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Comparing the Chlorine Disinfection of Detached Biofilm Clusters with Those of Sessile Biofilms and Planktonic Cells in Single- and Dual-Species Cultures▽†

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Although the detachment of cells from biofilms is of fundamental importance to the dissemination of organisms in both public health and clinical settings, the disinfection efficacies of commonly used biocides on detached biofilm particles have not been investigated. Therefore, the question arises whether cells in detached aggregates can be killed with disinfectant concentrations sufficient to inactivate planktonic cells. *Burkholderia cepacia* and *Pseudomonas aeruginosa* were grown in standardized laboratory reactors as single species and in coculture. Cluster size distributions in chemostats and biofilm reactor effluent were measured. Chlorine susceptibility was assessed for planktonic cultures, attached biofilm, and particles and cells detached from the biofilm. Disinfection tolerance generally increased with a higher percentage of larger cell clusters in the chemostat and detached biofilm. Samples with a lower percentage of large clusters were more easily disinfected. Thus, disinfection tolerance depended on the cluster size distribution rather than sample type for chemostat and detached biofilm. Intact biofilms were more tolerant to chlorine independent of species. Homogenization of samples led to significantly increased susceptibility in all biofilm samples as well as detached clusters for single-species *B. cepacia*, *B. cepacia* in coculture, and *P. aeruginosa* in coculture. The disinfection efficacy was also dependent on species composition; coculture was advantageous to the survival of both species when grown as a biofilm or as clusters detached from biofilm but, surprisingly, resulted in a lower disinfection tolerance when they were grown as a mixed planktonic culture.

In natural, industrial, and medical settings, bacteria attach to surfaces and grow in biofilm communities. These microorganisms are surrounded by a matrix of extracellular polymeric substances and may contain channels that allow for the diffusion of oxygen, nutrients, and metabolites (10, 33). As a result of their habitat, the physiological characteristics of biofilm-associated cells are different from those of free-floating planktonic cells in terms of growth rates, the production of extracellular polymeric substances (EPS), and expression of genes associated with the biofilm state (7).

Most importantly, biofilm cells have been shown to be significantly more tolerant to antibiotics and disinfectants than their planktonic counterparts (7, 11, 32). Increased resistance may be the result of limited diffusion of disinfectants into the biofilm due to the gel-like EPS matrix that protects the deeper layers of cells from damage (9). Additionally, it has been found that areas of low metabolic activity and oxygen limitation in the interior of the biofilm are associated with the regions that withstand antibiotics and disinfectants most effectively (21, 35). Cells in a biofilm display different phenotypes, therefore allowing for the differentiation of the biofilm into complex multicellular structures and optimizing survival during treatment with antibiotics or disinfectants (8, 27).

Biofilms are also characterized by the active or passive detachment of cell and clusters. Depending on the species composition and mechanical biofilm stability, increased shear forces can lead to detachment of biofilm clumps, which may be enhanced when treated with oxidizing disinfectants such as chlorine (29, 30). It has also been proposed that cells and clusters detach actively as a response to nutrient starvation by returning some cells into the bulk flow and thus optimizing nutrient supply for planktonic cells and the remaining biofilm cells (6, 27). Detached cell clusters can cover a wide range of cluster sizes also, including single cells, and the cluster size distribution depends highly on the species composition and growth or treatment conditions (34, 36). Detached cells are thought to express a transitional phenotype between sessile and planktonic states during the first hours after detachment, displaying growth kinetics and cell surface properties similar to those of attached biofilm cells (26). Compared to planktonically grown cells, detached cells and clusters have increased tolerance to antibiotics (12) or disinfection with chlorine (32), but they are less resistant than the attached biofilm itself. The efficacy of the disinfection of particle-associated cells is closely linked to the size of the particles, and failure of treatment may occur if prefiltration is not present or insufficient amounts of disinfectant are added (37). Regrowth of these cells may present a risk to human health and is also relevant in industrial settings and virtually any liquid flow scenario when surviving

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TABLE 1. Species, growth conditions, and chlorine doses for experiments with 30 min of disinfection

Species composition[b]	Doses (ppm) for reactor type[a]:		
	Chemostat	Tubing reactor effluent	Biofilm
FS-3 (single species)	1, 2, 3, 4	1, 2, 3, 4, 5, 6, 8, 10	2, 4, 6, 8, 10, 20, 30
RB-8 (single species)	1, 2, 3, 4, 5	1, 2, 3, 4	2, 4, 6, 8, 10, 20, 30, 40
Coculture of FS-3 and RB-8	1, 2, 3, 4	1, 2, 3, 4, 5, 6	2, 4, 6, 8, 10, 20, 30, 40, 50, 60

[a] Chlorine doses were incrementally increased until the culture was inactivated completely.
[b] FS-3, *Burkholderia cepacia*; RB-8, *Pseudomonas aeruginosa*.

cells reattach to surfaces downstream of the disinfection site and form new biofilms.

Although detachment of aggregated cells from biofilms is of fundamental importance to the dissemination of contamination and infection in both public health and clinical settings, the disinfection efficacies of commonly used biocides on detached particles have not been adequately investigated.

The goal of this study was the evaluation of the chlorine susceptibility of detached cells and cell clusters of environmental isolates of *Burkholderia cepacia* (FS-3) and *Pseudomonas aeruginosa* (RB-8) in comparison to those of their planktonic cultures and attached biofilms grown as a single species. Another aspect was to grow the organisms in coculture to determine how the presence of a second bacterial species alters disinfection tolerance. Synergistic interactions of more than one species have been previously described as advantageous in disinfection studies (3). Some strains of *B. cepacia* and *P. aeruginosa* are known to utilize the same signal molecules (25) and have been shown to communicate on an interspecies level (19), which makes them good candidates for coculture.

MATERIALS AND METHODS

To compare the chlorine susceptibilities of planktonic cells, cells detached from biofilm, and attached biofilm, environmental isolates of *Burkholderia cepacia* (FS-3) and *Pseudomonas aeruginosa* (RB-8) were grown as single-species cultures and dual-species cultures (with coinoculation of both species into a reactor) in chemostats (CS) and biofilm tubing reactors (Table 1). Cluster sizes were analyzed for the chemostat samples and the tubing reactor effluent (TRE), and disinfection susceptibilities were assessed for all sample types (chemostat, tubing reactor effluent, and attached biofilm, as well as the homogenized control samples) and all species scenarios (single FS-3, single RB-8, dual FS-3, and dual RB-8).

Bacterial strains and media. Inoculation cultures of FS-3 and RB-8 were grown at room temperature (22 ± 2°C) for 18 h on a shaker in a defined sterile nutrient medium consisting of 0.1 g/liter glucose, 0.018 g/liter NH_4Cl, 3.93g/liter phosphate buffer (2.71 g/liter Na_2HPO_4 and 1.22 g/liter KH_2PO_4), and 2 ml/liter 0.1 M $MgSO_4$ (Fisher Scientific, Pittsburgh, PA). Glucose was used as the sole carbon source because it has little chlorine demand. To avoid secondary effects of the formation of chloramines during chlorine disinfection, the ammonia concentration was adjusted so it was completely used by the bacterial culture. The absence of total ammonia was confirmed using the salicylate method (AmVer low-range ammonia; Hach Lange, Loveland, CO). Alternatively, ammonia was removed (by replacement of medium with buffer) when collecting detached cells and clusters from the biofilm effluent.

Batch cultures for growth rate determinations. A small aliquot of overnight culture was transferred to fresh medium at the start of the growth curve. Samples were taken every 2 h, diluted appropriately, and plated on R2A agar (see "Bacterial quantification" below).

Chemostat cells. Planktonic cultures were grown in the defined medium mentioned above. The chemostat (BioSurface Technologies Corp., Bozeman, MT) had a volume of 170 ml, a flow of 0.5 ml/min, and a residence time of slightly over 6 h. The filled chemostat was inoculated with 3 ml of the overnight cultures (1.5 ml of each strain for dual-culture inoculation) and incubated for 12 h. This was followed by continuous flow for 36 h. To reduce biofilm formation on the walls of the chemostat, the operation time was limited to 48 h. The flow was then turned off, and the lid of the chemostat was removed for collection of a sample.

Biofilm and detached cells. Biofilms were grown in a biofilm tubing reactor which consisted of 45 cm of silicone tubing (ColeParmer Masterflex size 16; inner diameter of 0.31 cm, peroxide-cured silicone tubing) supplied with nutrient medium via a peristaltic pump. The total reactor volume was 3.4 ml, and the residence time was 6.8 min at a flow of 0.5 ml/min. The autoclaved, sterile reactor was filled with the medium described above and inoculated with 2 ml of overnight culture (1 ml of each strain for dual culture), followed by 3 h with no flow. After the flow was turned on, the biofilms were grown for 4 days at room temperature (22 ± 2°C). Since the residence time is below the doubling time of the strains, it was assumed that planktonic cells were washed out of the reactor and that cells and clusters found in the effluent were the result of detachment.

Sampling of detached clusters and cells. Immediately before collection of tubing reactor effluent samples, the nutrient medium was switched to buffer (defined medium without NH_4Cl and glucose). The tubing reactor was detached from the waste carboy and the effluent samples collected in a 50-ml sterile Falcon tube (Becton Dickinson, Franklin Lakes, NJ) on ice.

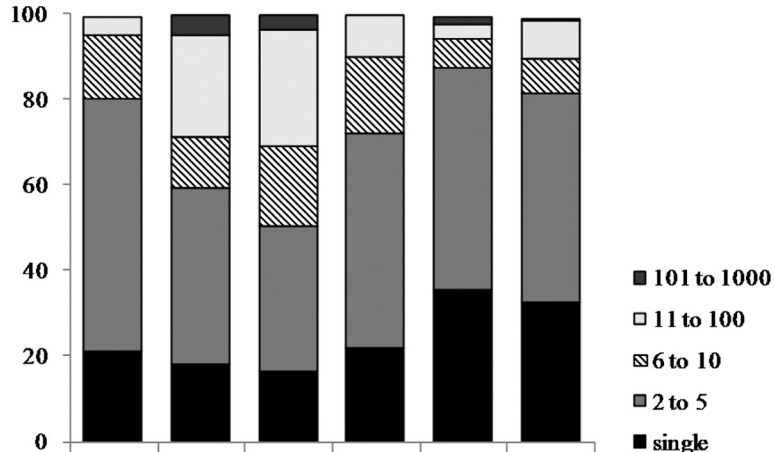
Sampling of attached biofilm. For destructive biofilm sampling, the tubing reactor was disconnected from the nutrient medium and emptied. The exposed outside of the tubing was ethanol disinfected. Small pieces were cut and individually submerged into 10 ml cooled buffer for quantification or treatment. For enumerating cells, biofilm was extruded from the tubing with the help of sterile tweezers. Detached biofilm was suspended in the buffer by pulse vortexing for 1 min, followed by removal of the tubing and shear homogenization at 20,500 rpm for 1 min. This novel method of biofilm removal was very repeatable, with low standard deviations/data variability (data not shown).

Homogenization. Prior to disinfection and for comparison with intact clusters, aliquots of the planktonic, tubing reactor effluent, and biofilm samples were shear homogenized (shear homogenizer from IKA Labortechnik, Staufen, Germany) at 20,500 rpm for 1 min. The homogenizer was sterilized between samples by flaming with ethanol.

Disinfection experiments. A sodium hypochlorite (Fisher Scientific, Pittsburgh, PA) stock was prepared daily, and the concentration was measured with the *n,n*-diethyl-*p*-phenylene diamine (DPD) colorimetric method (LaMotte DC1100 spectrophotometer and DPD chemicals; LaMotte, Chestertown, MD). Chlorine was added to samples according to a standard curve made with increasing amounts of fresh chlorine stock in medium without nitrogen or a carbon source or, alternatively, in filtered spent chemostat medium. Prior to the addition of chlorine, chemostat and tubing reactor effluent samples were standardized to 7 $\log_{10}$ CFU/ml by dilution with sterile buffer. The CFU of the attached biofilm were standardized by immersing a cut piece of tubing (1 to 4 cm in length) into 10 ml of sterile buffer, which resulted in approximately 7 $\log_{10}$ CFU/ml after homogenization in the buffer. Therefore, the CFU of the attached biofilm are also expressed as CFU/ml. Samples were treated in separate experiments for 30 min in a shaking incubator at room temperature with incrementally increasing doses of chlorine until no culturable cells could be detected on the agar plates. Neutralization was done with sodium thiosulfate (Fisher Scientific, Pittsburgh, PA).

Bacterial quantification. Samples were diluted in sterile phosphate-buffered saline (PBS) and plated on R2A plates (Difco R2A agar; Becton Dickinson, Franklin Lakes, NJ) using the drop plate method (15) and incubated for 48 h at 30°C. The two strains were easily distinguished from each other by the appearance of their colonies. Total loss of culturability was the concentration that resulted in no CFU in the undiluted treated sample. If no CFU were present, 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).

Microscopy and image analysis for cluster size determination. LIVE/DEAD BacLight staining (Invitrogen-Molecular Probes, Carlsbad, CA) was used to



	FS-3 CS	FS-3 TRE	RB-8 CS	RB-8 TRE	DUAL CS	DUAL TRE
101 to 1000	0.0	4.5	3.4	0.0	1.7	0.6
11 to 100	4.3	23.8	27.2	10.0	3.2	8.9
6 to 10	14.8	11.8	19.0	17.9	6.9	8.2
2 to 5	59.4	41.4	33.7	50.2	51.8	48.8
single	21.0	18.1	16.7	22.0	35.7	32.5

FIG. 1. Cell cluster size distributions of single and dual species of *B. cepacia* (FS-3) and *P. aeruginosa* (RB-8) from chemostat (CS) and tube reactor effluent (TRE) samples.

visualize the nondisinfected clusters collected on black polycarbonate membranes (Poretics, 0.22 μm by 25 mm). A B2A filter was used on the fluorescence microscope (Nikon Eclipse E800) and a 100× oil immersion lens (Nikon) to simultaneously capture the images of all cells. Captured images (30 random fields of view [6,445 μm^2]) were processed with the MetaMorph (Universal Imaging Corporation, Downingtown, PA) software to determine the number of single cells, number of clusters, and number of cells per cluster. The area of the flattened clusters and cells was calculated by converting pixels into μm^2 (1 pixel = 0.119 μm) and recording the occurrence and size of clusters. For each species a calibration was done to associate the size of the area with the number of bacteria in the cluster (36) as well as the extracellular polymeric substances. To determine the proportion of each cluster type, the area on the filter covered by each cluster type was divided by the total area of all clusters for each species.

Calculation of inactivation rate constants (*k* values). Inactivation rate constants were determined using additional disinfection experiments where cultures were sampled after 1, 2, 5, 10, 20, and 30 min. Inactivation curves were generated in Microsoft Excel by plotting the ln CFU/ml that survived the treatment. Due to the biphasic behavior of the disinfection curves, *k* values were calculated for the first minute (for chemostat and tube reactor effluent samples only), the first 2 min, and also for disinfection times longer than 2 min. The $k_{initial}$ values were calculated from the 2 time points using the equation $k = -\ln (N/N_0)/C \times t$, where N_0 is the initial CFU/ml, N is the CFU/ml after time t, and C is the concentration of disinfectant (4). For k values with multiple time points, 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 $k = -m/C$.

Statistical analysis. Cell numbers as CFU/ml were transformed to the log density (LD) $\log_{10}$ CFU/ml. Analyses of the disinfection susceptibilities were performed using log reductions (LR), defined as $LR = LD_{untreated} - LD_{dose}$, where $LD_{untreated}$ is the LD of the organisms when no disinfectant is applied and LD_{dose} is the LD of organisms which survive disinfection at some specified dose. The $LD_{untreated}$ for organisms grown as a single species was 7, but the RB-8 LDs were below 7 in the dual-species cultures (see Table 2).

For each pairwise species comparison (FS-3 versus RB-8, FS-3 versus dual FS-3, and RB-8 versus dual RB-8), an analysis of variance (ANOVA) was fit with reactor, species, and chlorine dose (1 ppm, 2 ppm, 3 ppm, and 4 ppm) as factors. Biofilm samples were not included in this analysis since LRs for 1 and 3 ppm were not measured. To account for the pairing of the dual FS-3 and dual RB-8 species grown in the same reactor, the difference between the LRs of the two species was 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.

The cell cluster analysis was performed separately for each of the cluster types (single, 2 to 5, 6 to 10, 11 to 100, and more than 100 cells) using an ANOVA with species (FS-3, RB-8, and dual, where dual refers to both dual FS-3 and dual RB-8 grown in a coculture), sample type (chemostat and detached), and the 2-way interaction as fixed effects. For the clusters of 6 to 10 and 11 to 100 cells, a weighted least-squares analysis was performed, since the variance of the proportions in these cases increased as the mean increased.

To determine the effect of homogenization on the cluster distributions, the cluster type proportion for the homogenized cells was subtracted from the proportion for the unhomogenized cells for each reactor. These differences were analyzed separately for each species using an ANOVA with cluster type (single, 2 to 5, 6 to 10, 11 to 100, and more than 100 cells as levels) and reactor (chemostat and detached) as fixed effects and experiment as a random effect.

All of the ANOVA models were fit in Minitab (version 16). The follow-up *t* tests and the weighted least-squares analyses were performed in R (version 2.11.0).

The extensive analyses described above were divided into seven categories: the LRs for FS-3 versus RB-8, FS-3 versus dual FS-3, RB-8 versus dual RB-8, dual FS-3 versus dual RB-8, homogenized versus unhomogenized, *k* values, and the cluster analyses. A Benjamini-Hochberg correction (2) was applied to each of these seven groups to maintain the false-discovery rate at either 5% or 10% for each group. Claims of statistical significance were made with respect to this correction, although the *P* values reported are unadjusted.

RESULTS

Cluster size distributions of single- and dual-species scenarios. Microscopic analysis of RB-8 (*P. aeruginosa*) and FS-3 (*B. cepacia*) as single strains and in coculture showed that chemostat-grown cells do not always exist as single cells. Up to 52% of cells were present in small clusters containing between 2 and 5 cells (Fig. 1). Organisms were also present as single

TABLE 2. Initial cell concentrations in the chemostat, tube reactor effluent, and attached biofilm for single-species and dual-species experiments

Sample type	Species[a]	Log density (t = 0)[b]		n
		Avg	SD	
Chemostat	FS-3 only	7.00	0.11	5
	RB-8 only	7.01	0.13	6
	Dual species FS-3	7.02	0.04	3
	Dual species RB-8	5.73	0.22	3
Tubing reactor effluent	FS-3 only	7.07	0.22	15
	RB-8 only	6.88	0.24	5
	Dual species FS-3	7.14	0.30	7
	Dual species RB-8	5.74	0.48	7
Biofilm	FS-3 only	7.42	0.09	10
	RB-8 only	7.28	0.10	5
	Dual species FS-3	7.13	0.14	6
	Dual species RB-8	5.43	0.36	6

[a] FS-3, *B. cepacia*; RB-8, *P. aeruginosa*; dual-species FS-3 and dual-species RB-8, strains in coculture.
[b] Log densities and standard deviations are expressed as $\log_{10}$ CFU/ml.

cells (up to 36% of all cells) and some larger clusters (up to 30%). Similarly, mainly smaller clusters detached from the tubing biofilm. FS-3 and RB-8 grown as single species resulted in cluster distributions with approximately 15 to 20% single cells, 50 to 75% smaller clusters of 2 to 10 cells, and 10 to 30% larger clusters of 11 or more cells. However, when grown in coculture (for the chemostat as well as the tubing reactor effluent), FS-3 plus RB-8 produced 30 to 35% single cells, 45 to 55% smaller clusters of between 2 and 10 cells, and only 15 to 20% large clusters of 11 cells or more. The proportion of large clusters (11 to 100 cells) was reduced in coculture, while more small clusters (2 to 5 cells) were present than in the single-species samples in the chemostat as well as the tubing reactor effluent samples (Fig. 1). Coaggregation and autoaggregation studies done with washed cells of both species according to the method of Rickard et al. (23) showed that this mechanism did not appear to be responsible for cluster formation (data not shown).

Statistically significant differences in cluster size were detected between the single-species clusters (both FS-3 and RB-8) and the clusters from the coculture, with more single cells in cocultures and more clusters containing 6 to 10 cells in single-species cultures (see Table S1 in the supplemental material). On average, there were no significant differences in cluster size proportions between the chemostat and tubing reactor effluent for any of the species.

Initial cell numbers and species distribution in cocultures for disinfection studies. Cultures were normalized to approximately 7.0 $\log_{10}$ CFU/ml so that all disinfection experiments started with similar $\log_{10}$ densities, except for RB-8 grown in coculture (Table 2). The log density for RB-8 in coculture was smaller on average than that for either RB-8 or FS-3 grown as a single species or for FS-3 grown in coculture ($P \leq 0.0015$). In coculture, FS-3 was the most abundant strain in the three different scenarios: chemostat, biofilm effluent, and attached biofilm (Table 2). *B. cepacia* (FS-3) had a slightly higher growth rate than RB-8, i.e., 0.198 ($\pm$ 0.02) h^{-1} and 0.173 ($\pm$ 0.02) h^{-1}, respectively, but the two growth rates were not statistically different ($P = 0.409$). Due to these differences in initial cell log densities, the log reduction was used as the measure of susceptibility to the disinfectant.

Disinfection susceptibilities of planktonically grown cells, detached biofilm cells, and attached biofilm samples (sample type comparison). Chlorine treatment at concentrations of below 10 ppm of chemostat and tubing reactor effluent samples for 30 min inactivated all single-species and dual-species samples (Fig. 2).

FS-3 in a single-species chemostat culture was inactivated with 3 ppm of chlorine within 30 min of exposure. Eight ppm resulted in total loss of culturability in the TRE. The mean LRs in the CS and the TRE were compared for 1 to 4 ppm chlorine, and the chlorine susceptibility for the CS samples was significantly higher than the chlorine susceptibility for the TRE sample in the single-species FS-3 ($P < 0.001$) (Fig. 2).

Concentrations of 4 ppm in the chemostat and 3 ppm in the tubing reactor effluent inactivated all RB-8 samples. Both species in coculture were readily inactivated with 2 ppm of free chlorine when grown in the CS. In the TRE, 5 ppm inactivated all detached cells and clusters. There was a significant difference between the CS and TRE for dual-species FS-3 over all 4 doses ($P < 0.001$), with the CS samples being more susceptible to chlorine treatment. The dual RB-8 CS samples were not more susceptible in coculture than the TRE samples.

Although there were no statistically significant differences among the initial mean log densities for each of the species (except for RB-8 in the coculture [Table 2]), the disinfection susceptibility was generally less for cells and clusters that had been grown planktonically than for cells and clusters that detached from the biofilm (Fig. 2). As an exception, RB-8 alone was less tolerant than RB-8 in coculture when the cells were detached from the biofilm.

As anticipated, the biofilm required doses of chlorine approximately 10 times higher than those used for the tubing reactor effluent and the planktonic cells (Fig. 3).

Disinfection susceptibilities of *B. cepacia* and *P. aeruginosa* as single species and in coculture (species comparison). RB-8 and FS-3 were grown as single species and in coculture in the CS and the TRE to test for differences in disinfection susceptibility depending on species composition. Figure 4 shows representative disinfectant doses of the experiments for CS, TRE, and biofilm.

As anticipated, CS cultures were readily inactivated by low concentrations of chlorine. RB-8 had lower $\log_{10}$ reductions in the CS when grown as single species or in coculture, indicating that it is more tolerant than FS-3 in the CS (mean log reduction difference of 0.5 over the tested doses [Table 3]). On the other hand, growth in coculture was of no benefit to FS-3 and RB-8, so that 2 ppm led to total loss of culturability of the CS culture.

In the TRE, the single species FS-3 was more tolerant to the chlorine treatment than the single species RB-8 (Fig. 4; Table 3 shows mean $\log_{10}$ reduction differences for all species and sample types). The $\log_{10}$ reductions of FS-3 and dual FS-3 were generally similar at 1 and 2 ppm, and the $\log_{10}$ reductions for RB-8 in coculture were much lower, at 1 and 2 ppm, than those for the single species RB-8 (Fig. 4). These significant differ-

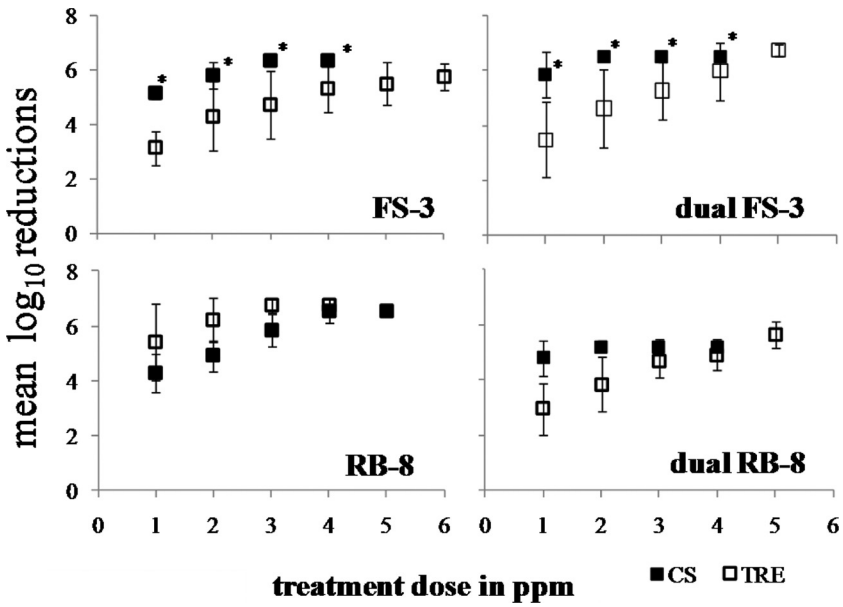


FIG. 2. Log_{10} reduction (LR) comparison among reactors per species after chlorine treatment. Each point represents the mean LR over multiple experiments. Error bars indicate one standard deviation of the LR ($n \geq 3$). Significance at 5% is indicated by an asterisk. FS-3, *B. cepacia*; RB-8, *P. aeruginosa*.

ences in species within the same sample type exist between FS-3 and RB-8 and between dual RB-8 and RB-8 (mentioned above) as well as both dual species ($P < 0.01$). The only significant difference in log_{10} reductions in the CS was between both dual species ($P < 0.01$). There was a significant interaction between dose, species, and reactor ($P = 0.037$) for dual RB-8 and RB-8 (Table 3). Due to this interaction, the mean LR for RB-8 was significantly larger than the mean LR for dual RB-8 for 4 ppm only in the CS, but there was no significant difference when pooled across all doses.

Complete loss of culturability of attached biofilms required about 10 times the concentration needed for the inactivation of CS cultures. RB-8 in the single-species biofilm displayed the highest tolerance to chlorine at low concentrations (2 ppm [Fig. 4]) and single FS-3 was significantly more susceptible than single RB-8 over all tested doses. FS-3 grown in coculture displayed better survival and lower log_{10} reductions than the single FS-3, especially at higher disinfectant concentrations (10, 20, and 30 ppm). Although RB-8 in coculture did not reach an initial cell density of 7 log_{10} CFU/ml, the low initial biomass did not result in decreased tolerance. In coculture, RB-8 displayed mean lower log_{10} reductions than single RB-8.

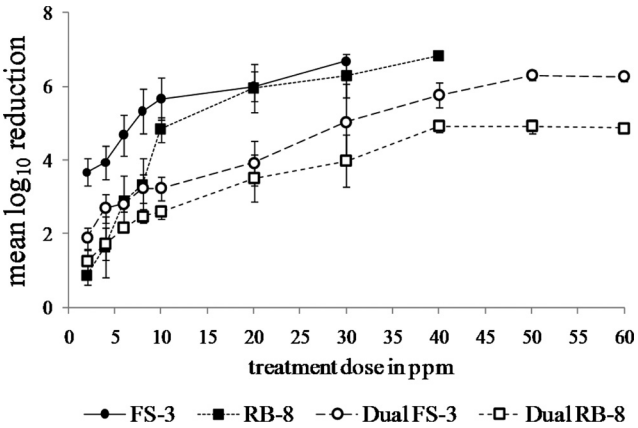


FIG. 3. Log_{10} reduction (LR) comparison among the biofilm sample types after chlorine disinfection. Each point is the mean LR over multiple experiments. Error bars indicate one standard deviation of the LR ($n \geq 3$). See Table 1 for all chlorine treatment doses. FS-3, *B. cepacia*; RB-8, *P. aeruginosa*.

Additional experiments were done to determine if the two species produced compounds that impacted disinfection susceptibility. When each species was exposed to cell-free chemostat effluent from the other species, no decreased tolerance to chlorine for either species could be found. For FS-3, there was no difference ($P = 0.468$), and for RB-8, the cells were slightly and significantly less susceptible ($P = 0.034$).

Homogenization of cells and clusters. Because multiple methods for measuring the amount of EPS in clusters were unsuccessful (data not shown), an alternative method for investigation of the importance of the matrix was used. Homogenization was chosen as an indirect measurement of matrix because it will disrupt cell clusters and can shear off the EPS.

Shear homogenization at 20,500 rpm was unable to disrupt smaller cell clusters but somewhat reduced the number of larger clusters. Homogenized single FS-3 from the TRE had significantly higher proportions of smaller cluster sizes than when not homogenized ($P \leq 0.01$), while single FS-3 CS samples did not show a significant difference in cluster proportions. Homogenized RB-8 grown as a single species also exhibited a higher percentage of smaller cluster sizes than the unhomogenized samples, but these differences were not statistically significant. Homogenized TRE samples of FS-3 and RB-8 in coculture had a higher proportion of cells contained in clusters with 2 to 5 cells and a lower proportion of cells in clusters of 11

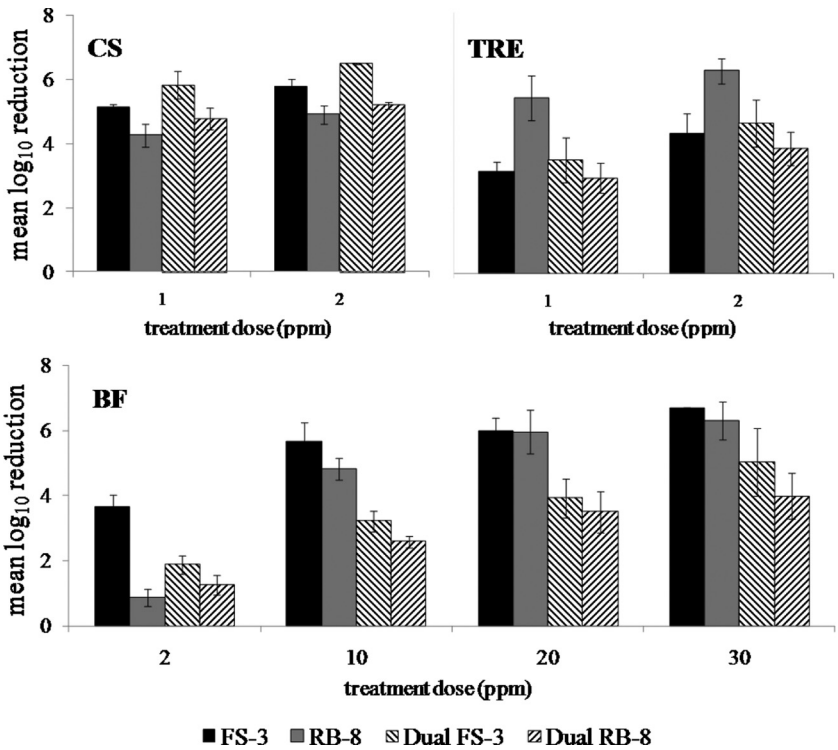


FIG. 4. Log_{10} reduction (LR) comparison among FS-3 (*B. cepacia*), RB-8 (*P. aeruginosa*), dual FS-3 (dual-species *B. cepacia*), and dual RB-8 (dual-species *P. aeruginosa*) for each reactor (chemostat, tubing reactor effluent, and biofilm [BF]) across multiple representative doses (not all doses are shown). The top of each bar represents the mean LR. Error bars indicate one standard deviation of the LR ($n \geq 3$).

to 100 cells (see Table S2 in the supplemental material). Although there were only few statistically significant differences, the number of single cells and small clusters generally increased and the occurrence of large clusters was reduced after shear homogenization (see Table S2 in the supplemental material).

Disinfection of homogenized samples. Initial log_{10} densities were not significantly different after homogenization ($P \geq 0.313$) except for the single species RB-8, which displayed higher log_{10} densities following the homogenization ($P < 0.001$).

Homogenized samples did not show chlorine susceptibilities in the chemostat that were significantly different from those of unhomogenized samples ($P > 0.056$) (see Fig. S1 in the supplemental material). FS-3, dual FS-3, and dual RB-8 were more susceptible to chlorine in TRE samples after homogenization ($P \leq 0.001$). Biofilm samples were always significantly ($P \leq 0.001$) more susceptible to disinfection after mechanical removal from the silicone surface followed by shear homogenization (see Fig. S2 in the supplemental material).

TABLE 3. Mean log_{10} reduction differences among species for each reactor (averaged over all tested doses)[a]

Sample type	Species pair			
	FS-3 - RB-8	DL FS-3 - FS-3	DL RB-8 - RB-8	DL FS-3 - DL RB-8
CS	0.5	0.45	−0.29	1.23*
TRE	−1.92*	0.47	−2.18*	0.73*
BF	1.45*	−1.88*	− 1.16*	0.73*

[a] An asterisk indicates significance at 5%.

***k* values.** To compare the disinfection rate constants, k values were calculated using two different methods: $k_{initial}$ was calculated using only the initial minute (i.e., using LR at times $t = 0$ and $t = 1$) in the CS and the TRE, and k_{end} was calculated over the period from 2 to 30 min. The k values describe the reaction constants, which are dependent on the microorganism, the type and concentration of the disinfectant, and the exposure time. The higher the k values, the faster the inactivation of cells is over a given time and concentration. The disinfection reaction with chlorine occurs mainly in the first minutes, with a significant decrease of log density, followed by little or no change at the later time points so that $k_{initial}$ was always greater than k_{end} (Fig. 5). Therefore, $k_{initial}$ captured the most important information.

Sample type comparison of *k* values. The disinfection rate for FS-3 in the first minute ($k_{initial}$) in chemostat samples was significantly higher than the $k_{initial}$ of FS-3 in the tubing reactor effluent ($P = 0.028$), while the $k_{initial}$ of RB8 in the chemostat was significantly lower than the $k_{initial}$ of RB8 in the tubing reactor effluent ($P < 0.001$) (see Table S3 in the supplemental material). There was no significant difference between the disinfection rates in the first minute for chemostat and tubing reactor effluent samples for both dual species FS-3 and RB-8. All $k_{initial}$ values for the biofilm were below 1.0 and were different from chemostat and tubing reactor effluent sample k values (data not shown).

The disinfection rates from 2 min to the end of the experi-

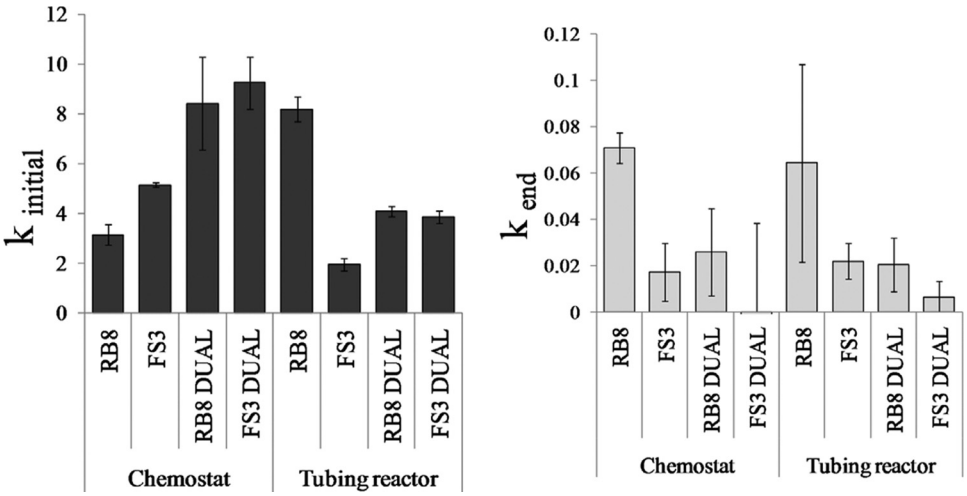


FIG. 5. $k_{initial}$ values for all species in the chemostat (CS) and the tubing reactor effluent (TRE) and k_{end} values for all species. Error bars indicate one standard deviation. Note the difference in *y* axis values.

ment (k_{end}) were not significantly different between the chemostat and tubing reactor effluent samples for all 4 combinations of species.

Species comparison of *k* values. See Tables S3 and S4 in the supplemental material for *k* value differences and significance levels. In chemostat samples, the disinfection rate in the first minute ($k_{initial}$) of FS-3 was higher than the $k_{initial}$ of RB8. The $k_{initial}$ for FS-3 was lower than that for the dual FS-3, while the $k_{initial}$ for RB8 was lower than that in the dual-species scenario, demonstrating that both single species were more tolerant in the chemostat than the dual species within the first minute of disinfection. In tubing reactor effluent samples, the $k_{initial}$ for FS-3 was lower than the $k_{initial}$ for RB8 and the $k_{initial}$ for the dual RB-8 was also lower than the $k_{initial}$ for RB8 indicating that single-species RB-8 detached cells and clusters were more susceptible to chlorine than single-species FS-3 and also RB-8 in coculture.

For the disinfection rate from the second minute to the end of the experiment (k_{end}), significant but small species differences could be found only between FS-3 and FS-3 in coculture for chemostat samples.

DISCUSSION

Cluster size distribution in dual- and single-species cultures. To assess whether particle size is a factor in disinfection tolerance, the cluster size distribution for cells grown in the chemostat was analyzed and compared to that for detaching biofilm clusters. Interestingly, chemostat-grown cells were not present exclusively as single cells but rather mostly as clusters. This result has been shown previously (18). Other studies have also found that cell-to-cell adhesion is independent of the pH of the medium and therefore cell surface charge (5, 31). Singh and Vincent (31) found that clumping is enhanced in low-nutrient solutions due to enhanced capsule formation, making cells "stickier." Rickard et al. (22) also reported that aggregation increases with the age of the cultures, which is supported by Clark (5), who hypothesized that dead cells cluster more easily. In the present study, the cluster size distributions of detached biofilm particles and particles from the planktonic culture were comparable, with single cells and small to medium-size clusters occurring most frequently. Larger clusters were rarer but still accounted for a large proportion of cells. The presence of the EPS matrix in the biofilm results in clusters in the biofilm effluent when pieces of the intact biofilm detach or slough off. However, detachment characteristics are species dependent and can range from single cells to large clusters, as was observed by Fux et al. (12). In the single-species cultures (CS and TRE), a high number of cells (up to 50%) were contained in medium-size and larger clusters (6 cells and more). However, when FS-3 and RB-8 were grown together, the majority (up to 90%) of cells were present as single cells and in small clusters of up to 5 cells (Fig. 1). The occurrence of low proportions of large clusters suggests that the two species do not attract each other via adhesins or receptors as described by Rickard et al. (24). This was confirmed using the amended method of Rickard et al. (23), where no coaggregation of FS-3 and RB-8 was seen. The species also did not autoaggregate in sterile deionized water.

Initial cell numbers and species distribution in coculture. *B. cepacia* (FS-3) was the dominant species in the coculture when comparing the initial species distributions in all three experimental scenarios (Table 2). *B. cepacia* has a slightly higher growth rate than *P. aeruginosa* (RB-8), which can explain the differences in the species distribution. However, after 4 days of growth in the biofilm tubing reactor, RB-8 remains established in the attached biofilm and in the reactor effluent, illustrating that an organism with a lower growth rate can persist in the biofilm Additional studies using cell extracts from chemostat-grown RB-8 and FS-3 showed that these organisms do not produce substances that influence the disinfection tolerance of the other strain (data not shown). However, the possibility for interaction via cell-to-cell signaling or other mechanisms cannot be excluded.

Disinfection of planktonically grown cells compared to detached biofilm cells. Although initial cell numbers were very similar in all cultures, chlorine tolerance differed from species to species and from CS to TRE samples. Generally, TRE samples had more large clusters than chemostat samples. In contrast to the case for *B. cepacia*, when *P. aeruginosa* was grown as a pure

culture in a chemostat, it produced more large clusters These results indicate that increased resistance may be directly linked to more cells being contained in larger clusters and thus being shielded from disinfection Even though cluster size and log reductions were not measured in the same experiments simultaneously, the correlation between disinfection and cluster sizes was assessed by pairing the proportion of large clusters with the log reduction for each species in each reactor system. A multiple regression was then fit across all doses of chlorine, and four separate simple linear regression models were fit for each dose. The results suggested that at low doses, those species in reactor systems with a higher proportion of clusters with more than 10 cells were less susceptible to chlorine disinfection, but the conclusion is not statistically significant. However, the lack of statistical evidence does not preclude the importance of cell cluster size, since the general trend is in this direction. Another aspect of cell clusters is the presence of extracellular polymeric substances that have been shown to be protective in a disinfection scenario. More organic substances in the EPS react with the available chlorine, making less of it available for disinfection, as has been reported by DeBeer et al. (9) and Xu et al. (38). The reaction of the biofilm matrix with the available chlorine also results in a retardation of diffusion into the biofilm. Xu et al. (38) entrapped bacteria in alginate beads to model the disinfection of cells surrounded by a matrix and noticed that the presence of such substances decreased disinfection susceptibility.

In addition it has been shown that when they are in the CS, cells are actively growing and are therefore more susceptible to disinfection than cells in the stationary phase (1, 20). Growth rates in the biofilm depend highly on the location within the biofilm and the availability of nutrients. Biofilm layering also influences the physiological states of the cells in the clusters and thus alters disinfection susceptibility (16).

Lower growth rates and nutrient limitation are factors in making biofilms more tolerant to disinfection than planktonic samples and tubing reactor effluent samples (21, 35). The attachment to a surface (silicone tubing in this study) allows chlorine to attack the biofilm from only one side, and chlorine is reacting with the EPS matrix (as described above) before it can reach cells in lower layers of the biofilm.

Disinfection of *B. cepacia* and *P. aeruginosa* as single species and in coculture. The disinfection susceptibilities of the two strains RB-8 and FS-3 depend on whether each is grown as a single-species culture or in coculture. Single species in the CS were more likely to survive disinfection than the coculture in the CS (Fig. 4).

In contrast, the two species in detached biofilm clusters responded more similarly to disinfection when grown as a coculture, which leads to better survival of RB-8 and more efficient killing of FS-3 than for the single species. Interestingly, the dual-species biofilm reactor effluent had a higher percentage of single cells and smaller clusters (>80%) than the single species (Fig. 1), but the tolerance to chlorine was increased (Fig. 4). This outcome suggests that the disinfection efficacy depends largely on the amount and occurrence of larger clusters in the samples, but other protective mechanisms may become a factor when the two strains are grown in coculture. The presence of the second species has been shown to alter the composition and viscosity of the EPS matrix and thus the diffusivity, slowing the penetration of chlorine dioxide into the biofilm (3). Physiological changes can occur when two species are able to share protective mechanisms by transferring conjugative plasmids (13) or support each other by complementing enzymes that are necessary to manage environmental challenges, as observed in dental biofilms maintaining a stable pH (28). Interspecies competition experiments showed that neither strain produces substances that make the other species more susceptible to disinfection.

Coculture is beneficial for survival during disinfection of attached biofilms. Single-species biofilms were readily inactivated with 30 ppm of chlorine, while dual-species biofilms required up to 80 ppm of chlorine. At low doses (2, 4, 6, and 8 ppm), the responses of the single- and dual-species biofilms were somewhat similar, but the dual-species biofilm was more resilient at higher concentrations (10, 20, and 30 ppm and higher if applicable). An explanation is the presence of tolerant specialized survivor cells (persister cells) in the biofilm community that are known to occur in bacterial populations (17), and their development may have been triggered by the dual-species growth. The cocultured biofilm was also the thinnest at 23 μm (±4 μm) but also the most resistant to disinfection, supporting the possibility of protective interactions.

Additional experiments and image analysis of cryosectioned biofilms showed that RB-8 biofilms were the thickest on average, at 28 μm (±4 μm). FS-3 biofilms were 26 μm (±4 μm) thick. Biofilm thickness depends on the cell size, the amount and type of EPS produced, the shear forces to which the biofilm is exposed during growth, and the availability of nutrients (6, 30, 33). Some strains and phenotypes of *P. aeruginosa* are well known for the production of increased amounts of extracellular substances (14). The environmental *P. aeruginosa* strain used in this study also produced large, glossy colonies with undefined borders that were visible after 24 h, while *B. cepacia* formed well-defined small colonies that were visible only after 48 h of incubation. These observations suggest the presence of increased amounts of extracellular polymeric substances and explain why RB-8 biofilms were slightly thicker under these growth conditions.

Mechanical disruption. Homogenization alone did not result in lower cell numbers, and control samples were not impaired in their growth after homogenization. However, recovery of cells after disinfection was negatively affected. Potential mechanisms are the removal of extracellular polymeric substances or injury to cell membranes that allowed better penetration of chlorine. Since disinfection efficacy is not always directly related to the cluster sizes in the samples, other factors, such as the physiological state of the cells, may have an effect.

***k* values.** The analysis of the disinfection rates $k_{initial}$ and k_{end} for all sample types and species revealed that $k_{initial}$ was about 10 times larger than k_{end} in all scenarios, showing that the majority of the disinfection occurs within the first minute of treatment with chlorine, with a remaining subpopulation. The $k_{initial}$ values were an excellent indicator of the chlorine tolerance of the samples. The $k_{initial}$ values for RB-8 and FS-3 in coculture were very similar, which supports findings from the disinfection experiments with incrementally increasing chlorine doses. Figure 5 shows that species and sample types with high initial k values also resulted in high $\log_{10}$ reductions after 30 min of chlorine exposure compared to other species and sample types. The k_{end} values were not statistically different, which confirms that most of the killing occurs within the first minute of exposure.

Conclusion. In this study, disinfection tolerance in single-species cultures was associated with the occurrence of larger cell clusters in either the CS or TRE samples. Single RB-8 TRE samples were less tolerant than the CS samples that contained a higher proportion of larger clusters than the tubing reactor effluent. In contrast, single FS-3 in the TRE was more tolerant than the CS samples that had a lower proportion of large cell clusters. This suggests that cells in samples with similar initial log densities are more resistant to chlorine disinfection when the relative number of large clusters is high than samples with a lower number of large clusters.

When RB-8 and FS-3 were grown in coculture, the relative number of larger clusters was reduced compared to that for the single-species samples, but the tolerances of the TRE and biofilm samples were at least as large as those for single-species samples. This indicates that other factors, such as the physiological state or cell-to-cell signaling, may play an important role, especially in multispecies cultures. The k values for the first minute of exposure to chlorine were a good indicator of chlorine tolerance when comparing different species and sample types.

This research suggests that species composition not only influences the cluster characteristics of cells in suspension and when detaching from biofilm but also determines chlorine susceptibility. Thus, disinfection studies with monospecies cultures may not sufficiently describe the disinfection tolerance observed in multispecies scenarios.

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