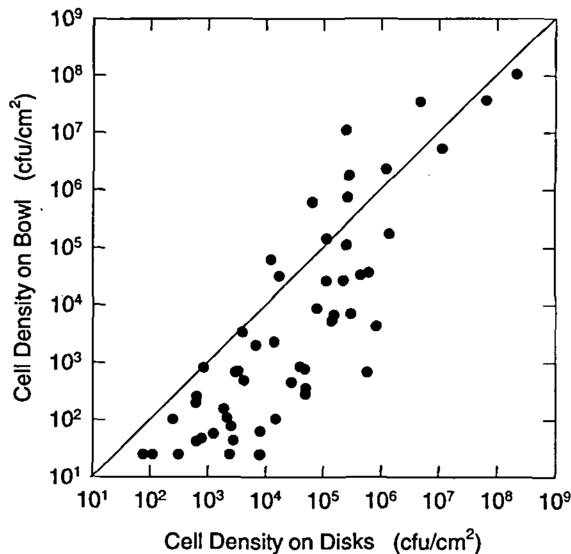

FIGURE 3 Correlation between cell densities on bowl surface and on ceramic sampling disks.

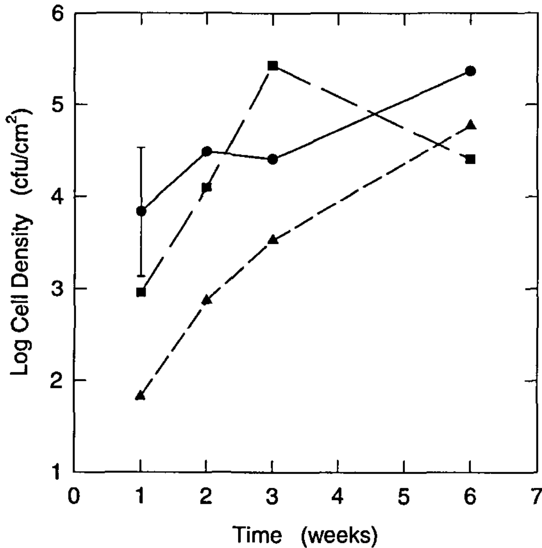

FIGURE 4 Cell densities averaged over all water levels for toilets A (▲); B (●); and C (■). The error bar shows the SE of the mean.

TABLE I Fouling rates (day^{-1}) with SEs in parentheses

Position	*Toilet*			
	Toilet A	*Toilet B*	*Toilet C*	*Average*
High	0.26 (0.081)	0.20 (0.056)	0.39 (0.079)	0.24 (0.06)
Middle	0.26 (0.14)	0.00 (0.069)	0.46 (0.050)	0.24 (0.10)
Low	0.34 (0.084)	0.09 (0.084)	0.36 (0.059)	0.28 (0.11)
Average	0.28 (0.006)	0.095 (0.07)	0.41 (0.06)	

Biofilm viable cell areal densities at the end of 6 weeks ranged from approximately 2×10^3 to 1×10^7 cfu cm^{-2}, depending on the toilet and the zone (Table II). The extent of fouling was highest below the water level and lowest above the water level (Table II; Figure 5). No similar pattern was evident between specific fouling rates and water level (Table I). The specific fouling rate was not obviously related to the endpoint accumulation of biofilm after 6 weeks. For example, toilet B had the lowest specific fouling rate during the first 3 weeks, but was ultimately the most heavily fouled.

The estimated components of variation indicate that variation in log cfu cm^{-2} among toilets is about twice as large as the variation between disks within a toilet at any given time and water level. Variability among toilets

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TABLE II Biofilm accumulation after 6 weeks expressed as $\log_{10}$ of cfu cm^{-2}. The approximate SE for these measurements is 0.7. (The SE is an average over all time points, obtained by fitting a linear statistical model, and is the same for each data point)

Position	*Toilet*			
	Toilet A	*Toilet B*	*Toilet C*	*Average*
High	4.09	3.59	3.27	3.65
Middle	5.06	5.44	4.82	5.11
Low	5.18	7.06	5.14	5.79
Average	4.78	5.36	4.41	4.85

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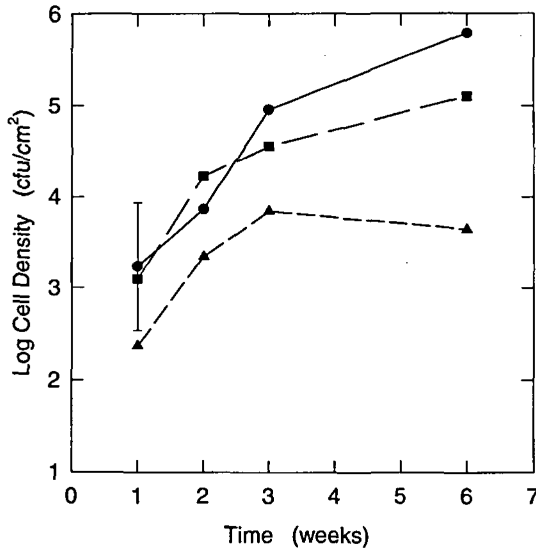

FIGURE 5 Cell densities averaged over all toilets for each of the water levels H (▲); M (■); and L (●). The error bar shows the SE of the mean.

may be somewhat exaggerated because of the quick accumulation and rapid drop of cell densities in toilet C. Accelerated accumulation in this case (Table I) may be a result of chlorine neutralization in the toilet. The drop coincides with the time that one of the occupants of residence C was taking a 10 d course of antibiotic.

Biofilms became visible to the naked eye when they reached cell densities of approximately 10^5 cfu cm^{-2}. For example, fouling was visible by eye in toilet B, zone L at 3 weeks. In contrast, in toilet A, fouling was first apparent at 5 weeks, in zone H. Toilet C on the other hand, failed to show visual evidence

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of biofilm accumulation at all, other than a small, isolated patch at front and center, zone M, which became visible at 7 weeks. Although these visibility thresholds all occurred at or near a cell count of 10^5 cfu cm^{-2}, it is not clear whether visibility of the biofilms was due to accumulation of cells or other organic or abiotic materials.

Taking the order of magnitude of the cell density in a biofilm to be 10^{11} cfu cm^{-3}, a biofilm with an areal density of 10^5 cfu cm^{-2} would only be a fraction of a monolayer, *i.e.* 0.01 μm on average. Since a biofilm this thin would not be expected to be visible, it may be that the biofilms were patchy or that they accumulated colored abiotic material that made them more visible. The thickness of a 13-week old biofilm, with an average cell density of approximately 10^7 to 10^8 cfu cm^{-2}, ranged from a few to 20 μm (Figure 6). This is consistent with the order of magnitude average thickness calculated as above, which would predict a thickness of approximately 1 to 10 μm.

The original experimental design for this study called for replacing pulled disks at weeks 1 and 3. In laboratory experiments following this field study, it was shown that such replacement disks tended to take on the fouling characteristics of the environment instead of fouling as if placed in a clean toilet. In other words, a disk replaced at week 5 and pulled at week 7 would be as fouled as if it had been in place for 7 weeks, rather than showing two week old growth. This casts doubt on the replacement-disk data collected at weeks 5 and 7. Accordingly, these data sets were not used in subsequent data analysis.

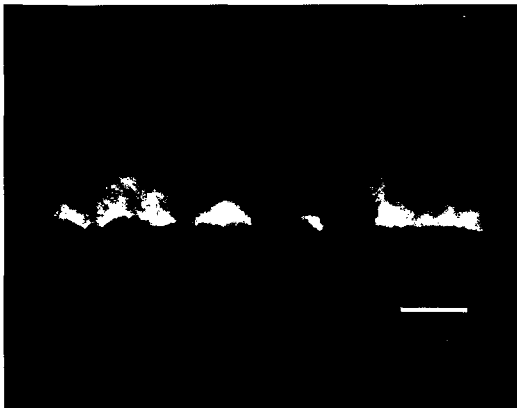

FIGURE 6 Photomicrograph of a toilet biofilm cross-section. This specimen was collected from a 13-week old toilet bowl biofilm accumulated on a porcelain-ceramic sampling disk located at the water line. The average areal cell density was 10^7–10^8 cfu cm^{-2}. Bar = 20 μm.

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Relationship Between Biofilm and Bulk Water

Relevant chemical and microbiological water quality parameters are summarized in Table III. Bowl water temperatures fluctuated within the range of approximately 4 to 12°C, with an average of 8.9°C. The true average bowl temperatures are probably somewhat higher, as the temperature rises during long periods of time without flushing, and the temperatures recorded during this experiment were not time-averaged. The average pH was 7.38. The average residual chlorine measured at the beginning of sampling sessions (at least 30 min after the last flush) was undetectable in the toilet with neutralizer and 0.42 $mg\,l^{-1}$ in the other two toilets. The neutralizing effect was complete in toilet C except for an isolated period of a few days when the dispenser failed to function properly. Residual chlorine in bowl water immediately after flushing was 0.9 to 1 $mg\,l^{-1}$ in the unamended toilets and in all the tap water samples collected. Heterotrophic plate counts for all three bowls yielded average cell densities of 4×10^3 $cfu\,ml^{-1}$. It might be expected that since water samples were taken immediately after flushing, planktonic cell count data would be more representative of the incoming water than of the attached bacterial community. However, planktonic cell counts in the bowl water were positively correlated with biofilm areal cell densities (Figure 7). This correlation suggests that flushing has a sloughing effect on the biofilm, causing cells to be rapidly deposited into the bowl water.

Microbial Identification

Of the approximately 50 toilet bowl isolates collected and frozen, 32 were retrieved and examined further. Nineteen isolates grew at rates that

TABLE III Toilet bowl water quality parameters

Parameter	*Toilet*		
	A	*B*	*C*
pH	7.0–7.61	6.87–7.64	7.06–7.7
Temperature (°C)	6.3–15.9	4.5–12.9	4.5–12.3
Total chlorine ($mg\,l^{-1}$) (average for bowl water immediately after flushing)	0.9	0.9	0.2
Total copper ($mg\,l^{-1}$)	0.028	0.023	0.018
Iron	0.08	0.11	0.10
Manganese	<0.001	<0.001	<0.001
Lead	<0.001	0.001	0.002
Planktonic cells ($cfu\,ml^{-1}$)	3.44×10^3	5.33×10^3	5.37×10^3

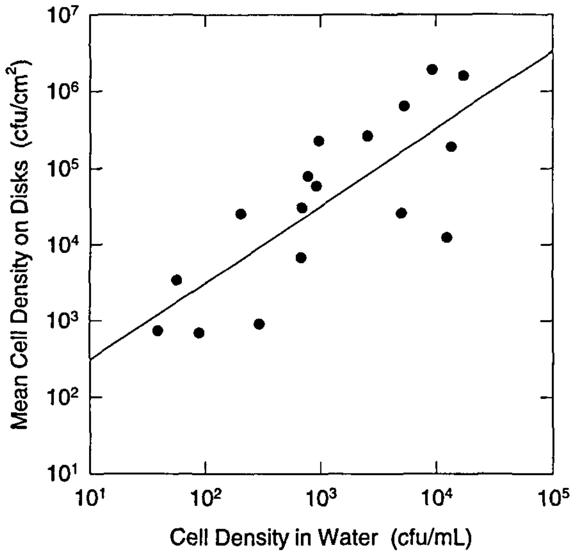

FIGURE 7 Correlation between cell densities on ceramic sampling disks and in toilet bowl water.

TABLE IV Toilet biofilm microbial identification

Profile	*Number of positive identifications*
Unreliable identification	3
Pseudomonas fluorescens	2
Pseudomonas putida	2
Chryseomonas luteola	2
Pseudomonas sp.	2
Sphingomonas paucimobilis	1
Pseudomonas vesicularis	1

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were compatible with API tests; 12 were found to be Gram-negative and 7 Gram-positive. The Gram-negative group was characterized, and pseudomonads and pseudomonas-like organisms dominated (Table IV). Efforts to quantify the enteric component of the microbial community using MacConkey's and MUG provided less than 1% positive response per sample studied. The groups of bacteria that predominated in the toilet bowl biofilms studied do not in general appear to be organisms of fecal origin, but rather those that would tend to be populous in similar aquatic environments.

SUMMARY AND CONCLUSIONS

Methods were developed and applied to characterize the accumulation of microbial biofilm in toilet bowls. Biofilm formed readily on toilet bowl surfaces. After 7 weeks the extent of biofouling ranged from 10^3 to 10^7 cfu cm^{-2} with the heaviest accumulation being as thick as tens of microns in places. The threshold for visibility of toilet bowl biofilm to the unaided human eye was approximately 10^5 cfu cm^{-2}. Specific fouling rates averaged 0.23 d^{-1} during the first 3 weeks of biofilm growth, a rate that is consistent with a periodically fed but otherwise oligotrophic environment. Biofilm accumulated despite the presence of a total chlorine residual of nearly 1 mg l^{-1}. This shows that bacteria even in very thin biofilms, in which transport limitation of chlorine delivery is unlikely, are able to mount protective defenses against this antimicrobial agent. Variability in the fouling within different zones of a toilet bowl was less than the variability in fouling that occured between different toilets. In conducting a field study, therefore, it is recommended that multiple toilets be sampled. Preliminary characterization of the bacterial component of the biofilm revealed pseudomonas-like organisms. This indicates that toilet bowl biofilm bacteria are not of fecal origin but rather are typical water-borne species.

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