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Monika Schwering^a, Joanna Song^a, Marie Louie^b, Raymond J. Turner^a & Howard Ceri^a

^a Department of Biological Sciences, Biofilm Research Group, University of Calgary, Calgary, Canada.

^b Provincial Laboratory for Public Health, Alberta Health Services, Calgary, Canada.

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Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection

Monika Schwering^a, Joanna Song^a, Marie Louie^b, Raymond J. Turner^{a*} and Howard Ceri^{a*}

^aDepartment of Biological Sciences, Biofilm Research Group, University of Calgary, Calgary, Canada; ^bProvincial Laboratory for Public Health, Alberta Health Services, Calgary, Canada

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A model biofilm, formed of multiple species from environmental drinking water, including opportunistic pathogens, was created to explore the tolerance of multi-species biofilms to chlorine levels typical of water-distribution systems. All species, when grown planktonically, were killed by concentrations of chlorine within the World Health Organization guidelines (0.2–5.0 mg l⁻¹). Higher concentrations (1.6–40-fold) of chlorine were required to eradicate biofilm populations of these strains, ~70% of biofilms tested were not eradicated by 5.0 mg l⁻¹ chlorine. Pathogenic bacteria within the model multi-species biofilms had an even more substantial increase in chlorine tolerance; on average ~700–1100 mg l⁻¹ chlorine was required to eliminate pathogens from the biofilm, 50–300-fold higher than for biofilms comprising single species. Confocal laser scanning microscopy of biofilms showed distinct 3D structures and multiple cell morphologies and arrangements. Overall, this study showed a substantial increase in the chlorine tolerance of individual species with co-colonization in a multi-species biofilm that was far beyond that expected as a result of biofilm growth on its own.

Keywords: biofilms; drinking water; chlorine resistance; community biofilms; water microbiology

Introduction

Traditional drinking water systems are composed of three distinct sections: source water obtained from surface or ground water, primary treatment processes composed of various filtration and/or disinfection steps, and the water distribution system which maintains the integrity of the treated water as it is transported to the consumer. The maintenance of a residual disinfectant (also known as secondary disinfection) is required throughout the distribution system. The World Health Organization (WHO) guidelines for drinking water quality stipulate that a residual level of 0.2 mg l⁻¹ chlorine be maintained throughout distribution systems, with a maximum of 5.0 mg l⁻¹ (WHO 2011). Residual chlorination is designed to prevent the re-growth of bacteria, eliminate any organisms that may enter a system, and retard the growth of biofilms within that system. Despite this precaution, biofilms still occur within the distribution system lines (Flemming 2002; Martiny et al. 2003; Wingender & Flemming 2004; Obst & Schwartz 2007). Microbiological monitoring only includes testing of planktonic indicator organisms, and does not account for biofilms present within the system, within which, it is estimated that up to 95% of the bacterial biomass of the system may be found (Flemming 2002).

Growth as a biofilm can confer a number of advantages to microorganisms. These include protection from or

increased resistance/tolerance to a number of antimicrobial agents at concentrations 10–1000-fold higher than those tolerated by planktonic cells (Ceri et al. 1999; Mah & O'Toole 2001; Hoiby 2010). Biofilms have also been shown to have increased resistance or tolerance to many different antibiotics (Hoyle & Costerton 1991; Stewart 2002), environmental toxins such as heavy metals (Harrison et al. 2007), and disinfectants such as quaternary ammonia compounds (Campanac et al. 2002) and chlorine (LeChevallier et al. 1988a). Characteristics contributing to the antimicrobial resistance of biofilms include a physico-chemical diffusion barrier created by extracellular polymeric substances (EPS); a reduced growth rate, in part due to oxygen and nutrient limitation; the activation of general stress response genes; higher rates of mutations and horizontal gene transfer, as well as a different overall metabolic physiology (Mah & O'Toole 2001; Harrison et al. 2007; Ceri et al. 2010; Bridier et al. 2011).

Biofilms begin to establish on the materials of new distribution systems after only a few weeks of use, although growth rate is dependent on the prevailing conditions within a particular system (Obst & Schwartz 2007). A high degree of biofilm heterogeneity and microcolony formation has been described on water system pipe materials (Percival et al. 1998; Boe-Hansen et al. 2003; Martiny et al. 2003; Wingender & Flemming

*Corresponding authors. Email: turnerr@ucalgary.ca; ceri@ucalgary.ca

2004) with mature biofilms covering ~76% of the surface (Martiny et al. 2003). A study examining the different characteristics of water systems that may influence the disinfection efficiency of chlorine (age, attachment, nutrients, and surface) found that attachment to a surface as a biofilm was the most important factor in increased disinfection resistance and tolerance (LeChevallier et al. 1988a).

Despite primary and secondary disinfection steps, a number of pathogenic or opportunistically pathogenic strains of bacteria are occasionally found within water systems. Microbiological parameters for routine monitoring of drinking water do not regulate for all pathogens but rather select specific indicator organisms that signify a contamination event or problem with a disinfection step. These indicator organisms include *Escherichia coli* and other coliform bacteria. In addition to *E. coli*, this group is represented by *Enterobacter cloacae*, which is often isolated from soil and untreated water and, of the *Enterobacter* species, has the highest pathogenic potential, causing 60–75% of all *Enterobacter* infections (Hennigs et al. 2011). Coliforms and *E. coli* have significance in a water system as potential pathogens as well as indicators of the possible presence of other microorganisms. Other bacteria of concern, especially in clinical settings, include opportunistic pathogens *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. Non-indicator organisms present in drinking water systems are often referred to as heterotrophic plate count (HPC) bacteria, and *P. aeruginosa* is often enumerated as part of the HPC in routine monitoring. *S. maltophilia*, has a high level of intrinsic antibiotic resistance to imipenem and other beta-lactam antibiotics, and has been identified as of considerable concern in certain environments as it may act as a reservoir for antimicrobial resistance genes (Bollet et al. 1995; Crossman et al. 2008; Nyc & Matejkova 2010).

Alongside any opportunistic pathogens, many other species of bacteria coexist in the biofilm community. A number of studies have reported on the identification of organisms present within various drinking water systems (Williams et al. 2004; Eichler et al. 2006; Hong et al. 2010). Bacteria identified routinely include: Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, specifically the Alphaproteobacteria Sphingomonadaceae (Bereschenko et al. 2010), *Methylobacterium* sp. and Bradyrhizobiaceae and Betaproteobacteria *Acidovorax* sp. and *Burkholderia* sp.; Actinobacteria including *Mycobacterium* spp; *Nitrospira* spp; Bacteroidetes and Planctomycetes (Kalmbach et al. 1997; Kalmbach et al. 1999; Martiny et al. 2003; Schmeisser et al. 2003; Tokajian et al. 2005). Some studies reported that ~70–80% of the community of microorganisms present within a water system could be represented by only 6–8 operational taxonomic units (Kalmbach et al. 1997;

Simoes et al. 2007). Adhesion deficient strains of bacteria are also able to form more proficient biofilms in the presence of heterotrophic organisms (Buswell et al. 2001; Castonguay et al. 2006; Gao et al. 2008). Some of the organisms detailed above would probably not be cultivable using standard methods for the isolation of HPC bacteria from water systems, due to the selective effects of media type, incubation length, and incubation temperature (Allen 2004). Discussions on whether limits should be set on the maximum allowable concentration of HPC organisms in water systems reason that the disinfectant levels required to eliminate them would be high enough to produce by-products that would be more hazardous to the general population than the organisms themselves (Hardalo & Edberg 1997; Allen 2004).

It is important to understand how the growth of a biofilm community in a drinking water system may lead to microorganisms surviving residual chlorination in order to be able to retard the formation of these biofilms and respond faster to the contamination of water systems. Biofilms can become a reservoir for pathogenic organisms leading to further or long-term contamination of the system. This in turn may require expensive disinfection measures, advisory notices to consumers to boil water, and illness in the serviced community. The overall objective of this study was to examine the growth and survival of single- and multi-species biofilms of environmental microorganisms isolated from drinking water systems, both with and without clinical important species. More specifically, the effects of chlorination at residual levels similar to drinking water distribution systems were used as a guide. This allowed the evaluation of changes in the survival of individual species as well as biofilms comprising multiple species.

Materials and methods

Bacterial strains and culture media

The bacterial strains used in this study were collected from treated drinking water distribution systems, with the exception of the *E. coli* strain MEC-8 which was isolated from an untreated private well. The low nutrient growth medium Reasoner's 2A (EMD, Gibbstown, NJ, USA) as R2A agar and R2B broth, was used to cultivate and recover biofilms (Reasoner & Geldreich 1985). Three selective differential media were used to recover and identify opportunistic pathogens from multi-species biofilms. *E. coli* MEC-8 and *E. colaceae* MTC-21 were isolated using differential coliform (DC) agar (Oxoid, Nepean, ON, Canada) amended with Cefsulodin (Sigma-Aldrich, St Louis, MO, USA) as per the manufacturer's directions. Oxidation-fermentation agar (Difco, Becton, Dickinson & Company, Sparks, MD, USA) with lactose

(OFL; Sigma-Aldrich) was used for recovery and differentiation of *P. aeruginosa* and *S. maltophilia* from mixed-species biofilms.

Cultivation of biofilms in the Calgary biofilm device

The Calgary biofilm device (CBD) was utilized to culture both single- and multi-species biofilms (Ceri et al. 2001). Bacterial strains were cultured from freezer stocks on R2A. Cells from a second subculture on R2A were suspended in 2–3 ml of sterile saline to a turbidity equal to 1.0 McFarland standard, measured visually. For the inoculation of a single-species biofilm, the suspension was then diluted 1:30 in R2B. For the inoculation of a multi-species biofilm, a suspension of each strain of bacteria was added to R2B at a dilution of 1:100. A 150 µl aliquot of the diluted inoculum in R2B was added to each well of a 96 well microtiter plate. The polystyrene pegs of the CBD microtitre plate lid were submerged in the inoculum and the plate was incubated at 25 °C on a gyratory shaker set to 125 rpm. For single-species biofilms grown longer than 24 h, growth medium was replenished by adding 150 µl of R2B to a new microtiter plate and moving the CBD into the new medium. Single-species biofilms grown on the CBD were evaluated for equivalency across all areas of the device by comparing cell viability from pegs across rows and columns of the CBD. Statistical analysis was performed on the data to evaluate whether the biofilm growth was statistically different between the pegs of the CBD. A one-way analysis of variance (ANOVA) and Tukey's *post hoc* multiple comparison test were conducted on the CFU peg⁻¹ values using 'row' and 'column' as the treatment terms. There was no significant difference in the viable cell counts in terms of the location of the pegs on the CBD ($p > 0.05$) for any bacterial strain in the study. Similarly,

this one-way ANOVA was used to evaluate the significance of differences in susceptibility to chlorine for both mixed- and single-species biofilms.

The bacterial strains used in the assay, as well as the duration of growth, schedule for changing the medium and initial viable cell density (CFU peg⁻¹) for each isolate as a single-species biofilm are summarized in Table 1.

Multi-species biofilms were grown for 9 days, using the following inoculation schedule: day 0 *Bradyrhizobium* sp. B1–1; day 3 *Methylobacterium isbiliense* MWI-1 and *Mycobacterium* sp. MWI-2; day 5 *Sediminibacterium* sp. C0–3; day 7 *Novosphingobium subterraneum* FH-D, *Sphingomonas* sp. FH-G, *Blastomonas natatoria* FH-J, and *Variovorax* sp. A3–1; day 8 *Cupriavidus respiraculi* A3–2 and *Kocuria rhizophila* C0–6; day 9 one of four opportunistic pathogen strains, *E. coli* MEC-8, *E. cloacae* MTC-21, *P. aeruginosa* PAE-1, or *S. maltophilia* FH-W1. Biofilms were grown for 24 h after inoculation of the opportunistic pathogen before being exposed to chlorine, with the exception of multi-species biofilms with *P. aeruginosa* PAE-1, which were grown for 20 h only.

Exposure of the biofilm to chlorine and recovery

Mature biofilms were challenged with increasing concentrations of free chlorine prepared from sodium hypochlorite (Clorox, Brampton, ON, Canada) in a minimum biofilm eradication concentration assay (MBEC; Ceri et al. 2001). The concentration of free chlorine in a stock solution was measured in triplicate using a Hach DR/700 hand-held colorimeter (Hach, Loveland, CO, USA; method 52.05.1) and Hach DPD free chlorine reagent pouches. The stock was then diluted in PBS to obtain a working solution equal to the highest concentration of

Table 1. Bacterial strains used in single-species biofilm assays with the growth schedule and the final mean viable cell counts of the biofilms (CFU peg⁻¹).

Bacterial strain	Total incubation	Medium changed	Mean biofilm viable cell count (CFU peg ⁻¹)
Opportunistic pathogens			
<i>Escherichia coli</i> MEC-8	24 h	–	2.03×10^5
<i>Enterobacteriaceae cloacae</i> MTC-21	24 h	–	7.41×10^6
<i>Pseudomonas aeruginosa</i> PAE-1	20 h	–	5.78×10^6
<i>Stenotrophomonas maltophilia</i> FH-W1	24 h	–	2.23×10^6
Normal microflora			
<i>Variovorax</i> sp. A3–1	3 d	48 h	5.08×10^4
<i>Cupriavidus respiraculi</i> A3–2	48 h	–	4.15×10^6
<i>Bradyrhizobium</i> sp. B1–1	9 d	Every 72 h	3.02×10^4
<i>Sediminibacterium</i> sp. C0–3	4 d	48 h	2.81×10^5
<i>Novosphingobium subterraneum</i> FH-D	3 d	48 h	1.16×10^6
<i>Sphingomonas</i> sp. FH-G	3 d	48 h	7.22×10^5
<i>Blastomonas natatoria</i> FH-J	3 d	48 h	8.08×10^5
<i>Methylobacterium isbiliense</i> MWI-1	6 d	Every 48 h	8.61×10^4
<i>Mycobacterium</i> sp. MWI-2	6 d	Every 48 h	4.71×10^4

Note: Viable cell count $n = 48$.

chlorine to be used in the assay ($\leq 124 \text{ mg l}^{-1}$ free chlorine, depending on the organism under evaluation for single-species biofilms, $\leq 3000 \text{ mg l}^{-1}$ for multi-species biofilms). The 96 well microtiter challenge plate was created by performing regular dilutions of the working chlorine solution in PBS from the highest chlorine level to the lowest in columns 1–11. The last column contained 200 μl of PBS, as the null (zero) chlorine control. Once the biofilms had reached maturity, the CBD plate was removed from the gyratory shaker and the pegs with adherent biofilms were rinsed ($2\times$) in microtiter plates containing 200 μl of sterile saline per well for 1 min to remove loosely adherent cells and excess media from the biofilms. The CBD plate was then placed in the chlorine challenge solution for 10 min. After the challenge, the pegs were again rinsed ($2\times$) for 1 min per rinse in saline and then placed in the recovery plate. The recovery plate contained 200 μl R2B with 0.5% Tween 20, except for biofilms of *Novosphingobium subterraneum* FH-D which was found to be sensitive to the detergent. Chlorine levels were below detection limits after the addition of the R2B media; therefore, no other neutralizing agents were used in the recovery plate. Biofilms were sonicated (Ultrasonic cleaner 5510; Branson, Danbury, CT, USA) for 10 min in the recovery broth to remove cells from the pegs. For biofilms comprising single species, the null (0) chlorine control was serially diluted and 20 μl were spot plated on R2A agar to enumerate the CFUs peg^{-1} in the biofilm. For all other wells 40 μl of the sonicate ($1/5$ of the total volume) were spot plated on R2A agar to evaluate the survival of bacteria.

Multi-species biofilms were recovered using two different culture media: the growth medium R2A and one selective/differential plate medium for the specific opportunistic pathogen under observation (DC agar for *E. coli* MEC-8 and *E. cloacae* MTC-21; OFL for *P. aeruginosa* PAE-1 and *S. maltophilia* FH-W1). Recovery on R2A, DC, and OFL was determined as described above for single-species biofilms. Recovery on the two media types allowed the establishment of a MBEC value for the multi-species community as a whole (recovery on R2A agar) as well as a MBEC value for the population of the specific opportunistic pathogen under study within the community of normal flora (recovery on DC or OFL agar). The survival of opportunistic pathogens within a multi-species biofilm was also determined using a standard water testing broth media (section SIII, Supplementary information [Supplementary material is available via a multimedia link on the online article webpage]). This methodology revealed an even higher level of pathogen survival than the plating technique.

Planktonic chlorine exposure and recovery

For each isolate, a minimum inhibitory concentration (MIC) assay for chlorine was performed on suspended

planktonic cells, in addition to the MBEC assay performed on the biofilm ($n=8$). Cells were suspended in PBS to the equivalent of a 1.0 McFarland standard (as above) and 50 μl of the culture were diluted in 20 ml PBS ($\sim 10^5 \text{ CFU well}^{-1}$). The challenge plate was prepared as described above for the MBEC assay with the exception that only 100 μl of PBS with chlorine were added to each well and the concentrations of free chlorine were $2\times$ the desired final concentration. One hundred μl of the bacterial culture in PBS were added to each challenge well (to bring the total volume to 200 μl and dilute the chlorine to the correct final concentration) and left for 10 min. After exposure, 100 μl were moved from each challenge well and added to a new microtiter plate containing 100 μl $2\times$ R2B per well to neutralize the chlorine. Wells were then spot plated on R2A as described above to obtain the total initial bacterial numbers and survival at increasing chlorine concentrations.

Confocal laser scanning microscopy

Multi-species biofilms containing opportunistic pathogens, both chlorine treated and untreated, were visualized using Confocal laser scanning microscopy (CLSM). Biofilms were stained with the Live/dead® BacLight™ bacterial viability kit (Invitrogen, USA) containing propidium iodide and SYTO 9. Pegs were removed from the CBD lid, rinsed for 1 min with saline, challenged with 16 mg l^{-1} chlorine for 10 min (if applicable), rinsed again, stained for 10 min with the Live/dead stain and then rinsed a final time with saline for 1 min. They were then visualized on the Leica Microsystems (Concord, ON, Canada) DM IRE2 spectral confocal and multiphoton microscope with the Leica TCS SP2 acoustic optical beam splitter (AOBS). A stack of images of the biofilms was taken from the center section of the peg to create a 3D rendering of the biofilm. Images were then compiled and analyzed using Imaris x64 version 7.0.0 software (Bitplane Inc. Scientific Software, South Windsor, CT, USA).

Results

Environmental isolates

The organisms selected for use in this study were all isolated from biofilms and the planktonic phase of Canadian drinking water systems. They were identified via 16S rRNA gene sequencing and represent the taxonomic groups routinely found in drinking water systems, as well as four clinically relevant organisms. The optimal order of inoculation and incubation of species in the multi-species biofilm was determined experimentally using terminal-restriction fragment length polymorphism (TRFLP) to ensure the presence of all isolates within the final biofilm community. Further details on isolate

collection and selection, as well as the creation of the TRFLP library, can be found in the Supplementary information (section SI).

The chlorine tolerance of planktonic cells and single-species biofilms

Isolates selected to represent taxonomical groups were evaluated individually for their ability to survive chlorine disinfection in both planktonic and biofilm growth phases. Single-species cultures were evaluated at relatively low levels of chlorine, a range slightly larger than may be found within water distribution systems. The level of chlorine required to eliminate populations of environmental isolates, from the drinking water system, in planktonic vs biofilm form is shown in Figure 1 ($n=8$). The bacterial populations in the biofilms ranged between 4.2×10^6 CFU peg^{-1} (*C. respiraculi* A3-2) and 3.0×10^4 CFU peg^{-1} (*Bradyrhizobium* sp. B1-1) before any chlorine treatment. The MICs of chlorine for planktonic cells of all isolates fell within the range of allowable chlorine concentrations from the WHO of between 1.0 and 2.0 mg l^{-1} free chlorine. Additionally, when grown as biofilms, only three of the nine isolates were able to be eradicated (populations reduced to below detectable levels, <5 CFU peg^{-1}) using chlorine levels lower than the suggested maximum and had MBEC $<5.0 \text{ mg l}^{-1}$ (*C. respiraculi* A3-2; *Bradyrhizobium* sp. B1-1; and *Sediminibacterium* sp. C0-3). The difference between the survival of the planktonic and biofilm forms was the smallest for B1-1 (1.6-fold greater). The remaining six isolates were able to survive

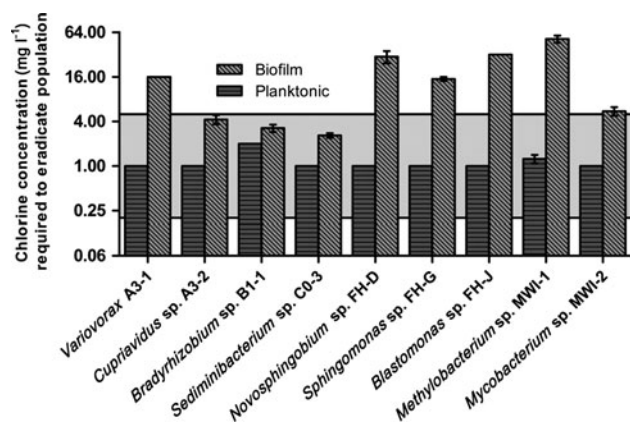


Figure 1. The MBEC (biofilm) values compared to the MIC (planktonic) values for isolates of normal drinking water flora grown as single species. The shaded area represents the WHO suggested range of allowable residual chlorine concentrations in drinking water distribution systems ($0.2\text{--}5.0 \text{ mg l}^{-1}$ free chlorine). $n=8$; error bars=standard deviation. A3-1=*Variovorax* sp.; A3-2=*Cupriavidus respiraculi*; B1-1=*Bradyrhizobium* sp.; C0-3=*Sediminibacterium* sp.; FH-D=*Novosphingobium subterraneum*; FH-G=*Sphingomonas* sp.; FH-J=*Blastomonas natatoria*; MWI-1=*Methylobacterium isbiliense*; and MWI-2=*Mycobacterium* sp.

chlorine levels $>5.0 \text{ mg l}^{-1}$. The three isolates from the family Sphingomonadaceae (*N. subterraneum* FH-D, *Sphingomonas* sp. FH-G, *B. natatoria* FH-J) as well as *Variovorax* sp. A3-1 had MBEC values well above the suggested maximum. Biofilms of *M. isbiliense* MWI-1 had the highest survival, and required $52 \pm 16.6 \text{ mg l}^{-1}$ free chlorine, $10\times$ the maximum allowable level and greater than 40-fold higher than the corresponding planktonic form. As expected all biofilms were able to survive significantly higher concentrations of chlorine than their planktonic counterparts ($p < 0.05$).

Results for the MIC and MBEC assays on the four opportunistic pathogen isolates followed a pattern similar to that of the normal water flora isolates (Figure 2). Like the normal microflora, all pathogenic isolates were able to survive significantly higher concentrations of chlorine when grown as biofilms than when planktonic ($p < 0.05$). The initial biofilm populations of the opportunistic pathogens studied ranged between 7.4×10^6 CFU peg^{-1} (*E. cloacae* MTC-21) and 2.0×10^5 CFU peg^{-1} (*E. coli* MEC-8). The MIC of chlorine for all pathogenic isolates in planktonic form fell within the range of values suggested by the WHO, as would be expected. Values to eliminate the populations fell within a small range, between $0.813 \pm 0.3 \text{ mg l}^{-1}$ (*P. aeruginosa* PAE-1) and $1.0 \pm 0 \text{ mg l}^{-1}$ chlorine (*S. maltophilia* FH-W1). MBEC values for these strains, by contrast, fell either just within

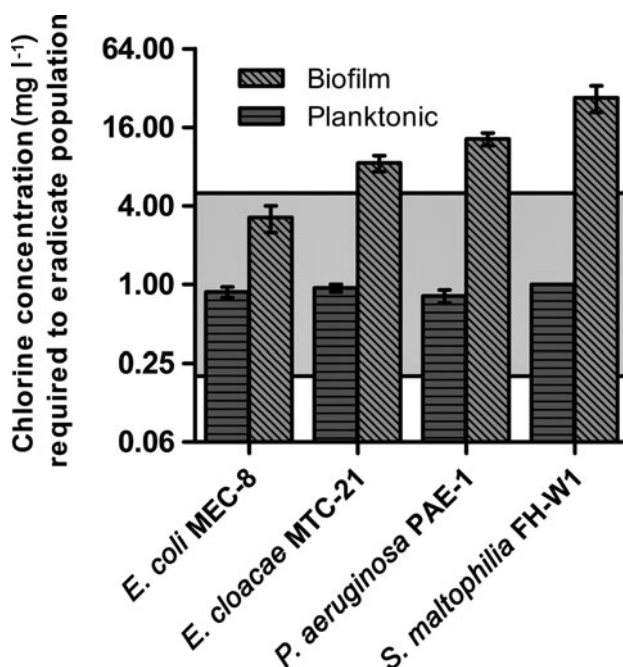


Figure 2. The MBEC (biofilm) values compared to the MIC (planktonic) values for single-species biofilms of opportunistic pathogen isolates. The shaded area represents the WHO suggested range of allowable residual chlorine concentrations in drinking water distribution systems ($0.2\text{--}5.0 \text{ mg l}^{-1}$ free chlorine). $n=14$; error bars=standard deviation.

or higher than the suggested maximum level of chlorine. Biofilms of *E. coli* MEC-8 could be eradicated with free chlorine levels $<5.0 \text{ mg l}^{-1}$ ($3.25 \pm 2.1 \text{ mg l}^{-1}$). Although still within the suggested range, the biofilms of *E. coli* MEC-8 were able to survive chlorine levels >3 -fold higher than its planktonic counterparts. The remaining three pathogenic organisms required >2 -fold the suggested maximum level of chlorine to eliminate the biofilm population, up to $27.0 \pm 17.6 \text{ mg l}^{-1}$ for *S. maltophilia* FH-W1.

In conclusion, the biofilm mode of growth consistently allowed bacterial populations to survive free chlorine concentrations higher than those tolerable by their planktonic counterparts. Between 1.6- and 40-fold, higher concentrations were required for biofilms and in many cases the amount of chlorine required was higher than the WHO guideline maximum value of 5.0 mg l^{-1} .

The chlorine tolerance of multi-species biofilm

Using a stepwise procedure of inoculation and subsequent strain addition over a 10 day period, we were able to generate a multi-species biofilm representing members of the natural drinking water community. Biofilms of mixed species reached an overall cell density of $10^6 \text{ CFU peg}^{-1}$, the distribution of which is shown in Figure S1. Organisms were originally isolated from treated drinking water systems, identified, and a subset were selected to represent a model multi-species water biofilm (further details are given in section SI of the Supplementary information). The success of this approach allowed a model multi-species community to be studied in a laboratory setting. The community was evaluated for its tolerance to chlorine exposure with and without the inclusion of opportunistic pathogens ($n = 14$). Further, the specific survival of the opportunistic pathogen within the whole biofilm community could be determined (Figure 3). A key finding of this research is the further increased tolerance of organisms occurring in a mixed-species community over their survival in a single-species biofilm.

When *E. coli* MEC-8 was incorporated into the model multi-species biofilm, the organism was found to be nearly 200-fold more resistant to chlorine disinfection than when in a single-species biofilm (Figure 3A). The concentration of chlorine required to reduce *E. coli* MEC-8 to below detectable levels ($<5 \text{ CFU peg}^{-1}$) was $643 \pm 43 \text{ mg l}^{-1}$, >125 times the suggested maximum level of chlorine in a water distribution system. In the multi-species biofilm with *E. coli* MEC-8, the entire biofilm community was only eradicated after treatment with $1000 \pm 74 \text{ mg l}^{-1}$ chlorine.

The survival of *E. cloacae* MTC-21 to chlorine in planktonic form, as a single-species biofilm, and as a member of the multi-species community is displayed in Figure 3B. As for *E. coli* MEC-8, the survival of *E. cloacae* MTC-21 increased substantially, ~ 100 -fold,

to $821.4 \pm 31.3 \text{ mg l}^{-1}$ chlorine when incorporated into the model biofilm. The community strains in this biofilm were able to survive approximately the same chlorine concentrations, $1357.1 (\pm 43.2) \text{ mg l}^{-1}$, as the control multi-species biofilm.

The third opportunistic pathogen to be incorporated into the model multi-species biofilm, *P. aeruginosa* PAE-1 exhibited the same trend of survival as was observed for *E. coli* MEC-8 and *E. cloacae* MTC-21 (Figure 3C). The multi-species biofilm with *P. aeruginosa* PAE-1 required treatment with $607.1 \pm 43.2 \text{ mg l}^{-1}$ chlorine to reduce *P. aeruginosa* PAE-1 survival to below detectable levels, a 47-fold increase in survival and 121-fold greater than the suggested maximum amount. In agreement with the biofilms incorporating *E. coli* MEC-8 and *E. cloacae* MTC-21, the normal flora of this multi-species biofilm required a higher chlorine treatment than the opportunistic pathogen ($1017.9 \pm 48.8 \text{ mg l}^{-1}$) chlorine, to be eliminated.

The same trends for the opportunistic pathogen and the biofilm community were repeated with the final pathogenic organism evaluated, *S. maltophilia* FH-W1 (Figure 3D). The highest concentrations of chlorine were tolerated by *S. maltophilia* FH-W1 both as a single-species biofilm ($13.0 \pm 1.5 \text{ mg l}^{-1}$, equal to *P. aeruginosa* PAE-1) and when incorporated into the model multi-species biofilm. As part of the multi-species consortia, $964.3 \pm 51.5 \text{ mg l}^{-1}$ of chlorine were required to reduce the population of *S. maltophilia* FH-W1 to below detection levels. This shows that the isolate was able to survive chlorine concentrations >190 -fold greater than the suggested maximum level. The normal microbiota of this biofilm was also able to survive slightly higher chlorine concentrations, $1500 \pm 58.6 \text{ mg l}^{-1}$.

An analysis of the population changes within the community of microorganisms present in the biofilm pre- and post-chlorine treatment was also performed using T-RFLP. Results are shown in section SII of the Supplementary information.

CLSM of multi-species biofilms

Biofilms were examined using CLSM and a bacterial viability stain after treatment with 16 mg l^{-1} chlorine, a concentration above WHO guideline levels but less than the MBEC values of the communities. An untreated control was also examined. Figure 4A shows a cross-section image of the model multi-species biofilm, inoculated with *E. coli* MEC-8, before any chlorine treatment. In the untreated biofilm, although some areas of stressed or injured cells (stained red) are evident, the majority of the cells are viable (stained green). A number of different cell morphologies and microcolonies are present, including cocci grouped into tetrads, larger groups of cocci, which have formed into less organized circular patterns, and bacilli that appear to be growing dispersed around

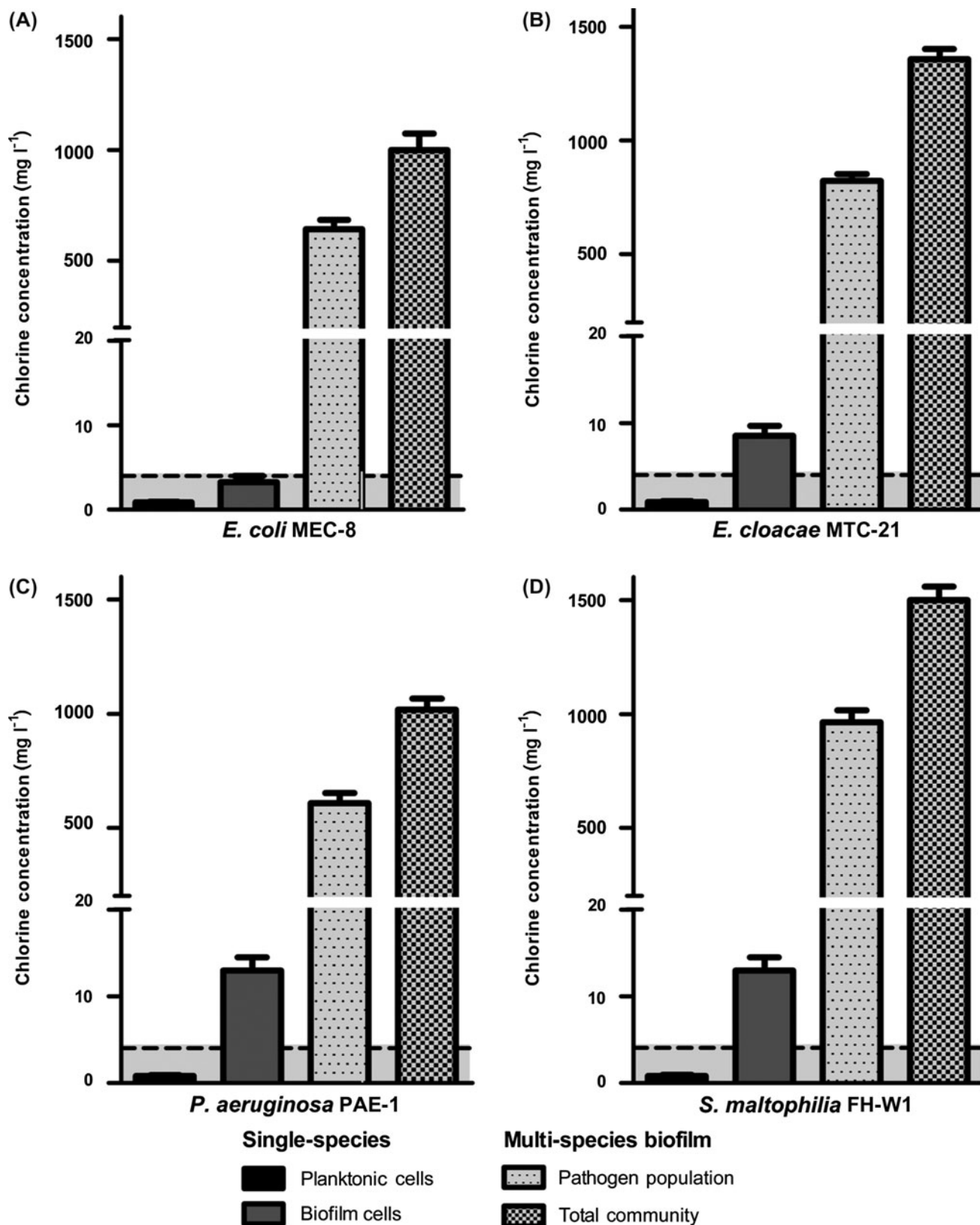


Figure 3. The MBEC values of the model multi-species biofilm with 4 opportunistic pathogens: *E. coli* MEC-8 (A), *E. cloacae* MTC-21 (B), *P. aeruginosa* PAE-1 (C), or *S. maltophilia* FH-W1 (D). The chlorine concentrations (mg l⁻¹) required to eliminate single-species planktonic (MIC) and biofilm cultures (MBEC) of each of the opportunistic pathogens are also shown for comparison. The gray shaded region indicates the range of chlorine concentrations suggested by the WHO (0.2–5.0 mg l⁻¹ chlorine). *n* = 14, error bars = standard deviation.

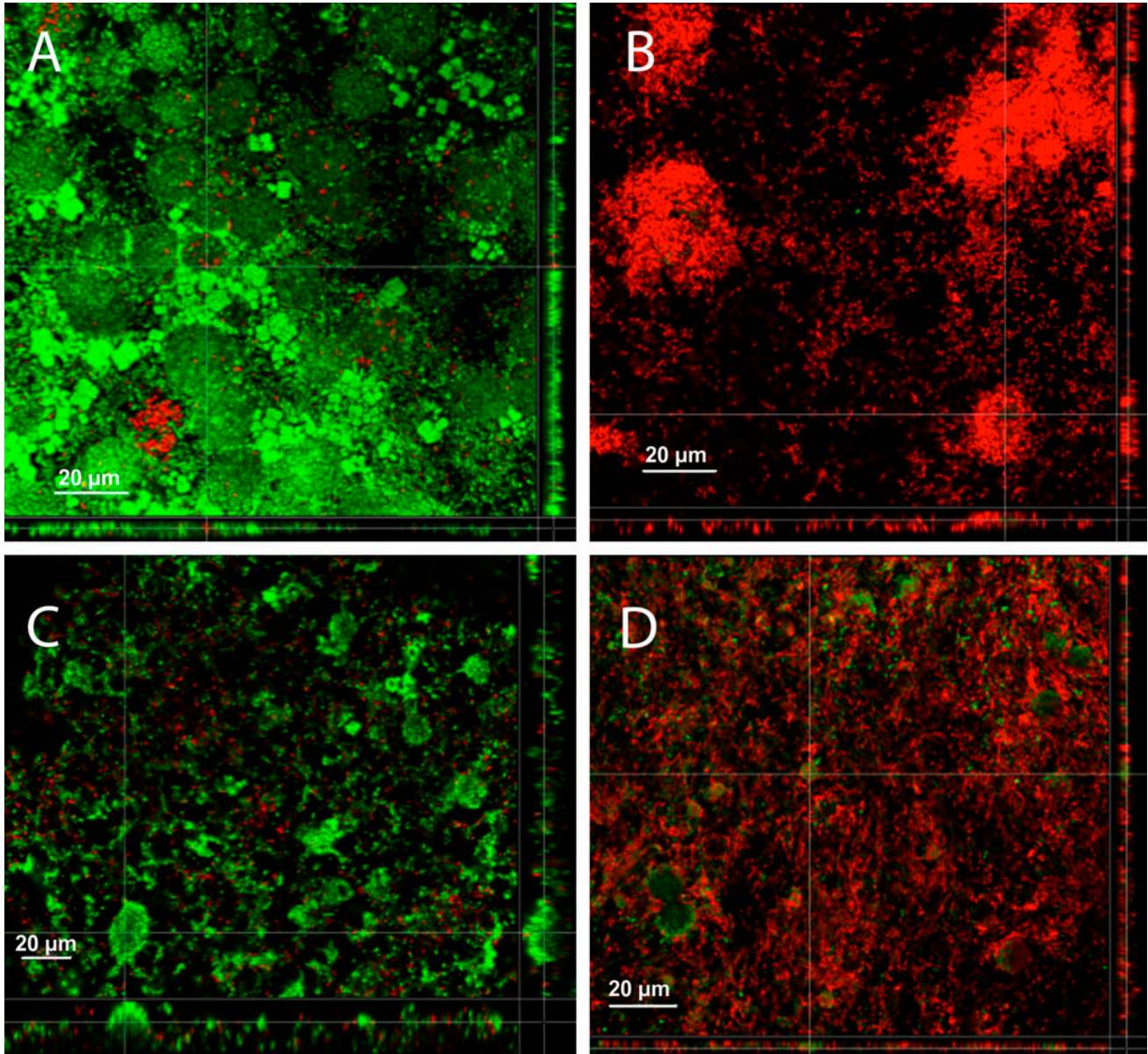


Figure 4. CLSM images of a multi-species water flora biofilm inoculated with: *E. coli* MEC-8 (A, B); *E. cloacae* MTC-21 (C, D); *P. aeruginosa* PAE-1 (E, F); or *S. maltophilia* FH-W1 (G, H) and stained with a live/dead stain. The biofilms have either undergone no treatment (A, C, E, G) or treatment with low levels of chlorination (B, D, F, H). Before treatment a 3D structure is visible within many of the biofilms as well as some microcolony formation where the biofilm appears to be thicker than the surrounding area. After treatment with chlorine for 10 min notable mortality can be observed, especially in A, with the main concentration of live cells appearing to be within the microcolonies. Additionally some cell clusters appear to be surrounded by red. This could indicate either a layer of dead cells, or possibly DNA that is encased in the EPS surrounding the cells. Either of these may act as a barrier between the live cells and the chlorine.

the microcolonies. When treated with chlorine (Figure 4B), the majority of cells are stained red (and therefore are likely to have been injured or stressed), and much of the diversity is lost. It is of note that some microcolonies are still present and the viable cells that are visible on the pegs appear to be contained within these groups. CLSM images of the untreated *E. cloacae* MTC-21 multi-species biofilm, like the images for *E. coli* MEC-8, contain mostly viable cells and a number

of different cell morphologies (Figure 4C). It is also possible to see *via* the cross-section of the biofilm that the microcolonies give the biofilm a 3D structure and the cells appear more densely packed together within these groups. Figure 4D shows the treated biofilm, in which many cells have been stressed or injured (stained red), but a higher proportion of surviving cells (stained green) occur within the microcolonies than in the surrounding areas. Images of the untreated multi-species biofilm with

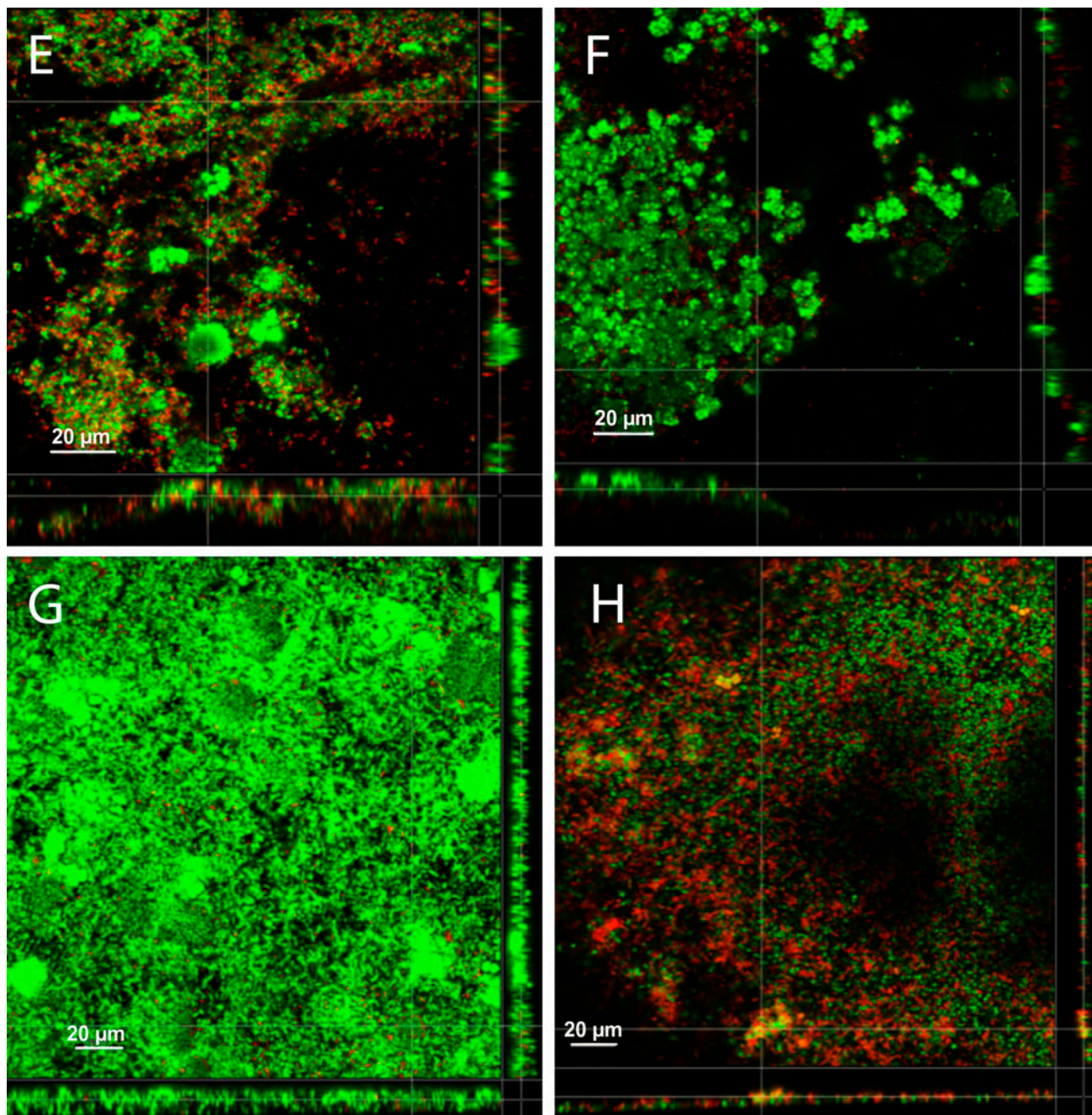


Figure 4. (Continued)

P. aeruginosa PAE-1 show that the biofilm has a distinct 3D structure, which is evident especially in the cross-section portion of the image (Figure 4E). Multiple microcolony variations are also visible. The treated biofilm with *P. aeruginosa* PAE-1 (Figure 4F) still maintains the 3D structure seen in the untreated image, as well as a high number of viable cells. A higher proportion of the cells have survived within the microcolonies *cf.* the surrounding areas of the biofilm. CLSM images of the untreated multi-species biofilm with *S. maltophilia* FH-W1 (Figure 4G) show that although the 3D structure

of the biofilm is not as complex as that seen in the biofilm with *P. aeruginosa* PAE-1, there is still a variety of cell types present which appear to be packed tightly together. Some microcolonies even appear to be surrounded by a red layer. There are approximately equal amounts of viable and stressed/injured cells visible and a number of different cell types are still visible in the treated biofilm (Figure 4H). It is of note that within the microcolonies of the treated biofilm, there seems to be about equal disinfection (red and green cells); this differs from the images of the other treated biofilms.

Discussion

The pattern of tolerance to chlorination between planktonic and biofilm populations of single species was similar for all the strains tested. Isolates of single species were consistently more resistant to chlorination as biofilms than when grown planktonically. This result is supported by numerous reports in the literature over the past few decades. Attachment to surfaces is considered the greatest factor in enhancing bacterial survival in water systems (LeChevallier et al. 1988a). However, what is notable in the present study, is that for the majority of organisms tested, the increased tolerance as a biofilm allowed them to survive past the highest level of chlorine directed for use by WHO guidelines. Although the planktonic resistance levels are similar for all the bacterial strains, the biofilm tolerance was more variable. This may be due to the different biofilm physiologies and development characteristics of the various species. For chlorination, diffusion into the biofilm is considered the primary limiting factor for disinfection (De Beer et al. 1994). Following this theory, it is possible that organisms with a higher susceptibility to chlorine are forming biofilms that are thinner, less dense or with lower EPS production. In support of this hypothesis, it was observed that the *Bradyrhizobium* sp. B1-1, which is a slow growing strain, showed little to no biofilm growth on the CBD peg after incubation for 9 days. For other, more resistant organisms, such as *M. isbiliense* MWI-1 and the Sphingomonadaceae (FH-D, FH-D and FH-J), biofilm growth was visible on the surface of the peg within 1–3 days.

The greater resistance of the biofilm populations to chlorination *cf.* their planktonic counterparts was also observed in the multi-species biofilm assay but to a greater extent. Multi-species biofilm communities composed of the natural microflora were able to survive chlorine concentrations up to 375 times the maximum legislated limit, and were 84-fold more resistant than the average biofilm tolerance of the single species. Additionally, community profiling indicated that all of the organisms used except for one (*Sediminibacterium* sp. C0-3) survived the chlorination treatment and thus had substantially increased resistance and/or tolerance as part of the community. This observation suggests that a mixed bacterial community provides some form of additional barrier beyond that of single-species biofilms. Some novel characteristics of morphologically distinct microcolonies were observed using CLSM, but there were no dramatic changes in the ultrastructure of the biofilms. This suggests that the increased tolerance is due to a physiological change in the species growing in the community that is beyond that of the species growing as an individual biofilm.

Their presence within the mixed-species community additionally increased the tolerance of the populations of

the opportunistic pathogens to far in excess of that conferred by their single-species biofilm. This suggests, even more so than the results from the single-species tolerance assays, that in a natural environment residual chlorination would be ineffective at eliminating pathogens growing as a part of the natural community biofilm. Examples exist in the literature to support the findings presented here, that multi-species biofilms often have increased biomass over each of their individual counterparts (Klayman et al. 2009) as well as increased resistance to antimicrobials due to some form of synergistic interaction (Burmolle et al. 2006).

CLSM was used to analyze the biofilms, to highlight differences in their structure and in the pattern of disinfection within the biofilms. It was observed on the cross-sections of the images that the cell density of the untreated biofilms was not constant throughout. This was most notable in the cross-section of a microcolony of the *E. cloacae* MTC-21 biofilm (Figure 3C). The ability of chlorine to diffuse into the biofilm has been reported as one of the main limitations to disinfection of drinking water system biofilms (LeChevallier et al. 1988b; Parent et al. 1996; Mah & O'Toole 2001), thus this higher cell density could act as a diffusion barrier. In images of the treated biofilms, this appears to be the case. After treatment with low-level chlorination, microcolonies are still visible on the CBD pegs and the viable (green) cells that are visible on the pegs appear to either be contained within these groups (*E. coli* MEC-8 multi-species biofilm) or the proportion of viable cells is higher (*E. cloacae* MTC-21 and *P. aeruginosa* PAE-1 multi-species biofilms). This suggests that the cells in the outer areas of the microcolony play a protective role for the inner cells.

In one of the treated biofilms (*E. cloacae* MTC-21 multi-species), groups of live cells are visible within a layer of red. While this color would normally indicate inactivated cells, the propidium iodide dye will bind DNA that is not protected by a membrane. It is possible that this is extracellular or eDNA, which for certain bacteria can be important in the development of the biofilm (Whitchurch et al. 2002; Flemming et al. 2007). It is therefore possible that the red dye has attached to eDNA that is part of the EPS surrounding the cells, which would serve to further protect the cells from chlorination. The penetration of chlorine into the biofilm matrix and ability of the disinfectant to reach the cells is a limiting factor for the disinfection efficiency of chlorine on biofilm communities. This is based on the reaction of the chlorine with organic molecules in the biofilm EPS as well as the diffusion of chlorine through the matrix (LeChevallier et al. 1988b; De Beer et al. 1994). However, these molecules also exist in single-species biofilms and therefore this does not completely explain the increased tolerance of multi-species biofilms.

Whether or not these opportunistic pathogens may pose a health risk if present in water system biofilms is still to be shown and cannot be deduced conclusively from this study, although these results demonstrate that these organisms do possess the ability to survive levels of chlorine far higher than those currently present in drinking water distribution systems.

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