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Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells

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Disinfection efficacy testing is usually done with planktonic cells or more recently, biofilms. While disinfectants are much less effective against biofilms compared to planktonic cells, questions regarding the disinfection tolerance of detached biofilm clusters remain largely unanswered. Burkholderia cepacia and Pseudomonas aeruginosa were grown in chemostats and biofilm tubing reactors, with the tubing reactor serving as a source of detached biofilm clusters. Chlorine dioxide susceptibility was assessed for B. cepacia and P. aeruginosa in these three sample types as monocultures and binary cultures. Similar doses of chlorine dioxide inactivated samples of chemostat and tubing reactor effluent and no statistically significant difference between the $\log_{10}$ reductions was found. This contrasts with chlorine, shown previously to be generally less effective against detached biofilm particles. Biofilms were more tolerant and required chlorine dioxide doses ten times higher than chemostat and tubing reactor effluent samples. A second species was advantageous in all sample types and resulted in lower $\log_{10}$ reductions when compared to the single species cultures, suggesting a beneficial interaction of the species.

Keywords: chlorine dioxide; disinfection; biofilm; detached biofilm; Burkholderia cepacia; Pseudomonas aeruginosa

Introduction

When bacteria become attached to surfaces in suitable aqueous environments, they proliferate and form microcolonies that eventually develop into biofilms. These bacterial communities produce extracellular polymeric substances (EPS) as a protective layer and are highly heterogeneous in their composition with regard to gene expression and nutrient requirements (Donlan and Costerton 2002; Davies 2003). Biofilms are much more tolerant to antibiotic and disinfectant treatment than planktonic cells partially due to the production of EPS material that serves as a protective layer which physically holds cells together but can also reactively deplete the chemicals and retard their diffusion to the cells (DeBeer et al. 1994: Donlan and Costerton 2002; Davies 2003; Steed and Falkingham 2006). In addition, it has been shown that biofilm cells express different protein profiles than planktonic cells and also display different growth rates as a result of limited nutrient and oxygen availability that can make them more tolerant (Walters et al. 2003; Rani et al. 2007).

In mixed species biofilms bacteria inhabit suitable niches in a particular microenvironment and may undergo symbiotic relationships between the different species (Møller et al. 1996, 1998). It has been suggested that cell to cell signaling in multispecies biofilms increases disinfection tolerance by boosting transcrip-tion of genes responsible for survival of oxidative stress (Loewen and Hengge-Aronis 1994), altering the composition and viscosity of the EPS (Burmølle et al. 2006), or influencing biofilm architecture and the spatial distribution of different strains (Cowan et al. 2000; Nielsen et al. 2000). These quorum sensing induced mechanisms enable bacteria to react to environmental challenges such as oxidative stress in a coordinated manner.

Additionally, environmental changes may trigger detachment of cells and clusters as part of the biofilm life cycle to guarantee survival of the community when conditions are no longer suitable for proliferation (Sauer et al. 2002; Hunt et al. 2004). Passive forms of detachment can occur when shear stress increases or certain chemicals alter the cohesion of the EPS (Simo˜ es et al. 2005, 2008; Davison et al. 2010). A wide range of cluster sizes detaches from biofilms, from single cells to large clusters containing more than 1000 cells while the proportions are dependent on the species composition and the growth conditions of the biofilm (Wilson et al. 2004; Behnke et al. 2011).

Detached clusters may behave similarly to biofilms when exposed to disinfectants especially shortly after

the detachment event. When returned to the bulk fluid, clusters may eventually shift back to protein expression profiles characteristic of planktonic growth when conditions are suitable (Rollet et al. 2009). These detached cells and clusters are of concern because they may survive disinfection challenges due to neutralization or complete consumption of the disinfectant by EPS constituents, followed by re-attachment on suitable surfaces downstream to continue growth (Costerton et al. 1995). Disinfectant doses used to kill planktonic free floating cells may not be sufficient to disinfect cells within detached biofilm clusters (Fux et al. 2004; Steed and Falkinham 2006).

Biofilm control often is performed using a strong oxidizing agent such as chlorine, ozone or chlorine dioxide. While chlorine is inexpensive and commonly used in a variety of industrial settings, chlorine dioxide, with high oxidation capability, has been described to be more effective than chlorine against biofilms (Mayack et al. 1984). In potable and waste water treatment applications, a number of researchers have commented on the significantly lower demand of the water for chlorine dioxide than for chlorine (Ridenour and Ingols 1947; Ingols and Ridenour 1948). Chlorine dioxide has been described to be more selective in polluted samples since it does not react with every compound that can be oxidized, but is more specific to organic compounds (Hoigné and Bader 1994). Benarde et al. (1967) reported that chlorine dioxide might specifically act against the protein synthesis of bacteria by attacking amino acids bonds. Jang et al. (2006) reported that chlorine dioxide at 25 ppm was able to penetrate biofilms up to 100 μm in thickness which suggests more effective killing for thinner biofilms.

The goal of this study was to determine the differential efficacy of chlorine dioxide against bacterial monocultures and co-cultures of environmental isolates *P. aeruginosa* (RB-8) and *B. cepacia* (FS-3). Co-culture of both strains is thought to be beneficial to the survival of the cells during treatment due to physical interactions or physiological adaptations of the two species. The EPS matrix may change with respect to diffusional properties or viscosity due to the presence of a second species in the biofilm caused by a shift in gene expression patterns. Other mechanisms that have been observed in multi-species biofilms are the conjugative transfer of tolerance factors (Ghigo 2001) and the protection of one species through the close spatial association with the other species (Cowan et al. 2000). Cultures grown as planktonic cells, biofilms, and detached biofilm clusters harvested from the biofilm effluent were tested and log reductions compared. Chlorine dioxide as a strong oxidizer has been demonstrated to act more specifically on cells and electron-rich centers of organic molecules and is thought to be especially effective against biofilms (Benarde et al. 1967; Mayack et al. 1984) which leads to the belief that chlorine dioxide at low concentrations will effectively disinfect biofilm samples, as well as rapidly inactivate planktonic cells and detached biofilm clusters.

Materials and methods

Two different standardized reactors were used to determine the chlorine dioxide tolerance of planktonic cells, cells detached from biofilm, and the attached biofilm. Environmental isolates of *Burkholderia cepacia* (FS-3) and *Pseudomonas aeruginosa* (RB-8) were grown as monocultures and mixed cultures in chemostats and biofilm tubing reactors.

Disinfection effectiveness was assessed for all sample types (chemostat, tubing reactor effluent, attached biofilm, and homogenized control samples) and all species scenarios (single FS-3, single RB-8, binary culture) and $\log_{10}$ reductions were compared. Additionally, the disinfection rates for all sample types and species scenarios were calculated by recording $\log_{10}$ reductions at various time points during disinfection.

Bacterial cultures and nutrient medium

Cultures for the inoculation of reactors were grown overnight on a shaker in a low nutrient sterile defined medium consisting of 0.1 g l^{-1} glucose, 0.018 g l^{-1} NH_4Cl, 3.93 g l^{-1} phosphate buffer (2.71 g l^{-1} Na_2HPO_4; 1.22 g l^{-1} KH_2PO_4), and 2 ml l^{-1} 0.1 M $MgSO_4$ (Fisher Scientific, Pittsburgh, PA) at room temperature (22 $\pm$ 2°C). The maximum growth rates of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 in the defined medium were 0.20 ($\pm$0.02) h^{-1} and 0.17 ($\pm$0.02) h^{-1}, respectively.

Planktonic cells from the chemostat

The chemostat (BioSurface Technologies Corp., Bozeman, Mont.) with a volume of 170 ml and a residence time of just over 6 h was run using a nutrient flow of 0.5 ml min^{-1}. The chemostat was filled with the defined medium mentioned above and then inoculated with 3 ml of the 18 h overnight cultures (1.5 ml of each strain for dual culture inoculation). Batch mode (no flow) for $\sim$2 residence times (12 h) was followed by continuous flow for another $\sim$6 residence times (36 h) to reach a steady state of the planktonic culture. To reduce biofilm formation on the walls of the chemostat, the operation time was limited to 48 h. The lid of the chemostat was removed for collection of a sample after the flow was turned off.

Biofilm reactor

Sterile, autoclaved silicone tubing with length of 45 cm and an inner diameter of 0.31 cm (ColeParmer Masterflex Size 16 peroxide-cured silicone tubing, reactor volume 3.4 ml) was filled with nutrient medium and then inoculated with 2 ml of overnight culture (1 ml of each strain for dual culture) using a sterile syringe and needle. The tubing reactor was run with a flow of 0.5 ml min^{-1} and a residence time of 6.8 min which was much lower than the doubling time of the two strains and thus avoided the possibility of a planktonic culture in the bulk fluid of the reactor. After inoculation, the cells were allowed to attach to the walls of the tubing for 3 h, under no flow, followed by growth for 4 days under flow conditions at room temperature (22 $\pm$ 2°C).

Sampling of detached clusters and cells from the biofilm reactor

For sampling of detached cells and clusters, the nutrient medium was switched to buffer (defined low nutrient medium without NH_4Cl and glucose) immediately before samples were taken. This was necessary to prevent chlorine dioxide decay and the production of any organic chlorine compounds that could impact the disinfection process. The waste carboy was detached from the tubing reactor and reactor effluent was collected in a sterile falcon tube (Becton Dickinson, Franklin Lakes, NJ) and kept on ice. Samples were handled carefully to avoid disruption of clusters.

Sampling of attached biofilm from the biofilm reactor

Before destructively sampling the biofilm tubing reactor, the exposed outside of the silicone tubing was disinfected with ethanol for several minutes. Then, the reactor was disconnected and the remaining nutrient medium in the reactor was discarded. Individual pieces of tubing were cut to desired length (1 to 1.5 cm) and submerged into 10 ml of chilled buffer prior to disinfection treatment or quantification. Attached biofilm was removed from the tubing for plate counting of controls and biofilm disinfection experiments. With the help of sterile forceps, biofilm was extruded from the silicone tubing and returned to the buffer for pulse-vortexing (1 min) followed by removal of the tubing and shear homogenization (20,500 rpm, 1 min) of the detached biofilm (shear homogenizer, IKA Labortechnik, Staufen, Germany).

The thicknesses of these biofilms were previously determined and found to be 26 μm ($\pm$4 μm) for FS-3, 28 μm ($\pm$4 μm) for RB-8 and 23 μm ($\pm$4 μm) for the dual species (Behnke et al. 2011).

Homogenization

Aliquots of the planktonic, tubing reactor effluent and biofilm samples were shear homogenized (shear homogenizer, IKA Labortechnik, Staufen, Germany) at 20,500 rpm for 1 min prior to disinfection, for comparison with intact clusters. The homogenizer was sterilized between samples by flaming with ethanol. Data on the characteristics of homogenized *vs* non-homogenized samples can be found in Behnke et al. (2011).

Chlorine dioxide disinfection

Chlorine dioxide tablets (Aseptrol® S10-Tab, provided by BASF) were used as a source for dissolved chlorine dioxide gas. A new stock (1 tablet in 200 ml of nanopure water) was prepared daily and the concentration was measured at 360 nm (molar absorption coefficient = 1250 l mol^{-1} cm^{-1}) using a Genesys spectrophotometer (Gauw et al. 1999). For the direct measurement, small aliquots of the chlorine dioxide solution were transferred to cuvettes and measured for absorbance. In the range of chlorine dioxide concentrations used in these experiments (0.5–10 ppm) the method generated linear standard curves with an average R^2 of 0.999. This method was found to be the most appropriate for this system in side-by-side comparisons with other analytical approaches (data not shown).

Chlorine dioxide concentrations were verified *via* standard curves and added to the samples in incrementally increasing amounts until no survivor colonies were visible on agar plates with the 0^{th} dilution.

Initial $\log_{10}$ numbers of the planktonic cells and detached particles were standardized to 7.0 $\log_{10}$ (CFU ml^{-1}) by dilution with sterile buffer before exposure to chlorine dioxide. This was accomplished by enumerating the number of cells in the chemostat and the detached particle samples by serial dilution and drop plating ($n \geq 3$) on R2A agar. The dilution factor was then calculated to standardize the initial cell numbers to 7.0 $\log_{10}$ CFU ml^{-1} for each disinfection experiment.

Attached biofilm was standardized by choosing the suitable length of tubing (1 to 1.5 cm) that resulted in ~7.0 $\log_{10}$ (CFU ml^{-1}) after homogenization in 10 ml of buffer. The $\log_{10}$ densities of attached biofilm are therefore also expressed in CFU ml^{-1}. With this approach initial cell number comparison to CFU ml^{-1} from the chemostat and detached cells/clusters was straightforward.

After addition of the initial dose of chlorine dioxide, samples were exposed for 30 min or other time points as designated in a shaking incubator at room temperature (22 $\pm$ 2°C) along with untreated control samples in clean glassware with minimal

oxidant demand (data not shown). At the end of 30 min, no measurable chlorine dioxide remained in the samples where the cells were added. Regardless of this, samples (10 ml) were neutralized by adding 100 μl of 6N sodium thiosulfate (Fisher Scientific, Pittsburgh, PA). Control experiments demonstrated no effect of neutralization on the culturability of either species (data not shown).

Bacterial counts

Serial dilutions in sterile pH 7.4 1 × PBS buffer (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24 g of KH_2PO_4 suspended in 1 l $_{of}$ purified water, chemicals by Fisher Scientific, Pittsburg, PA) were carried out prior to plating on R2A plates (Difco™ R2A Agar, BectonDickinson, Franklin Lakes, NJ) using the drop plate method (Herigstad et al. 2001). Agar plates were incubated for 48 h at 30°C. Colony appearance allowed for visual discrimination of the two strains on R2A plates. *P. aeruginosa* produced larger (compared to *B. cepacia*) colonies that were yellowish-green and glossy. *B. cepacia* colonies were smaller, very defined white-yellowish colonies.

Total inactivation was defined as the concentration that resulted in the absence of any colony forming units (CFU) in the undiluted treated sample (0^{th} dilution). If no CFU were present on the drops on the R2A plates, a 1 was substituted for one of the drops and then averaged over 3 drops (EPA guidance for data quality assessment QA/G-9, section 4.7, 1998).

Broken line regression/calculation of inactivation rate constants (k values)

Additional disinfection experiments were done with subjectively chosen sub-lethal doses of chlorine dioxide and sampled after 1, 2, 5, 10, 20, and 30 min, neutralized and plated to calculate inactivation rate constants. Inactivation curves were generated in Microsoft Excel by plotting the ln(CFU ml^{-1}) that survived the treatment *vs* time. Due to the bi-phasic behavior of the disinfection curves, k values were calculated for the first minute and also for disinfection times from 2 to 30 min. For k values with multiple time points (at least 3), a regression line was fit to ln(N) to obtain a slope 'm' and an intercept $\ln(N_0)$. The k value was subsequently found by setting $k = -m/C$. Chick (1908) described killing curves with

$$k = -\ln(N/N_0)/C * t$$

where 'N_0' is the initial CFUml^{-1} and 'N' is the CFUml^{-1} after time 't', and 'C' is the initial concentration of disinfectant.

Statistical analysis

For each pairwise species comparison (FS-3 *vs* RB-8; FS-3 *vs* dual FS-3, RB-8 *vs* dual RB-8), an ANOVA was fit with reactor, species and chlorine dioxide dose (reactor and species only for initial $\log_{10}$ densities) as factors. To account for the pairing of the dual FS-3 and dual RB-8 species grown in the same reactor, the difference between the $\log_{10}$ reductions (LRs) of the two strains were calculated for each reactor, and an ANOVA with reactor and dose was fit to these differences. This same paired analysis was used to compare homogenized and unhomogenized samples which were collected from the same reactor. To compare the LDs of the biofilm controls of the two species in a dual culture, a paired t-test was performed.

Inactivation rates (k values) were compared pairwise (FS-3 *vs* RB-8; FS-3 *vs* dual FS-3, RB-8 *vs* dual RB-8) by performing an ANOVA with reactor and species as factors. The pairing of dual FS-3 and dual RB-8 was accounted for by calculating the differences between these k values and an ANOVA was fit to these differences. All of the ANOVA models were fit in Minitab® (Version 16). The follow-up t-tests and the weighted least squares were performed in R (version 2.11.0).

The analyses described above were divided into seven categories for control of the false discovery rate: the LRs for FS-3 *vs* RB-8; FS-3 *vs* dual FS-3; RB-8 *vs* dual RB-8; dual FS-3 *vs* dual RB-8; homogenized *vs* unhomogenized; k values; and k values of homogenized samples. A Benjamini-Hochberg correction (Benjamini and Hochberg 1995) was applied to each of these seven groups to maintain the false discovery rate at 5%. Claims of statistical significance were made with respect to this correction, although the p-values reported are un-adjusted.

Results

In this study the differential efficacy of chlorine dioxide against bacterial monocultures and co-cultures of *P. aeruginosa* RB-8 and *B. cepacia* FS-3 was determined. Cultures grown as planktonic cells, biofilms, detached biofilm clusters harvested from the biofilm effluent and homogenized samples were tested and log reductions were compared. Additionally, disinfection rates were calculated for each sample type and species scenario.

Initial cell numbers and species distribution in co-culture for disinfection studies

The initial log densities (LD) of the two species as monocultures were compared to the two species grown

together as a co-culture (Table 1). The LDs of FS-3 and RB-8 monocultures were not statistically different from each other for the chemostat and the tubing reactor effluent samples (p-values > 0.05), but there was a difference between the LDs in the biofilm samples for FS-3 and RB-8 (p-value < 0.001). The cell numbers were slightly higher for cultures containing only RB-8. FS-3 as a monoculture compared to FS-3 in a dual culture only resulted in significantly lower LDs for the monoculture in the biofilm samples (p-value < 0.001). RB-8 as a monoculture biofilm had significantly higher LDs when compared to RB-8 in the dual species biofilm (p-value < 0.001) while the chemostat and tubing reactor effluent samples were similar at the 5% level.

Species distribution differences were reflected in the LDs of the co-culture. Both species were co-inoculated into the same reactor and grown for 48 h (chemostat) or 4 days (biofilm and biofilm reactor effluent). FS-3 was the most abundant species in all 3 sample types (chemostat, tubing reactor effluent, and biofilm) while the ratios between the species were different in the different samples. Significant differences between the species were observed in the tubing reactor effluent and the biofilm samples (p-value $= 0.004$, p-value < 0.001, respectively).

B. cepacia (FS-3) was dominant when co-cultured with *P. aeruginosa* (RB-8) when comparing the initial species distribution in the chemostat, tubing reactor effluent, and biofilm samples. *B. cepacia* has a slightly higher growth rate (not significantly different) than *P. aeruginosa*, but was able to establish a niche in the reactors without being out-competed even after 4 days in the tubing reactor. Simultaneously, RB-8 also continued to detach from the biofilm after 4 days.

Table 1. Starting mean $\log_{10}$ densities of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as binary cultures (co-culture of FS-3 and RB-8).

Sample type	Species	Mean	SD	n
CS	FS-3	7.03	0.06	4
	RB-8	7.07	0.58	4
	DL FS-3	6.48	0.74	4
	DL RB-8	6.29	0.19	4
TRE	FS-3	6.96	0.29	4
	RB-8	7.21	0.57	3
	DL FS-3	7.22	0.20	3
	DL RB-8	5.41	0.29	3
BF	FS-3	6.98	0.06	6
	RB-8	7.44	0.22	5
	DL FS-3	7.43	0.10	7
	DL RB-8	6.12	0.22	7

Note: One SD is shown. CS = chemostat; TRE = tubing reactor effluent; BF = biofilm; DL = dual (species in co-culture); SD = one standard deviation; n = number of replicates.

Disinfection of monocultures

Log_{10} reductions after addition of incrementally increasing doses of chlorine dioxide were recorded for each sample type (chemostat, tubing reactor effluent, and biofilm) and each species scenario (FS-3, RB-8, and binary culture) (Figure 1).

Chemostat samples of the FS-3 monoculture were more susceptible to chlorine dioxide disinfection compared to tubing reactor effluent although the differences were not statistically significant when all doses of chlorine dioxide were compared (p-value > 0.05). Mean $\log_{10}$ reductions for RB-8 in the chemostat and detached clusters were very similar to each other (p-value $= 0.99$). The biofilms with FS-3 and RB-8 were more tolerant to disinfection compared to the other 2 sample types (chemostat and tubing reactor effluent) with mean $\log_{10}$ reductions at least 2 logs below the $\log_{10}$ reductions of the chemostat and detached clusters (p-values < 0.001). The RB-8 biofilm displayed very low $\log_{10}$ reductions at low doses (0.5, 1.0, 1.5, and 2.0) and was eventually killed with 10 ppm of chlorine dioxide. FS-3 biofilms were more

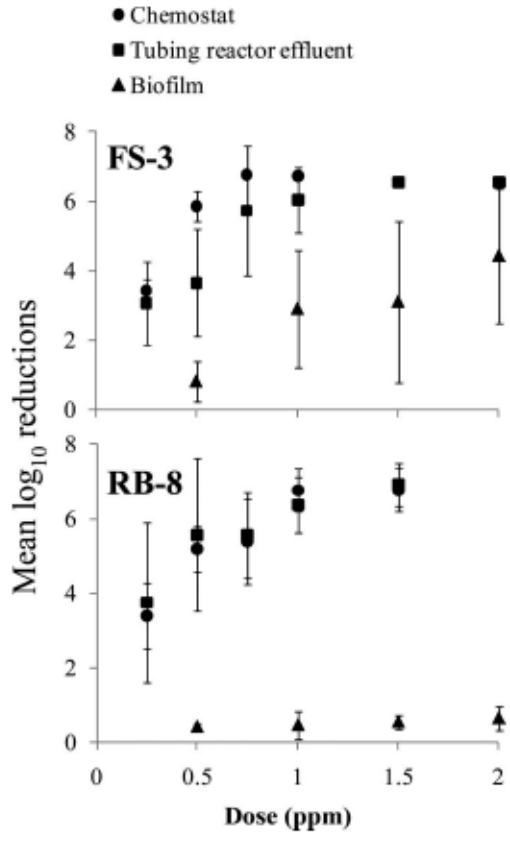

Figure 1. Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 monocultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one SD ($n \geq 3$).

susceptible to the disinfectant at these low doses although complete killing was also achieved with 10 ppm (Figure 2).

When comparing both species within one sample type there were no significant differences in the $\log_{10}$ reductions (p-values > 0.05) in the chemostat or tubing reactor effluent sample. However, the $\log_{10}$ reductions for FS-3 and RB-8 in the biofilm samples were significantly different over all treatment doses, with FS-3 being more susceptible (p-value < 0.001).

Disinfection of binary cultures

When FS-3 and RB-8 were co-inoculated into the chemostat or tubing reactor and grown together, CFUs could be counted separately due to differential colony morphologies on R2A. This allowed for the calculation of separate $\log_{10}$ reductions and comparison of the two species with each other and also with the same species in the single species scenario.

The mean $\log_{10}$ reduction patterns for the two species grown together after disinfection were similar to each other although the starting cell densities were lower for RB-8 (Figure 3, also see Table 1 for comparison). Tubing reactor effluent samples were generally more susceptible to chlorine dioxide than the chemostat samples when both species were grown together, but the differences were not significant (p-value $= 0.07$). The biofilm $\log_{10}$ reductions were significantly lower than the chemostat and detached cluster $\log_{10}$ reductions for both species (p-values < 0.001).

Comparison between monocultures and binary cultures

Generally, FS-3 and RB-8 had lower $\log_{10}$ reductions when grown in co-culture samples, meaning that single species cultures were more easily disinfected (Figure 4).

RB-8 in the dual species scenario has significantly lower $\log_{10}$ reductions for the chemostat and the tubing reactor effluent than the single species cultures (p-values < 0.001) but there was no significant difference for the biofilm samples of single and dual RB-8 (p-value $= 0.80$) despite the initial high tolerance of the single species RB-8 biofilm at low doses. FS-3 was also more tolerant when grown in co-culture and displayed significantly lower $\log_{10}$ reductions in the chemostat samples (p-value < 0.001) and biofilm samples than the single species FS-3 (p-value $= 0.005$) but no significant differences were found in tubing reactor effluent samples (p-value $= 1.0$).

Disinfection of homogenized aliquots

Aliquots of chemostat, tubing reactor effluent, and biofilm samples were shear homogenized for 1 min prior to treatment with chlorine dioxide. Untreated controls were homogenized to compare the initial $\log_{10}$ densities to non homogenized and untreated control samples. No significant differences were found between the homogenized and non homogenized initial $\log_{10}$ densities (p-values > 0.28) (Figure 5).

Homogenization of chemostat samples slightly increased susceptibility in all species types (FS-3,

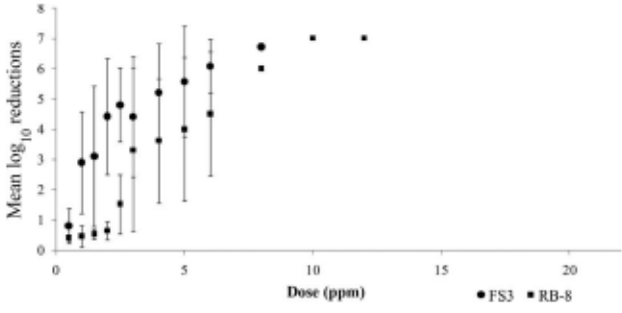

Figure 2. Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures in the biofilm. The bars represent one SD ($n \geq 3$).

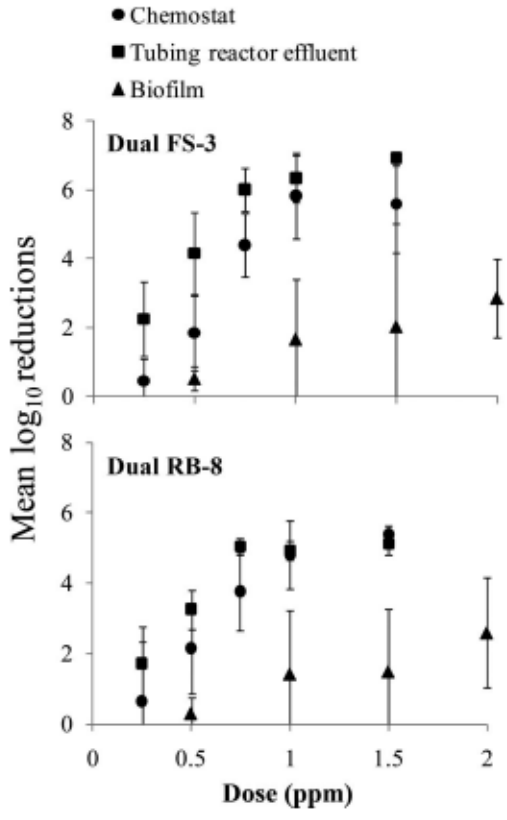

Figure 3. Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as dual cultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one SD ($n \geq 3$).

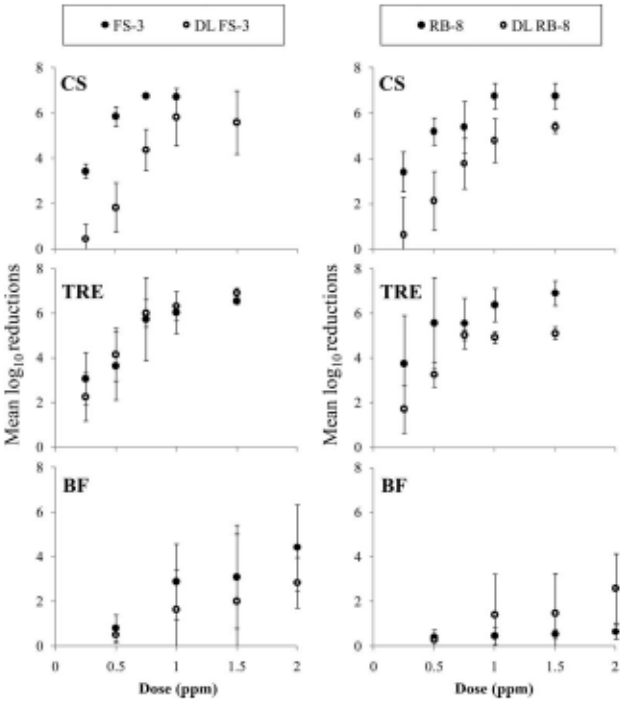

Figure 4. Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and binary cultures in comparison. Bars represent one SD ($n \geq 3$). DL = dual (species in co-culture).

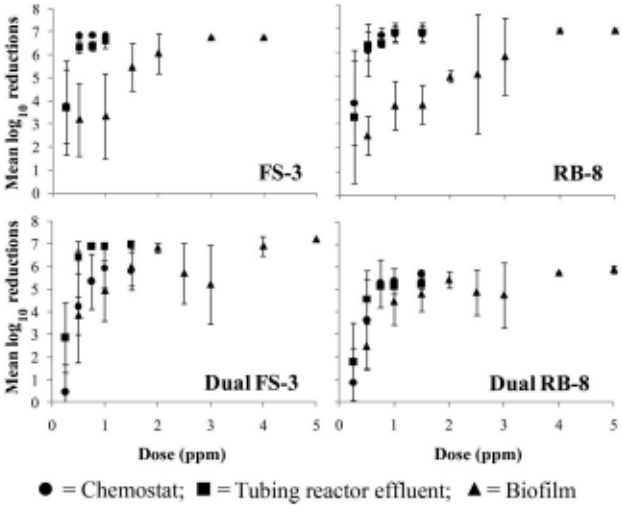

Figure 5. Mean $\log_{10}$ reductions of homogenized *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and dual cultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one SD ($n > 3$).

RB-8 and co-culture) with significant susceptibility increases for RB-8 and dual RB-8 (p-values < 0.02). None of the tubing reactor effluent samples increased significantly in susceptibility across all doses after homogenization while FS-3 and dual FS-3 in the tubing reactor effluent were significantly more

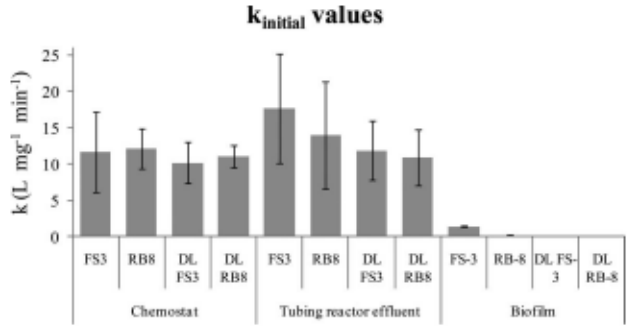

Figure 6. k values for the first minute ($k_{initial}$) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as dual cultures). Error bars represent one SD ($n \geq 3$). DL = dual (species in co-culture).

susceptible when treated with 0.5 ppm (p-values < 0.001). As expected, biofilm samples of all species were significantly more susceptible to chlorine dioxide treatment across all doses after biofilm has been extruded from the tubing, vortexed, and shear homogenized (p-values < 0.01) (Figure 5).

Disinfection rates (k values)

Using broken line regression, the concentration over time (Ct) graphs were analyzed to determine the two different linear disinfection rates (k values). The first k value ($k_{initial}$) describes the disinfection reaction rate of the first minute after addition of chlorine dioxide. Initially, the $\log_{10}$ reductions were high and then decreased considerably after 2 min. The remaining disinfection reaction (after minute 1) is described by the second k value (k_{end}).

Most of the killing of cells occurred within the first 2 min after addition of chlorine dioxide. Disinfection rates ($k_{initial}$) were very similar in the chemostat and tubing reactor effluent samples (p-values > 0.93), indicating that similar $\log_{10}$ reductions occurred within the same time period for all 4 species scenarios and both sample types (Figure 6). The dual species FS-3 and RB-8 resulted in a slightly lower $k_{initial}$ than the single species, but there were no statistically significant differences for any of the species or sample types (p-values > 0.05). The biofilm $k_{initial}$ were significantly lower than the $k_{initial}$ for all chemostat and tubing reactor effluent samples regardless of species (p-values < 0.03), indicating that the $\log_{10}$ reductions within the first minute of disinfection were much lower for all tested biofilms.

The k_{end} values indicate the disinfection rate from minute 2 to minute 30. Since most of the killing occurred within the first 2 min, the k_{end} were

significantly decreased, ranging from 0.1 $l\,mg^{-1}\,min^{-1}$ to approximately 0.3 $l\,mg^{-1}\,min^{-1}$ compared to the $k_{initial}$ that ranged from 10 $l\,mg^{-1}\,min^{-1}$ to 18 $l\,mg^{-1}\,min^{-1}$ (Figure 7). The k_{end} of the two dual species in the tubing reactor effluent were slightly higher than the other k_{end} values although no statistically relevant difference could be found. The k_{end} values were not significantly different from each other for any of the species or sample types (after Benjamini–Hochberg correction for false discovery) due to the high variability of the data.

Disinfection of homogenized control samples (k values)

Shear homogenization of aliquots of samples resulted in significantly higher $k_{initial}$ in the biofilm samples for all 4 species types (p-values < 0.018). This indicates that homogenization makes biofilm cells more susceptible to disinfection (Figures 8 and 9).

Surprisingly, for homogenized chemostat and tubing reactor effluent samples, $k_{initial}$ did not increase, but instead decreased by 3.73 $l\,mg^{-1}\,min^{-1}$ for chemostat samples and 8.10 $l\,mg^{-1}\,min^{-1}$ for tubing reactor effluent samples on average for all species. Homogenization of the co-culture led to significantly increased susceptibility in the tubing reactor effluent for the dual FS-3 (p-value $= 0.014$) and in the chemostat for dual RB-8 (p-value $= 0.02$) with the $k_{initial}$ being lower for homogenized samples.

The k_{end} values, on the other hand increased by 0.46 $l\,mg^{-1}\,min^{-1}$ for chemostat samples and 0.40 for tubing reactor effluent samples on average for all species. The k_{end} values increased for all chemostat samples after homogenization (p-values < 0.029), meaning that more inactivation occurred after 2 min than in the non homogenized samples. In the co-culture, dual FS-3 and dual RB-8 the k_{end} increased significantly in the tubing reactor effluent samples after homogenization as well (p-values < 0.02). None of the biofilm samples or single species FS-3 and RB-8 tubing reactor effluent k_{end} increased significantly after homogenization (p-values > 0.11) (Figure 10).

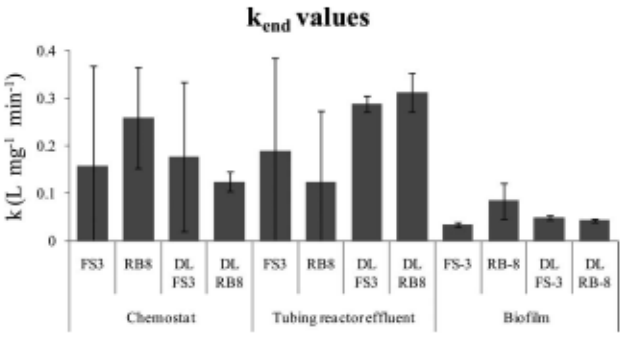

Figure 7. k values for minute 2 to minute 30 (k_{end}) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios. (FS-3 and RB-8 as monocultures and as a dual culture). Error bars represent one SD ($n \geq 3$). DL = dual (species in co-culture).

Discussion

Initial cell numbers

B. cepacia (FS-3) was dominant when co-cultured with *P. aeruginosa* (RB-8) when comparing the initial species distribution in chemostat, tubing reactor effluent, and biofilm samples (Table 1). *B. cepacia*

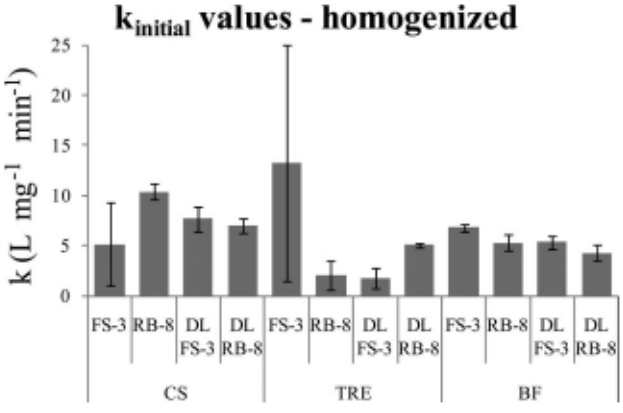

Figure 8. k values of homogenized samples for the first minute ($k_{initial}$) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as dual cultures). Error bars represent one SD ($n \geq 3$). DL = dual (species in co-culture).

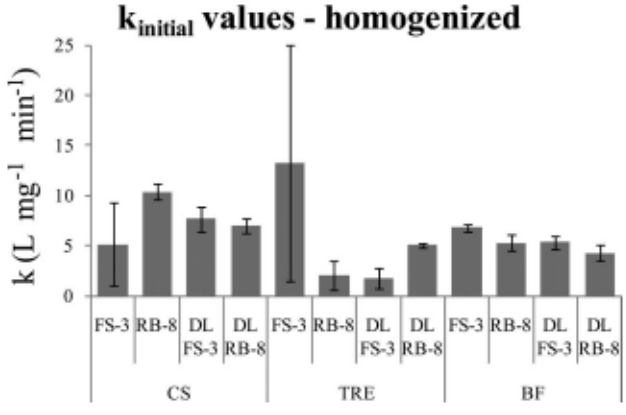

Figure 9. k values of homogenized samples for minute 2 to minute 30 (k_{end}) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as dual cultures). Error bars represent SD ($n \geq 3$). DL = dual (species in co-culture.)

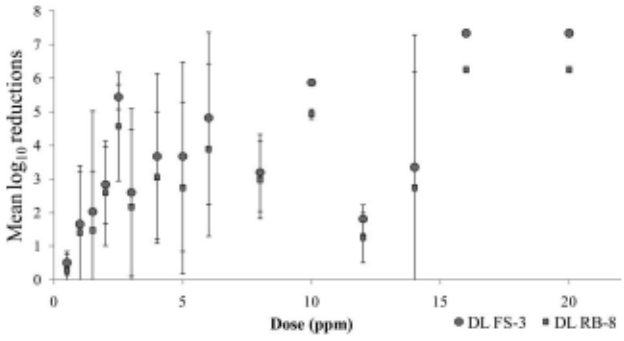

Figure 10. Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as a dual (DL) biofilm culture. The bars represent one SD ($n \geq 3$).

has a slightly higher growth rate (not significantly different) than *P. aeruginosa*, but *P. aeruginosa* was able to establish a niche in the reactors without being out-competed even after 4 days in the tubing reactor. Simultaneously, RB-8 also continued to detach from the biofilm after 4 days. This is not surprising, since other studies have shown that growth rate is not the sole predictor of population densities and species distributions in multi-species cultures (Banks and Bryers 1991; Komlos et al. 2005).

FS-3 and RB-8 have been tested for interspecies competition *via* the production of substances that impair the disinfectant susceptibility of the other species (Behnke et al. 2011). In the tests, spent medium (supernatant) cell-free extract of an FS-3 culture added to a RB-8 culture did not increase the susceptibility of a RB-8 chemostat culture to disinfection or *vice versa* compared to a control with PBS. Similarly, cell-free extracts of RB-8 did not affect the disinfection susceptibilities of FS-3. In addition, auto-aggregation and co-aggregation tests showed no interactions between the species (Behnke et al. 2011). All cell cultures from the chemostat and the tubing reactor effluent were standardized to 7 $\log_{10}$ by dilution with buffer, while biofilm $\log_{10}$ densities were adjusted by cutting the tubing into 1 to 1.5 cm long pieces of the silicone tubing before submerging them into 10 ml of buffer. Disinfection efficacy has been shown to be dependent on initial cell densities (Xu et al. 1995) and statistical analyses indicate that starting cell LDs were comparable for the performed tests.

Monocultures

FS-3 and RB-8 were individually grown in chemostats and tubing reactors. Planktonic cell samples were taken from the chemostat while attached and detached biofilm were harvested from the tubing reactor. After exposure to chlorine dioxide and neutralization of the samples, $\log_{10}$ reductions were assessed for both species in the three sample types. No significant differences between the $\log_{10}$ reductions between the chemostat and tubing reactor effluent samples were found for either species. The biofilm samples were significantly different from both the chemostat and effluent samples and this applied to both species. These results indicate that detached clusters were more similar to chemostat cells than biofilm cells; this contradicts findings by Rollet et al. (2009) who suggested a transitional phenotype for detached cells in the first hours after detachment. Disinfection experiments in this work were always performed within 1 h of sample collection so that the proposed transitional phenotype should still be expressed. The phenotypical switch from biofilm to planktonic state may occur much faster than initially proposed or it is possible that phenotype plays no role in protection from chlorine dioxide disinfection in the detached biofilm particles.

Interestingly, RB-8 biofilms were very tolerant to chlorine dioxide ($\log_{10}$ reductions < 0.5) below the concentration of 1 ppm while the FS-3 and binary biofilm responded to these low concentrations with higher $\log_{10}$ reductions.

Binary cultures

FS-3 and RB-8 were co-inoculated into the chemostat and tubing reactor to grow binary cultures. Due to the fact that both species resulted in distinct colony morphologies, CFUs could be counted separately for FS-3 and RB-8 and separate log reductions were calculated. The $\log_{10}$ reduction patterns of the two species grown in binary cultures were very similar to each other although the starting LDs for RB-8 were lower. Lindsay et al. (2002) have previously reported this behavior for binary cultures and concluded that co-cultured bacteria influence each other's disinfection susceptibilities and attachment characteristics. In this study the beneficial relationship between the two species was seen in biofilm samples as well as chemostat and tubing reactor effluent samples. However, this is not universal. Previous work using these same two organisms showed that co-culture in a chemostat resulted in decreased survival after a chlorine challenge (Behnke et al. 2011). These results suggest that the interaction between species will depend on the growth conditions and the disinfectant used.

In monocultures, planktonic cells were more susceptible or equally susceptible to chlorine dioxide when compared to detached clusters. In contrast to this, detached clusters from binary biofilms were more susceptible than chemostat cells although there was no statistically significant difference. A multitude of mechanisms and conditions can affect the disinfection

tolerance of bacteria so that further testing is required to identify the factors involved here.

Comparison of monocultures and binary cultures

When directly comparing the $\log_{10}$ reductions of FS-3 and RB-8 grown as monocultures and binary cultures, increased disinfection tolerance of the species in co-culture was observed. The $\log_{10}$ reductions for FS-3 in co-culture were slightly lower than the $\log_{10}$ reductions for the single species FS-3, meaning that the single species samples were less tolerant to disinfection. Similarly, RB-8 cultures were slightly more tolerant to disinfection when co-cultured with FS-3. In general, binary biofilm samples survived higher concentrations (up to 16 ppm) than single species biofilms (survived up to 10 ppm, data not shown). These results agree with the findings of other researchers who determined that binary cultures are more tolerant to sanitizer treatment than their single species counterparts (Leriche and Carpentier 1995; Elvers et al. 2002; Lindsay et al. 2002). Kara et al. (2006) showed that dual species biofilms of two oral bacteria were less susceptible to chlorhexadine. Using the same two organisms, subsequent work with five different antimicrobials showed increased survival for one of the species (Luppens et al. 2008). The increased survival was attributed to changes in gene expression when grown in co-culture. Cowan et al. (2000) additionally reported commensal relationships in binary biofilms that resulted in adaptive strategies and increased survival especially with respect to the spatial distribution of the species within the biofilm.

The mechanisms of increased disinfection tolerance in binary biofilms are to date mostly speculative, but studies suggest that cell to cell communication may play a role. *B. cepacia* and *P. aeruginosa* strains have previously been shown to be able to communicate (McKenney et al. 1995; Riedel et al. 2001) which may explain the beneficial effect of co-culture. The presence of the second species may alter the composition and viscosity of the EPS matrix and thus the diffusivity, slowing the penetration of chlorine dioxide into the biofilm (Burmølle et al. 2006). Physiological changes may occur when two species are able to transfer conjugative plasmids and thus share protective mechanisms (Ghigo 2001) or support each other by complementing enzymes that are necessary to manage environmental challenges as observed in dental biofilms maintaining a stable pH (Shu et al. 2003).

Disinfection of homogenized controls

Before chlorine dioxide treatment, aliquots of all samples were subjected to shear homogenization. It has been shown in previous work that homogenization did not disintegrate all clusters but was able to reduce the number of larger clusters and increase the number of smaller clusters and single cells in samples (Behnke et al. 2011).

Homogenization had the most significant effect on the disinfection tolerance of the biofilm samples for all tested species. The biofilm specific tolerance against disinfection was greatly reduced, but biofilm cells remained more tolerant than chemostat or tubing reactor effluent cells (Figures 2 and 10). Since homogenized biofilm cells remained more tolerant than chemostat and tubing reactor effluent samples, a protection mechanism beyond physical biofilm-specific factors such as attachment, EPS material, close proximity of cells, and reaction-diffusion limitations may play a role in biofilm disinfection (Stewart and Raquepas 1995; Chen and Stewart 1996; Stewart 2003). However, the chemostat and tubing reactor effluent disinfection tolerance did not change as significantly as the biofilm tolerance after homogenization (p-values $<$ 0.01). Differences between LRs of homogenized and unhomogenized chemostat samples were limited to RB-8 (p-value $= 0.02$) and dual RB-8 (p-value $= 0.0001$) as well as a single dose (0.5 ppm) in the tubing reactor effluent and chemostat samples for FS-3 and dual FS-3 (p-values all < 0.09).

Chlorine dioxide disinfection experiments with unhomogenized samples demonstrated that the tubing reactor effluent cells were similar to planktonic cells and different from biofilm cells with respect to $\log_{10}$ reductions. This set of control experiments with homogenized samples supports this finding because the tubing reactor effluent samples did not decrease significantly (across all doses) in tolerance after homogenization as was seen in the biofilm.

Disinfection rates

Chlorine dioxide added to cell culture samples of the chemostat and the tubing reactor effluent decayed slowly over the course of 30 min and no residual remained (data not shown). Most of the $\log_{10}$ reductions occurred within the first 2 min of the experiment with no significant subsequent reductions. As expected, based on disinfection experiments, no significant differences were found when comparing the chemostat and tubing reactor effluent $k_{initial}$ for all 4 species scenarios (FS-3 single, RB-8 single, FS-3 dual, RB-8 dual; p-values > 0.93). This indicates that cells in these samples were inactivated at similar rates within the first minute of exposure to chlorine dioxide. Regardless of species, the $k_{initial}$ of biofilm samples were significantly different from the chemostat and tubing reactor $k_{initial}$ (p-values < 0.03) which was also

supported by the results of the disinfection experiments (Figures 1 and 3). Biofilm inactivation may be slowed by reaction-diffusion limitations of chlorine dioxide into the biofilm where the disinfectant is simultaneously diffusing into the biofilm and reacting with biofilm constituents thus protecting the cells (Stewart and Raquepas 1995).

Disinfection rates of homogenized samples

The disinfection rates of homogenized aliquots of samples reflect findings from the disinfection experiments with homogenized samples. The $k_{initial}$ significantly increased when biofilms were homogenized before treatment with chlorine dioxide. Homogenization greatly decreases the influence of physical biofilm-specific properties such as the presence of EPS material, diffusion-reaction limitation, and possibly even cell to cell communication which has been demonstrated to be beneficial during disinfection challenges on biofilms (Burmølle et al. 2006).

Surprisingly, for homogenized chemostat and tubing reactor effluent samples, $k_{initial}$ did not increase, but instead decreased while k_{end} values increased for all species. This suggests that homogenization reduces the susceptibility of the cells to chlorine dioxide during the first two minutes.

Comparison with free chlorine

Behnke et al. (2011) demonstrated that chlorine disinfection of detached clusters may be dependent on cell cluster size. Clusters containing a higher number of cells were found to be more tolerant to chlorine treatment than cells in small clusters.

Cluster distribution in the present study also varied with reactor type and species where FS-3 chemostat samples contained ~5% large clusters (11 + cells) while the biofilm reactor effluent resulted in about 30% large clusters. The chemostat samples of RB-8 on the other hand had about 30% large clusters while the biofilm reactor effluent had a smaller percentage of large clusters (10%). In co-culture the percentage of large clusters was 5% in the chemostat and 10% in the biofilm reactor effluent samples. However, evidence for a relationship between the cluster sizes and the disinfection efficacy could not be found.

In disinfection studies with chlorine higher doses of the chemical are required to inactivate samples than are needed with chlorine dioxide, while the highest log reductions of cultures with chlorine dioxide occur at a slower rate (2 min) than the disinfection kinetics observed in experiments with chlorine (1 min). These differences can be attributed to the different modes of biocidal action and the reactivity of the two compounds with constituents of the biofilm matrix. Independent of the disinfecting agent, biofilms were always significantly more tolerant to treatment than planktonic cells or detached clusters. Other studies confirmed that mature drinking water biofilms were reduced by only minimal $\log_{10}$ reductions (0.5 to 1.5) after a single dose of chlorine dioxide (Gagnon et al. 2005), also suggesting protection and that several doses may be required to achieve higher log reductions in drinking water biofilms.

Conclusions

In this study, detached biofilm clusters were found to have similar $\log_{10}$ reductions and disinfection rates to chemostat cells and were very different from biofilm cells.

Binary cultures were less susceptible than single species cultures for all three sample types (chemostat, biofilm, and detached biofilm particles). This finding suggests that the two strains promoted overall disinfection survival and led to similar disinfection patterns, as previously described by Lindsay et al. (2002) and Luppens et al. (2008). Multiple studies have found similar results with binary cultures (Leriche and Carpentier 1995; Elvers et al. 2002; Lindsay et al. 2002), but mechanisms for this increased tolerance have not been sufficiently studied. Researchers have suggested a change in the EPS matrix constituents due to the presence of a second species can lead to viscosity and permeability changes (Skillman et al. 1999; Burmølle et al. 2006). Allison and Matthews (1992) found viscosity changes in *Burkholderia cepacia* and *Pseudomonas aeruginosa* dual biofilms that reduced diffusion of antibiotics which may also slow disinfection with chlorine dioxide.

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