DISINFECTION SUSCEPTIBILITIES OF DETACHED BIOFILM CLUSTERS COMPARED TO PLANKTONIC CELLS AND BIOFILMS IN SINGLE SPECIES AND DUAL SPECIES CULTURES

by

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ABSTRACT

Detachment of cells and clusters from biofilms is an important process in the dissemination of microorganisms in industrial, environmental, and clinical settings but the disinfection susceptibilities of these cell clusters have not been sufficiently characterized.

With the help of fluorescent microscopy and image analysis, naturally detaching cells and clusters from single species and dual species biofilms of *Burkholderia cepacia* and *Pseudomonas aeruginosa* grown in biofilm tubing reactors were analyzed for cluster size distributions and compared to the cluster sizes in chemostat cultures.

The commonly used oxidizing agents free chlorine, chlorine dioxide and dissolved ozone were used for disinfection experiments and susceptibilities of detached clusters, planktonic cells, and intact biofilms in single species and dual species cultures were determined. Additionally, disinfection rates were calculated for chlorine and chlorine dioxide disinfection for all sample types and species.

In experiments with chlorine as the disinfectant, a correlation between cluster sizes and disinfection efficacy was observed for single species only. Samples with the higher percentage of large clusters were more tolerant than samples with fewer large clusters. Chemostat samples and detached clusters from dual species reactors contained lower numbers of large clusters but were equally or less susceptible than their single species counterparts. Biofilms required chlorine doses up to ten times higher than chemostat or detached biofilm cells for total inactivation.

Chlorine dioxide disinfection was independent of cluster size so that chemostat cells and detached clusters were similar with respect to log reductions and disinfection rates. Dual species chemostat cells, detached clusters, and biofilms were more tolerant to chlorine dioxide than the single species samples. As with chlorine, biofilms required much higher chlorine dioxide doses for total inactivation.

Ozone was very efficient against *B. cepacia* chemostat cells and detached clusters but failed to inactivate biofilm samples with the concentrations used in this study.

In general, detached clusters were more similar to chemostat cells and very different from biofilms with respect to disinfection susceptibilities and disinfection rates suggesting that biofilm-specific physical and physiological protection mechanisms may be lost shortly after the detachment event or may be absent in small clusters.

CHAPTER 1

LITERATURE REVIEW

Biofilm Life Cycle

In most environments, bacteria occur as structured communities rather than single cells. These so called biofilms represent a lifestyle that allows bacteria to survive in virtually any environment and inhabit new niches by dispersal of cells. Biofilms take advantage of several survival and protection mechanisms such as production of extracellular polymeric substances (EPS), cell to cell communication and cell differentiation (Donlan 2002).

After single planktonic cells attach to surfaces with the help of flagella-mediated motility and weak molecular interactions, they form permanent bonds with the substratum by excreting extracellular polymers (Characklis 1990). In differential gene expression assays, surface-regulated switches from swimming motility to twitching motility have been shown to induce irreversible attachment in *Pseudomonas putida* (Sauer & Camper 2001). Microbial EPS is a gel-like substance that aids in the process of cell attachment, is highly diverse in chemical composition and may include a variety of polysaccharides, proteins, nucleic acids, phospholipids and mainly water (Wingender *et al.* 1999).

Following initial attachment, pili- and flagella-mediated movement may help in the formation of microcolonies on the substratum (O'Toole & Kolter 1998). As the biofilm matures, a complex architecture with channels and pores is generated and

bacteria grow away from the surface of initial attachment (Davies *et al.* 1998). This unique structure allows for the diffusion of oxygen, nutrients, and metabolites in and out of the biofilm (Donlan 2002, Stoodley *et al.* 1994). In addition, the characteristics of the biofilm become dramatically different from characteristics of planktonic cells. *P. aeruginosa* biofilms were shown to have very different protein profiles from cells in chemostats with respect to metabolism, phospholipid and LPS biosynthesis, membrane transport and secretion (Sauer *et al.* 2002). Nutrient requirements and growth rates of biofilm-associated cells change as a result of their natural habitat (Xu *et al.* 1998).

Another advantage of growth in close proximity for cells is cell-density dependent cell to cell communication (quorum sensing) that allows for concerted efforts such as gene regulation. Autoinducer molecules have been found in naturally occurring biofilms and laboratory biofilms (McLean *et al.* 1997; Davies *et al.* 1998) *P. aeruginosa* PAO1 biofilms require HSL (homoserine lactone) molecules in order to develop normal differentiated biofilms while quorum sensing mutants resulted in very thin biofilms.

Biofilms are also characterized by the active or passive detachment of cells and clusters which is not limited to mature biofilms (Bester *et al.* 2005). Depending on the species composition and mechanical biofilm stability, increased shear forces can lead to detachment of biofilm clumps, which may be enhanced when exposed to oxidizing agents such as chlorine (Simões *et al.* 2008; Simões *et al.* 2005). 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 remaining biofilm cells (Sauer *et al.* 2002, Hunt *et al.* 2004, O'Toole et al. 2000).

Detachment can cover a wide range of cluster sizes including single cells but frequency and size distribution highly depend on species composition and growth or treatment conditions (Wilson *et al.* 2004). Detached cells are thought to express a transitional phenotype between sessile and planktonic states during the first hours after detachment, displaying similar growth kinetics and cell-surface properties as attached biofilm cells (Rollet *et al.* 2009). Sauer *et al.* (2002) found that dispersing biofilm cells return to a planktonic mode of growth, thus completing the biofilm life cycle.

Mixed Species Biofilms

In mixed species biofilms, which occur in nature rather than monospecies biofilms, bacteria inhabit suitable niches in a particular microenvironment and also undergo symbiotic relationships between different bacterial species (Møller *et al.* 1996, Møller *et al.*1998). Since complex mixed species biofilms are more difficult to study, researchers often investigate dual species biofilms with respect to species dominance, growth rates, competition, symbiotic interactions, and communication strategies (Banks & Bryers 1991; Komlos *et al.* 2005; Burmølle *et al.* 2006; Riedel *et al.* 2001; McKenney *et al.* 1995).

Dual species biofilm population dynamics are usually different from population dynamics in batch cultures. Banks and Bryers (1991) found that the organism with the faster planktonic growth rate was the dominant species in a dual species biofilm while the second species stayed established and even increased in numbers as the biofilm matured. However, planktonic growth rates do not always determine species dominance in a biofilm as shown by Komlos *et al.* (2005) who determined substrate concentration in the

growth medium to be the most important factor. Independent of growth rates and species dominance, mixed species biofilms are generally more resistant to disinfection challenges than their single species counterparts (Burmølle *et al.* 2006). Factors that may influence the increased tolerance are the production of more biomass and thicker biofilms along with changes in the EPS matrix that may slow diffusion through increased viscosity (Burmølle *et al.* 2006). Ghigo (2001) suggests that conjugative transfer of plasmids may be responsible for the dispersal of defense mechanisms within a biofilm. Where survival mechanisms are not exchanged via conjugation, enzyme complementation may support species diversity in a biofilm due to enzyme production of one of the species that allows survival of other species as shown in dental biofilm consortia (Shu *et al.* 2003). As a response to environmental changes, two species have been shown to re-organize spatially within a biofilm to take advantage of the protective mechanisms offered by one of the species (Cowan *et al.* 2000; Nielsen *et al.* 2000).

Synergistic relationships between two strains have been recognized in dual species biofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia* which are common environmental strains but also key pathogens in cystic fibrosis infections. Depending on the strain, *P. aeruginosa* and *B. cepacia* are able to communicate via *N*-acylhomoserine-lactone molecules as shown by Riedel *et al.* (2001) using unidirectional signaling with *P. aeruginosa* excreting molecules and *B. cepacia* receiving them. The pathogenicity of clinically relevant *B. cepacia* in cystic fibrosis infections is influenced by the presence and signal molecule secretion of *P. aeruginosa* in terms of virulence factor production (McKenney *et al.* 1995).

Biofilm Control

Biofilm control has become an important issue in water quality management, food processing plants, industrial water lines and many other applications. In these settings, biofilms are undesirable because of contamination and health risks, system failure due to hydraulic problems, contamination of end-products, degradation of materials (biofouling), and aesthetic reasons (Flemming 2002).

Studies have recognized that cells in biofilms are much more tolerant to disinfection than planktonic free-floating cells due to a variety of reasons. Since biofilm cells display different phenotypes, they allow for the differentiation of the biofilm into complex multicellular structures that optimizes survival during treatment with antibiotics or disinfectants (Davies *et al.* 1998, Sauer *et al.* 2002). Increased resistance may also be the result of limited diffusion or neutralization of disinfectants due to the gel-like EPS matrix that protects the deeper layers of cells from damage (DeBeer *et al.* 1994; Steed & Falkinham 2006; Donlan & Costerton 2002; Davies 2003). Additionally, it has been found that areas of low metabolic activity and oxygen limitation in the interior of the biofilm correlate with the regions that withstand antibiotics and disinfectants most effectively (Walters *et al.* 2003; Rani *et al.* 2007, Huang *et al.* 1995).

More recently, studies have considered the existence of so-called persister cells which are thought to be deeply dormant cells that are survive disinfectant treatment but are different from resistant or simply non-growing organisms (Keren *et al.* 2004).

Disinfection solutions and biofilm control strategies largely depend on the application and system characteristics. Factors to be considered are range of application,

availability, price and by-product formation (Mara & Horan 2003). If water is to be treated, the chemical composition of the water may influence the choice of disinfectant. Free chlorine, chlorine dioxide, chloramines, ozone and UV light are possible disinfectants for water. Chlorine-based oxidants are the only disinfectants that deliver a continuing level of disinfection known as residual disinfectant which is required by law for some utilities that disinfect water (Alternative Disinfectants and Oxidants Guidance Manual, EPA 815-R-99-014, April 1999).

Inactivation of Detached Cell Clusters

As part of the natural biofilm life cycle or as a result of cell to cell signaling, cell clusters can actively detach from biofilms to initiate further biofilm growth downstream and possibly guarantee survival of the community (Sauer *et al.* 2002, Hunt *et al.* 2004). Detachment can occur in very young (6 h) biofilms and is not limited to mature biofilms (Bester *et al.* 2005). Passive forms of detachment can occur when shear stress increases or certain chemicals alter the cohesion of the EPS (Simões *et al.* 2008; Simões *et al.* 2005, Davison *et al.* 2010). Cluster sizes detaching from biofilm range from single cells to large clusters containing more than 1000 cells and the proportions are dependent on species composition and growth conditions of the biofilm (Wilson *et al.* 2004).

Compared to planktonically grown cells, detached cells and clusters show increased tolerance to antibiotics (Fux *et al.* 2004) or disinfection with chlorine (Steed & Falkinham 2006), but are less resistant than the sessile biofilm. These cell clusters may not be inactivated properly if insufficient amounts of disinfectant are applied which can also be dependent of particle size (Winward *et al.* 2008). Detached clusters may behave

similarly to biofilms when exposed to disinfectants especially shortly after the detachment event (Rollet *et al.* 2009).

Surviving cells in clusters may initiate re-growth of the biofilm downstream of the disinfection site which can present a risk to human health in water treatment and food processing and is also relevant in other industrial settings. Additionally, detaching cell clusters can contain a number of bacteria that may represent an infective dose of a pathogen which is typically not found in a bulk fluid when cells are suspended and diluted (Hall-Stoodley & Stoodley 2005).

Chlorine Disinfection

To control microbial growth in industrial settings, oxidizing agents like chlorine, chlorine dioxide, or ozone are frequently used because they are inexpensive and easily accessible. Free chlorine is one of the most frequently used disinfectants in water treatment because it readily available as gas, liquid or powder, is highly soluble in solution (7,000 ppm), and thus easy to apply. Another important characteristic of free chlorine is its ability to leave a residual in disinfected water which offers a continuing level of protection. A disadvantage of chlorine is the production of halogenated disinfection byproducts, for example trihalomethanes and halogenated acidic acids that are considered a risk to human health with respect to potential for cancer and reproductive /developmental effects (Richardson *et al.* 2007).

Free chlorine is a very effective and fast-acting disinfectant that inactivates a wide range of bacteria at low concentrations (Ridgway & Olson 1982) but is less effective against protozoan oocysts (Venczel *et al.* 1997), viruses like the Noro virus (Keswick *et*

al. 1985), Hepatitis A (Mbithi *et al.* 1990), and the coxsackievirus which needs up to 5,000 ppm of chlorine for inactivation (Sattar *et al.* 1989).

Chlorine is a strong oxidizer and attacks cells in multiple ways. When it comes in contact with the bacterial membrane it can inhibit membrane mediated active transport processes like the update of glucose as a carbon source (Camper & McFeters 1979). Venkobachar *et al.* (1977) reported leakage of marcomolecules along with proteins and nucleic acids from the cells suggesting permeability changes of the membrane. Phosphate and oxygen uptake have also been shown to be inhibited by chlorine treatment (Venkobachar *et al.* 1977). Once in the cells, free chlorine is able to attack nucleic acids by introducing single- and double-stranded lesions into the bacterial chromosome (Shih & Lederberg 1976a) and can lead to mutations (Shih & Lederberg 1976b). In addition, protein synthesis may be inhibited (Benarde *et al.* 1967) and amino acids are oxidatively decarboxylated (Pereira *et al.* 1973).

Lisle *et al.* (1998) proposed that chlorine first reacts with extrinsic components (e.g., the capsule and outer membrane) thus reducing the concentration of the disinfectant. After extracellular targets have been overwhelmed the chlorine can diffuse into the cytoplasmic membrane and cause oxidative damage which induces repair mechanisms (heat shock proteins and redox regulon) in the cells. This theory is supported by Camper and McFeters (1979) who demonstrated that low amounts of chlorine can sub-lethally injure cells that subsequently recover under suitable conditions.

Recent studies have found that virtually all bacteria are much more tolerant to disinfection when they are associated with biofilms (see background “Biofilm Control”).

Biofilms and their constituents have a high chlorine demand due to the presence of compounds that react with chlorine (Lu *et al.* 1999). DeBeer *et al.* (1994) showed that chlorine simultaneously reacts with EPS constituents while it diffuses into the biofilm which decreases the concentration of chlorine within the biofilm compared to the bulk fluid.

Chlorine Dioxide Disinfection

Chlorine dioxide is a yellowish-green gas and potent oxidizing disinfectant that is used in water treatment. Volk *et al.* (2002) reported that disinfection with chlorine dioxide significantly reduces the formation of trihalomethanes that are commonly formed during chlorine disinfection.

Compared to chlorine, chlorine dioxide does not react with ammonia nitrogen, amines or other oxidizable organic matter and is also independent of pH (Aieta *et al.* 1980). In potable and waste water treatment applications, researchers have commented on the significantly lower demand of the water for chlorine dioxide than for chlorine (Ingols & Ridenour, 1948, Ridenour & Ingols 1947). On the other hand more chlorine dioxide is required to maintain a given residual over a longer period of time in water compared to chlorine (Aieta *et al.* 1980).

Although chlorine and chlorine dioxide are both oxidizing agents, their modes of action on cells differ slightly from each other. Berg *et al.* (1986) observed that no macromolecules leaked out of the cells after chlorine dioxide treatment, but a release of potassium ions indicated a loss of permeability of the membranes and the destruction of the trans-membrane ionic gradient.

In comparison to free chlorine, chlorine dioxide has a greater bactericidal activity (Benarde *et al.* 1965) as well as being significantly more effective against viruses (Aieta *et al.* 1980). Chlorine dioxide has been shown to damage nucleic acids and the protein capsid in viruses (Li *et al.* 2004). Reactivity of chlorine dioxide with viral proteins and bacterial protein synthesis has been shown previously (Benarde *et al.* 1967; Noss *et al.* 1985).

Chlorine dioxide, with a high oxidation capability, has been described to be more effective than chlorine against biofilms (Mayack 1984). Jang *et al.* (2006) reported that chlorine dioxide at 25 ppm was able to penetrate biofilms up to 100 µm in thickness after 15 minutes exposure time which indicates fast penetration of the disinfectant into the biofilm.

Ozone Disinfection

Ozone is an unstable gas created from O_2 via corona discharge or ultraviolet light methods and can be produced directly where needed. O_3 is only slightly soluble in water (1.05 g/L at 0 °C) compared to chlorine and chlorine dioxide, but can be brought into solution using gas diffusers for disinfection purposes (Roth & Sullivan 1981). It has been successfully used in water treatment facilities in Europe for several decades and also has been recognized by the food industry as an efficient and safe disinfectant (Glaze 1987). O_3 is often a preferred disinfectant because it does not produce trihalomethanes that have been identified as harmful disinfection by-products arising from the disinfection with chlorine (Bull 1982). However, other non-halogenated disinfection by-products such as a variety of ketones, aldehydes, and carboxylic acids have been identified (Richardson *et*

al. 2000). Since O_3 is very reactive it is often used for the oxidation of organic and inorganic pollutants in water, making ozonation a suitable approach for meeting treatment requirements.

Once dissolved in water, O_3 decays quickly into free radicals and reacts with organic substances as well as cells and cell constituents. It has been reported that ozone damages bacterial membranes (Scott & Leshner 1962) and then diffuses into the cells to react with cell constituents (Ishizaki *et al.* 1987). Hamelin *et al.* (1978) observed single stranded breaks in DNA of treated bacteria which resulted in extensive DNA damage followed by cell death. Ishizaki *et al.* (1987) also proposed chromosomal DNA damage as a major factor in bacterial killing by ozone. In the gaseous or aqueous phase, O_3 has a wide range of activity as a powerful disinfectant against bacteria (Kim & Yousef, 2008), fungi (Lezcano *et al.* 2000), protozoa (Rennecker *et al* 1999), and viruses (Vaughn *et al.* 1987; Katzenelson & Biedermann 1976).

Although ozone is very efficient at killing suspended cells at low concentrations, much higher concentrations can be necessary to kill biofilm cells. Studies determined that the disinfection efficacy of ozone on biofilms depends on the applied concentration as well as the exposure time (Viera *et al.* 1999a, Viera *et al.* 1999b). O_3 reacts with and may remove extracellular polymeric substances (EPS) from cells so that biofilms display a higher degree of tolerance to the disinfectant due to reaction-diffusion limitations (Tachikawa *et al.* 2009).

Lezcano *et al.* (1999) recognized that species composition of the biofilm is a crucial factor that determines ozone dose and exposure time, with environmental strains

being more tolerant to ozone treatment than regularly used, commercially available lab strains.

Concluding Remarks

Disinfectants are commonly judged on their efficacy against planktonic cells or more recently, biofilm cells. While it has been noted that disinfectants are orders of magnitude less effective against cells in biofilms compared to planktonic cells, questions regarding the disinfection tolerance of detached biofilm clusters remain largely unanswered.

In order to appropriately test for trends in the disinfection tolerance of detached particles, a few aspects have to be considered. Studies suggested that mixed species cultures, in particular biofilms, are more tolerant to disinfection challenges (Burmølle *et al.* 2006) so that not only monocultures should be subjected to treatment.

Cell to cell communication is common in environmental samples (Fuqua *et al.* 1996) and has been shown to not only impact biofilm architecture, dispersal, and EPS production (Irie & Parsek 2008), but also may increase the transcription of the *rpoS* gene (Kojic & Venturi 2001) that codes for a central regulator system involved in survival of starvation and environmental challenges such as oxidative stress (Loewen & Hengge-Aronis 1994).

Environmental isolates of *Burkholderia cepacia* and *Pseudomonas aeruginosa* are good candidates for co-culture disinfection studies since it has been demonstrated that some *Burkholderia* and *Pseudomonas* strains may be able to communicate via signaling molecules and thus be more tolerant to biocide challenges (Riedel *et al.* 2001).

Increased tolerance in binary biofilms may lead to detached particles from binary biofilms with similar tolerance to disinfection compared to their single species counterparts.

Additionally, cluster sizes of detached particles have been shown to be a crucial factor in disinfection efficacy (Winward *et al.* 2008), with larger clusters presenting a barrier during disinfection due to slowed diffusion into the middle of the cluster (deBeer et al. 1994, Stewart & Raquepas 1995). To allow for the correlation of disinfection results with detached cluster sizes, microscopic analysis of detached particles was performed as an integral part of this study.

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CHAPTER 2

COMPARING THE CHLORINE DISINFECTION OF DETACHED BIOFILM CLUSTERS WITH SESSILE BIOFILMS AND PLANKTONIC CELLS IN SINGLE AND DUAL SPECIES CULTURES

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Abstract

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 has 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 co-culture. 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 co-culture, and *P. aeruginosa* in co-culture.

The disinfection efficacy was also dependent on species composition; co-culture 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 grown as a mixed planktonic culture.

Introduction

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 (Donlan 2002, Stoodley *et al.* 1994). As a result of their habitat, the physiological characteristics of biofilm-associated cells are different from 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 (Davies 2003).

Most importantly, biofilm cells have been shown to be significantly more tolerant to antibiotics and disinfectants than their planktonic counterparts (Steed & Falkinham 2006; Donlan & Costerton 2002; Davies 2003). Increased resistance may be the result of limited diffusion of disinfectants into the biofilm due to the gel-like EPS matrix that protect the deeper layers of cells from damage (DeBeer *et al.* 1994). 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 (Walters *et al.* 2003; Rani *et al.* 2007). 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 (Davies *et al.* 1998, Sauer *et al.* 2002).

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 (Simões *et al.* 2008; Simões *et al.* 2005). 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 (Sauer *et al.* 2002, Hunt *et al.* 2004). Detached cell clusters can cover a wide range of cluster sizes also including single cells and the cluster size distribution highly depends on species composition and growth or treatment conditions (Stoodley *et al.* 2001; Wilson *et al.* 2004). Detached cells are thought to express a transitional phenotype between sessile and planktonic states during the first hours after detachment, displaying similar growth kinetics and cell-surface properties as attached biofilm cells (Rollet *et al.* 2009). Compared to planktonically grown cells, detached cells and clusters have increased tolerance to antibiotics (Fux *et al.* 2004) or disinfection with chlorine (Steed & Falkinham 2006), but are less resistant than the attached biofilm itself. Efficacy of the disinfection of particle-associated cells is closely linked to the size of the particles and may lead to failure of treatment if pre-filtration is not present or insufficient amounts of disinfectant are added (Winward *et al.* 2008). 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 cells re-attach 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 their planktonic cultures and attached biofilms grown as a single species. Another aspect was to grow the organisms in co-culture 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 (Burmølle *et al.* 2006). Some strains of *B. cepacia* and *P. aeruginosa* are known to utilize the same signal molecules (Riedel *et al.* 2001) and have been shown to communicate on an interspecies level (McKenney *et al.* 1995) which makes them good candidates for co-culture.

Initially, the occurrence and size distribution of clusters detaching from biofilms grown in tubing reactors were characterized. Three different biofilms were considered; a single species *B. cepacia* (FS-3), single species *P. aeruginosa* (RB-8), and a co-culture of both species. The organisms were grown in a low nutrient growth medium to simulate low nutrient environments. This was followed by the evaluation of the chlorine susceptibility of the single and dual species in 3 different experimental scenarios: grown as planktonic cultures in a continuously stirred chemostat, grown as biofilms attached to the inner walls of silicone tubing, and as detached cells and cell clusters from the effluent

of the tubing reactor. This represented three physiological states: planktonic, biofilm and detached as single species and as a dual culture.

Materials and Methods

To compare the chlorine susceptibilities of planktonic cells to cells detached from biofilm, as well as attached biofilm, environmental isolates of *Burkholderia cepacia* (FS-3) and *Pseudomonas aeruginosa* (RB-8) were grown as single species cultures and dual species cultures (co-inoculation of both species into a reactor) in chemostats and biofilm tubing reactors (see Table 2.1). Cluster sizes were analyzed for the chemostat samples and the tubing reactor effluent, 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

Cultures of FS-3 and RB-8 were grown at room temperature (22 ± 2°C). Inoculation cultures were grown for 18 h on a shaker in a low nutrient sterile defined medium consisting of 0.1 g/L glucose, 0.018 g/L NH_4Cl, 3.93g/L phosphate buffer (2.71 g/L Na_2HPO_4; 1.22 g/L KH_2PO_4), and 2 ml/L 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 chloramines during chlorine disinfection, the ammonia concentration was adjusted so it was completely used by the bacterial culture. For experiments where reactor effluent or chemostat samples were used,

total ammonia measurements using the salicylate method (AmVer™ Low Range Ammonia, Hach Lange, Loveland, CO) at 655nm confirmed the absence of ammonia in the spent and filtered medium. Alternatively, ammonia was removed (replacement of media with buffer) when collecting detached cells and clusters from the biofilm effluent.

Batch Culture for Growth Rates

Overnight cultures were grown in the defined medium described previously. A small aliquot was transferred to fresh medium at the start of the growth curve. Samples were taken every 2 hours, diluted appropriately and plated on R2A agar (see section on bacterial quantification).

Chemostat Cells

Planktonic cultures were grown in the defined medium mentioned above. The chemostat (BioSurface Technologies Corp., Bozeman, Mont.) had a volume of 170 ml and the residence time was set to be slightly over 6 hours using a nutrient flow of 0.5 ml/min. The chemostat was filled with medium 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 2 doubling times (12 hours) was followed by continuous flow for another 6 doubling times (36 hours) 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 hours. The flow was then turned off and the lid of the chemostat was removed for collection of a sample.

Biofilm and Detached Clusters and Cells

Biofilms were grown in a biofilm tubing reactor which consisted of 45 cm of silicone tubing (ColeParmer Masterflex Size 16 peroxide-cured silicone tubing) supplied with nutrient medium via a peristaltic pump. The tubing has an inner diameter of 0.31 cm with a total reactor volume of 3.4 ml and a residence time of 6.8 min when the flow rate was set at 0.5 ml/min. The autoclaved, sterile reactor was filled with the medium described before and inoculated with 2 ml of overnight culture (1 ml of each strain for dual culture), followed by an attachment period of 3 h with no flow. After the flow was turned on the biofilms were grown for 4 days at room temperatures (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 cells and clusters found in the effluent were the result of detachment.

Sampling of Detached Clusters and Cells Immediately before collecting 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 before cutting the tubing into smaller pieces to provide a reproducible cell number for the disinfection experiments. The pieces were 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 another minute.

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, IKA Labortechnik, Staufen, Germany) at 20,500 rpm for 1 min. The homogenizer was sterilized between samples by flaming with ethanol.

Disinfection Experiments

Sodium hypochlorite (Fisher Scientific, Pittsburgh, PA) was used as a source for free chlorine. A chlorine stock was prepared daily and the concentration was measured with the DPD colorimetric method (LaMotte spectrophotometer DC1100 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 carbon source, or, alternatively in filtered spent chemostat medium. To achieve total inactivation, samples were treated in separate experiments with incrementally increasing doses of chlorine until no culturable cells could be detected on the agar plates. 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 (1-4 cm length) into 10 ml of sterile buffer that resulted in approximately 7 $\log_{10}$ (CFU/ml) after homogenization in the buffer. Therefore, the CFU of the attached biofilm are also

expressed in CFU/ml. Samples were exposed to chlorine for 30 minutes in a shaking incubator at room temperature along with untreated control samples. Neutralization was done with sodium thiosulfate (Fisher Scientific, Pittsburgh, PA).

Bacterial Quantification

Samples were appropriately diluted in sterile1x PBS buffer (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24g of KH_2PO_4 suspended in 1 L purified water, chemicals by Fisher Scientific, Pittsburg, PA) and plated on R2A plates (Difco™ R2A Agar, BectonDickinson, Franklin Lakes, NJ) using the drop plate method (Herigstad et al. 2001). The incubation period was 48 hours at 30°C. The two strains were easily distinguished from each other by the appearance of their 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).

Microscopy and Image Analysis
for Cluster Size Determination

LIVE/DEAD® BacLight™ stain (Invitrogen – Molecular Probes, Carlsbad, CA) was added to samples from chemostats and tube reactor effluents and incubated for 15 min. Then, the samples were filtered onto a black polycarbonate membrane (Poretics®, 0.22 µm, 25 mm). Fluorescent samples were visualized using a fluorescent microscope (Nikon Eclipse E800, software: MetaVue® for Fluorescence (Universal Imaging Corporation, v 7.4), and a 100x oil immersion lens (Nikon). Image analysis was done

with the MetaMorph® (Universal Imaging Corporation, Downingtown, PA) software to determine the number of single cells, number of clusters, and the number of cells per cluster. Thirty random fields of view (6445 μm^2) were recorded using MetaVue® software and analyzed with the MetaMorph® software. 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 (Wilson *et al.* 2004). This also takes into account the amount of extracellular polymeric substances produced by the bacteria. To get a measure of the proportion of each cluster type out of the entire population of cells, 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)

To calculate inactivation rate constants additional disinfection experiments were done where chlorine was added to cultures and sampled after 1, 2, 5, 10, 20, and 30 minutes, neutralized and plated. Inactivation curves were generated in Microsoft Excel by plotting the ln(CFU/ml) that survived the treatment. Due to the bi-phasic behavior of the disinfection curves, k values were calculated for the first minute (for chemostat and tube reactor effluent samples only), the first 2 minutes, and also for disinfection times longer than 2 minutes. The $k_{initial}$ values were calculated from the 2 time points using the following equation:

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

where 'N_0' is the initial CFU/ml, 'N' is the CFU/ml after time 't', and 'C' is the concentration of disinfectant (Chick 1908). 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

The density of organisms were reported as CFU/ml, and then log 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 was applied, and LD_{dose} is the LD of organisms which survived disinfection at some specified dose. The $LD_{untreated}$ for organisms grown as single species was 7, but the RB-8 LDs were below 7 in the dual species cultures (Table 2.2).

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 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 - 5, 6 - 10, 11 - 100, and 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 co-culture), sample type (chemostat and detached), and the 2-way interaction as fixed effects. For the 6 - 10 and 11 - 100 cell clusters, 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 - 5, 6 - 10, 11 - 100, and 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 were performed in R (version 2.11.0).

The extensive analyses described above were divided into seven categories: 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 the cluster analyses. A Benjamini-Hochberg correction (Benjamini & Hochberg 1995) 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 un-adjusted.

Additional Experiments

Biofilm Thickness To measure the thickness of the biofilm, the tubing was cut into smaller sections and then cut lengthwise using a clean, sterile razorblade. The exposed biofilm was subsequently covered with OCT embedding medium (Tissue-Tek, Sakura Finetech U.S.A., Inc., Torrance, CA) and frozen on dry ice. Using sterile forceps, the silicone tubing was carefully removed and the biofilm sectioned into 5 µm slices in a cryostat (Leica CM1850) and viewed under transmitted light and a 20x or 4x objective (Nikon). Thickness was calculated using the MetaMorph® (Universal Imaging Corporation, Downingtown, PA) software. After four days of growth at continuous flow, the RB-8 biofilm was the thickest at 28 µm (±4 µm), followed by the FS-3 biofilm at 26 µm (±4 µm). The dual species biofilm was thinner than the single species biofilms at 23 µm (± 4 µm). There was a significant difference between the dual species biofilm and the RB-8 biofilm (p-value = 0.0018).

Interspecies Competition Testing was done to determine whether *P. aeruginosa* or *B. cepacia* produced substances that were harmful to the other species and therefore made them more susceptible to chlorine disinfection. For that purpose two individual chemostat reactors were run separately with *B. cepacia* (FS-3) and *P. aeruginosa* (RB-8) at the conditions described before. Two centrifuge tubes with 20 ml of the cultures were mixed with 20 ml sterile buffer and 20 ml cell-free spent medium of the other culture

respectively. After incubation at room temperature for 6 hrs, samples were treated with 1 and 2 ppm of chlorine at the conditions described before. Surviving cells were quantified on R2A agar. No decreased tolerance to chlorine for either species could be found in the presence of the other species' supernatant ($p = 0.468$ for FS-3). For RB-8 the LRs were significantly lower after the addition of FS-3 supernatant ($p = 0.034$) (data not shown).

Auto- and Co-Aggregation of Bacterial Strains Auto-aggregation was tested as a control to assess whether the two strains have a tendency to interact with each other or themselves without EPS being present. Experiments were done according to the amended method of Rickard *et al.* (2000). Batch cultures were washed with sterile deionized water three times and brought to an OD_{650nm} of 1.5, then identical volumes were mixed together, vortexed for 10 seconds and gently rolled for 30 seconds. The scoring criteria were as follows: 0, no flocs in suspension; 1, very small uniform flocs in a turbid suspension; 2, easily visible small flocs in a turbid suspension; 3, clearly visible flocs which settle, leaving a clear supernatant; 4, very large flocs of co-aggregates that settle almost instantaneously, leaving a clear supernatant. Control tubes of each isolate on their own were also included to visually assess auto-aggregation. Auto-aggregation was scored by using the same criteria, and the score was deducted from the co-aggregation score. Only turbidity could be observed in the experiments indicating that the strains did not auto-aggregate or co-aggregate with each other (a co- and auto-aggregation score of 0 was always observed).

Results

To determine the chlorine susceptibility of detached cells and clusters compared to chemostat grown cells and biofilms, single species and dual species cultures of *Burkholderia cepacia* FS-3 and *Pseudomonas aeruginosa* RB-8 were treated with incrementally increasing amounts of chlorine until total inactivation was reached (Table 2.1). Additionally, cluster size distribution was recorded for chemostat and detached cluster samples and correlated to the disinfection results.

Table 2.1 Species, growth conditions, and chlorine doses for experiments with 30 minute disinfection. Chlorine doses were incrementally increased until the culture was inactivated completely. FS-3 = *Burkholderia cepacia*; RB-8 = *Pseudomonas aeruginosa*.

Species composition	Reactor type (doses in ppm)		
	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
Co-culture 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

Cluster Size Distribution of Single and Dual Species Scenarios

Microscopic analysis of RB-8 (*P. aeruginosa*) and FS-3 (*B. cepacia*) as single strains and in co-culture 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 - 5 cells (Figure 2.1). Organisms were also present as single 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 - 20% single cells, 50 - 75% smaller clusters of 2 - 10 cells, and

10 - 30% larger clusters of 11 or more cells. However, when grown in co-culture (for the chemostat as well as the tubing reactor effluent), FS-3 plus RB-8 produced 30-35% single cells, 45-55% smaller clusters between 2 - 10 cells, and only 15-20% large clusters of 11 cells or more. The proportion of large clusters (11- 100 cells) was reduced in co-culture while more small clusters (2-5 cells) were present when compared to the single species samples in the chemostat as well as the tubing reactor effluent samples (Figure 2.1). Co-aggregation and auto-aggregation did not appear to be responsible for cluster formation as determined by experiments done with both species.

Statistically significant differences in cluster size were detected between the single species clusters (both FS-3 and RB-8) and the clusters from the co-culture, with more single cells in co-culture, and more clusters containing 6 to 10 cells in single species cultures (Table 2.4, supplemental materials). On average, there were no significant differences in clusters size proportions between the chemostat and tubing reactor effluent for any of the species.

Initial Cell Numbers and
Species Distribution in Co-culture
for Disinfection Studies

All reactors were inoculated with overnight cultures containing 8.02 ± 0.07 $\log_{10}$(CFU/ml) for FS-3 and 8.16 ± 0.12 $\log_{10}$(CFU/ml) for RB-8. After growth in the reactor, 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 co-culture (Table 2.2). For all three sample types (chemostat, tubing reactor effluent, and biofilm), the only significant difference was that the log density for RB-8 in co-culture

was smaller on average than either RB-8 or FS-3 grown as a single species, or FS-3 grown in co-culture (p-value ≤ 0.0015). In co-culture, FS-3 was the most abundant strain in the three different scenarios: chemostat, biofilm effluent, and attached biofilm (Table 2.2). *B. cepacia* (FS-3) had a slightly higher growth rate than RB-8 with 0.198 (± 0.02) hr^{-1} and 0.173 (± 0.02) hr^{-1}, respectively, but the two growth rates were not statistically different (p-value = 0.409) in the defined low nutrient medium. Due to these differences in initial cell log densities, the log reduction was used as the measure of susceptibility to the disinfectant.

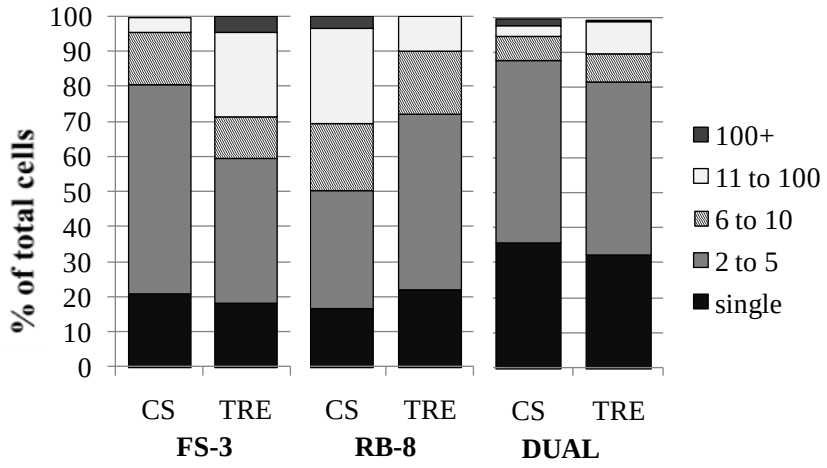

Figure 2.1 Cell cluster size distribution of single and dual species of *B. cepacia* (FS-3) and *P. aeruginosa* (RB-8), from chemostat (CS) and tube reactor effluent (TRE) samples.

Table 2.2 Initial cell concentrations in the chemostat, tube reactor effluent, and attached biofilm in the single species and dual species experiments; log densities and standard deviations are expressed as $\log_{10}$ (CFU/ml), n≥ 3; FS-3 = *B. cepacia*; RB-8 = *P. aeruginosa*. Dual species FS-3 and dual species RB-8 = strains in co-culture.

Sample type	Species	Average log densities (t=0)	Standard deviation	n
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 in $\log_{10}$ (CFU/ml)	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
Biofilm in $\log_{10}$ (CFU/cm^2)	FS-3 only	8.25	0.09	10
	RB-8 only	8.13	0.10	5
	Dual species FS-3	8.14	0.14	6
	Dual species RB-8	6.45	0.36	6

Disinfection Susceptibilities of Planktonically Grown Cells Compared to Detached Biofilm Cells and Attached Biofilm Samples (Sample Type Comparison)

Chlorine treatment at concentrations below 10 ppm of chemostat and tubing reactor effluent samples for 30 minutes inactivated all single species and the dual species samples (Figure 2.2).

FS-3 in a single species chemostat culture was inactivated with 3 ppm of chlorine within 30 minutes of exposure. Eight ppm resulted in total inactivation 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 of the TRE sample in the single species FS-3 (p-value < 0.001) (Figure 2.2).

Four ppm in the chemostat and 3 ppm in the tubing reactor effluent inactivated all **RB-8** samples. Significant differences (at the 10% level) in chlorine susceptibility over 1 – 4 ppm could only be found for RB-8 samples treated with 2 and 3 ppm where CS samples were more tolerant to disinfection than TRE samples (1 ppm: p-value 0.29; 2 ppm: p-value 0.08; 3 ppm: p-value 0.06; 4 ppm: p-value 0.86).

Both species in **co-culture** are 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-value <0.001) with the CS samples being more susceptible to chlorine treatment. The dual RB-8 CS samples were only significantly more susceptible at the 10% level at doses 1 and 2 compared to the TRE samples (1ppm: p-value 0.06; 2 ppm: p-value 0.09).

Although there were no statistically significant differences among the initial mean log densities for each of the species (except for RB-8 in the co-culture, see Table 2.2), the disinfection susceptibility was generally less for cells and clusters that had been grown planktonically than cells and clusters that detached from the biofilm (Figure 2.2). As an exception, RB-8 alone was less tolerant than RB-8 in co-culture when the cells were detached biofilm.

As anticipated, the biofilm required doses of chlorine approximately ten times higher than that used for the tubing reactor effluent and the planktonic cells (Figure 2.3).

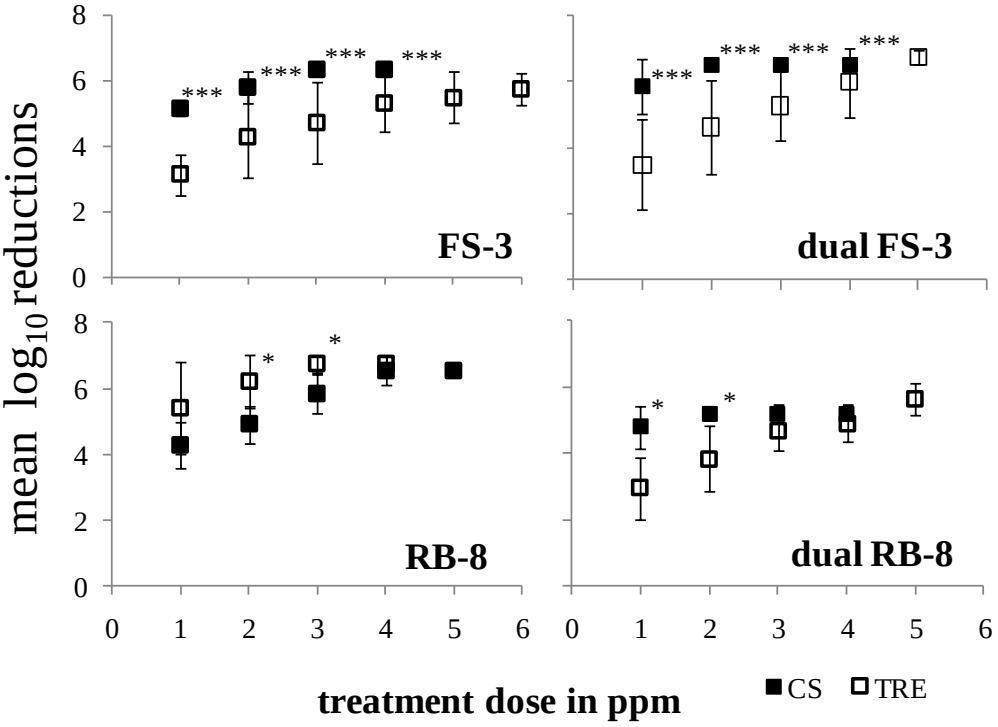

Figure 2.2 Log_{10} reductions after 30 minute chlorine treatment (LR) comparison among reactors per species. Each point represents the mean LR. The bar and whisker indicates one standard deviation of the LR ($n \geq 3$). Significance at 1% is indicated by***, significance at 5% is indicated by **, and significance at 10% is indicated by *. FS-3 = *B. cepacia*; RB-8 = *P. aeruginosa.*

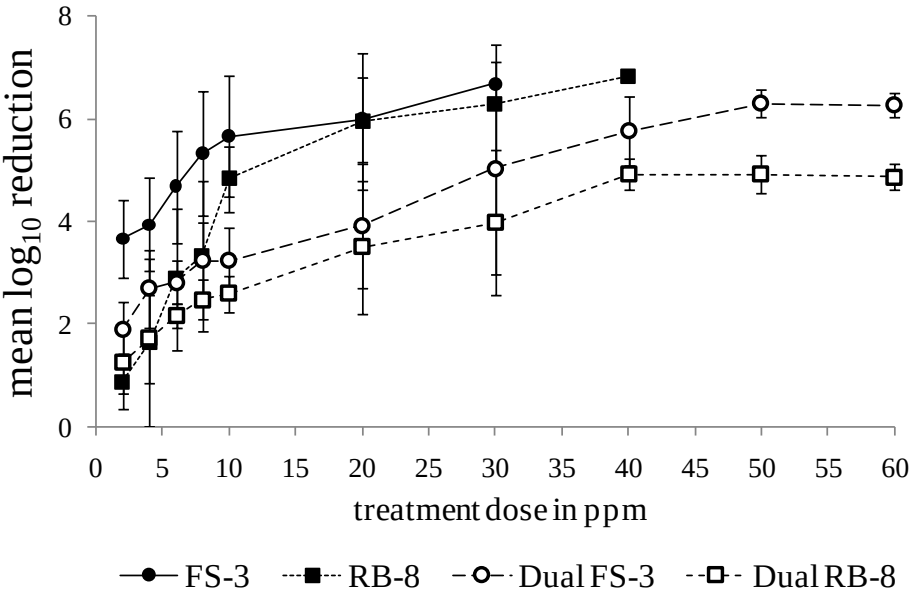

Figure 2.3 Mean $\log_{10}$ reductions for 30 minute biofilm chlorine disinfection. The bar and whisker indicates one standard deviation of the $\log_{10}$ reduction ($n \geq 3$). See Table 2.1 for all chlorine treatment doses. FS-3 = *B. cepacia*; RB-8 = *P. aeruginosa*.

Disinfection Susceptibilities of *B. cepacia* and *P. aeruginosa* as Single Species and in Co-culture (Species Comparison)

RB-8 and FS-3 were grown as single species and in co-culture in the CS and the TRE to test for differences in disinfection susceptibility depending on species composition. Figure 2.4 shows representative disinfectant doses of the experiments for CS, TRE, and BF.

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 co-culture indicating that it is more tolerant than FS-3 in the CS (Table 2.3). On the other

hand, growth in co-culture was of no benefit to FS-3 and RB-8 so that 2 ppm led to total inactivation 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 (See Figure 2.4; Table 2.3 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 co-culture were much lower at 1 and 2 ppm compared to the single species RB-8 (Figure 2.4). These significant differences in species within one 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-values < 0.01). The only significant difference in $\log_{10}$ reductions in the CS was between both dual species (p-values < 0.01). There was a significant interaction between dose, species, and reactor (p-value = 0.04) for dual RB-8 and RB-8 (Table 2.3). 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 inactivation 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 in Figure 2.4) and FS-3 was significantly more susceptible than single RB-8 over all tested doses. FS-3 grown in co-culture 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 co-culture did not reach an initial cell log density of 7 $\log_{10}$(CFU/ml)

the low initial biomass did not result in decreased tolerance. In co-culture, RB-8 displayed mean lower $\log_{10}$ reductions than single RB-8.

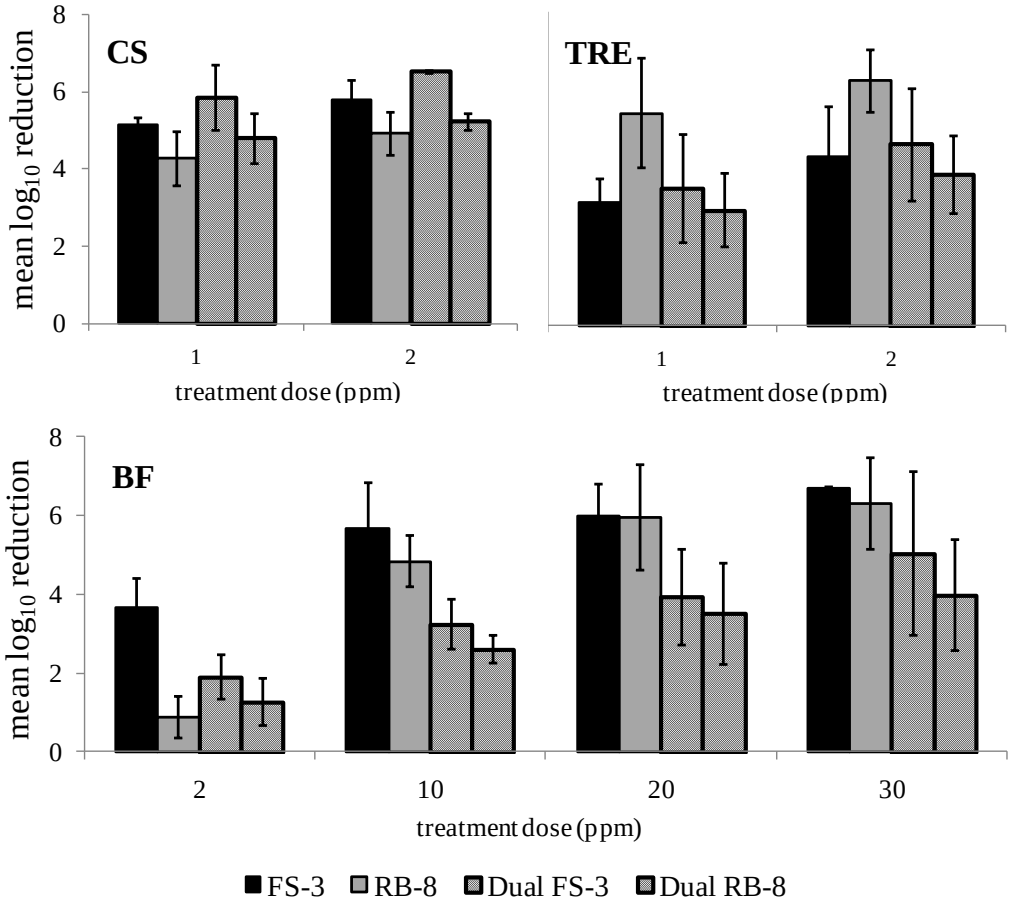

Figure 2.4 Log_{10} reduction (LR) comparisons among species FS-3 (*B. cepacia*); RB-8 (*P. aeruginosa*), Dual FS-3 (dual species *B. cepacia*); Dual RB-8 (dual species *P. aeruginosa*) for each reactor (chemostat, tubing reactor effluent, and biofilm) across multiple representative doses (not all doses shown). The top of each bar represents the mean LR. The bar and whiskers indicate one standard deviation of the LR ($n \geq 3$).

Table 2.3 Mean $\log_{10}$ reduction (LR) differences among species for each reactor (averaged over all tested doses).

	Species pair			
sample type	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***

Significance at 1% is indicated by***, significance at 5% is indicated by **, and significance at 10% is indicated by *. † indicates that although there is no significant LR difference between DL RB-8 and RB-8 when pooled across all doses, the mean LR for RB-8 was significantly larger than the mean LR for dual RB-8 at a dose of 4ppm only.

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. Therefore, an indirect method for assessing the importance of cell clustering and numbers on disinfection was necessary. Homogenization was chosen because it will disrupt cell clusters and potentially release the matrix that binds the cells together.

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-value < 0.01) while single FS-3 CS samples did not lead to a significant difference in cluster proportions. Homogenized RB-8 grown as a single species also exhibited a higher percentage of smaller cluster sizes compared to the unhomogenized

samples, but these differences were not statistically significant at the 10% level. Homogenized TRE samples of FS-3 and RB-8 in co-culture resulted in a higher proportion of cells being contained in clusters with 2 – 5 cells and a lower proportion of cells in cluster of 11-100 cells (Table 2.5, supplemental materials). 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 (Table 2.5, supplemental materials).

Disinfection of Homogenized Samples

Initial $\log_{10}$ densities were not significantly different after homogenization (p-values ≥ 0.313) except for the single species RB-8 which displayed higher $\log_{10}$ densities following the homogenization (p-value <0.001).

Homogenized samples did not show significantly different chlorine susceptibilities in the chemostat compared to unhomogenized samples (p-values > 0.056) (Figure 2.6, supplemental materials). FS-3, as well as dual FS-3 and dual RB-8 were more susceptible to chlorine in TRE samples after homogenization (p-values ≤ 0.001). Biofilm samples were always significantly (p-values ≤ 0.001) more susceptible to disinfection after mechanical removal from the silicone surface followed by shear homogenization (Figure 2.7, supplemental material).

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 minutes. 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 mainly occurs in the first minutes with a significant decrease of log density followed by little or no change in the later time points so that $k_{initial}$ was always bigger than k_{end} (Figure 2.5). Therefore, $k_{initial}$ captured the most important information.

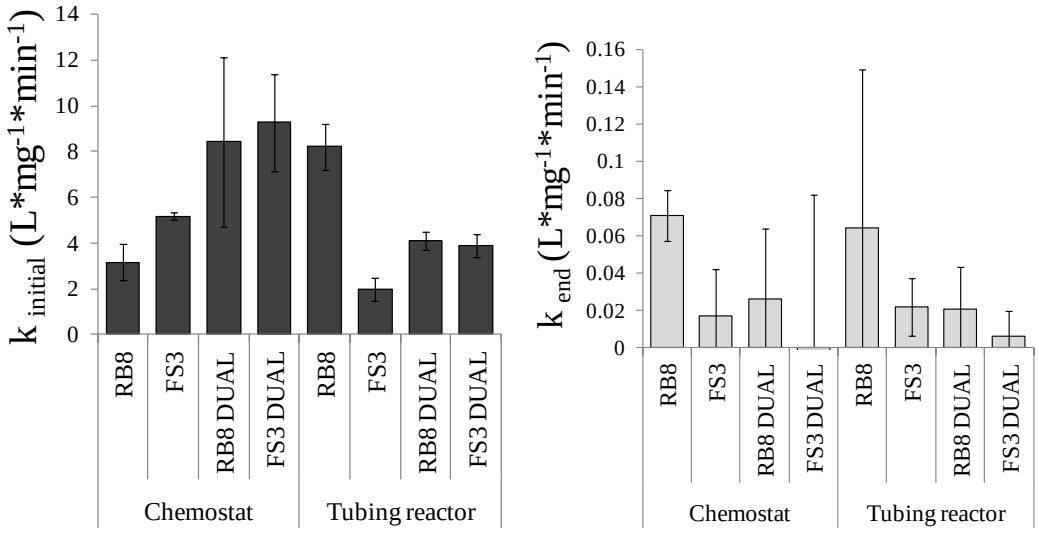

Figure 2.5 $k_{initial}$ values for all species in the chemostat and the tubing reactor effluent and k_{end} values for all species. The bar and whisker indicates one standard deviation. Note the difference in y-axis values.

Sample Type Comparison of k Values: The disinfection rate of FS-3 in the first minute ($k_{initial}$) in chemostat samples was significantly larger than $k_{initial}$ of FS-3 in the tubing reactor effluent (p-value = 0.03) while $k_{initial}$ of RB8 in the chemostat was

significantly smaller than $k_{initial}$ of RB8 in the tubing reactor effluent (p-value <0.001) (Table 2.6, supplemental materials). There was no significant difference between the disinfection rate in the first minute for chemostat and tubing reactor effluent samples for both dual species FS-3 and RB-8. All $k_{initial}$ for the biofilm were below 1.0 $L*mg^{-1}*min^{-1}$ and were different from chemostat and tubing reactor effluent sample k values (Figure 2.8, supplemental materials).

The disinfection rates after 2 minutes to the end of the experiment (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 Table 2.6 and 2.7, supplemental materials for k value differences and significance levels). In chemostat samples, the disinfection rate in the first minute ($k_{initial}$) of FS-3 was larger than the $k_{initial}$ of RB8. The $k_{initial}$ value for FS-3 was smaller than $k_{initial}$ of the dual FS-3 while $k_{initial}$ for RB8 was smaller than 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 smaller than the $k_{initial}$ for RB-8, and $k_{initial}$ for the dual RB-8 was also smaller than the $k_{initial}$ for RB-8 indicating that single species RB-8 detached cells and clusters were more susceptible to chlorine when compared to single species FS-3 and also RB-8 in co-culture.

For the disinfection rate from the second minute to the end of the experiment (k_{end}), significant but small species differences could only be found between FS-3 and FS-3 in co-culture 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 detaching biofilm clusters. Interestingly, chemostat grown cells were not exclusively present as single cells in the chemostat, but rather most of the cells formed clusters in solution. This result has been shown previously (Krometis *et al.* 2009). Previous studies have also found that cell to cell adhesion is independent of the pH of the medium and therefore cell surface charge (Singh, 1987; Clark 1958). Singh & Vincent (1987) found that clumping is enhanced in low nutrient solutions due to enhanced capsule formation, making cells 'stickier'. Rickard *et al*. (2002) also reported that aggregation increases with the age of the cultures which is supported by Clark (1957) 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-sized 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 in Fux *et al.* (2004). In the single species cultures (CS and TRE), a high number of cells (up to 50%) were contained in medium sized and larger clusters (6 cells and more). However, when FS-3 and RB-8 were grown together the majority (up to 90% of all cells) of cells were present as single cells

and in small clusters up to 5 cells (Figure 2.1). The occurrence of low proportions of large clusters may indicate that the two species do not attract each other via adhesins or receptors as described by Rickard *et al.* (2003). After testing the aggregation behavior of the two species using the amended method of Rickard *et al.* (2000), co-aggregation of FS-3 and RB-8 can be excluded. The species also did not auto-aggregate in sterile deionized water. Since auto- and co-aggregation is only tested on thoroughly washed cells where EPS has presumably been removed, cluster formation in the chemostat may be due to EPS production which makes cells "stickier".

Initial Cell Numbers and Species Distribution in Co-culture

B. cepacia (FS-3) was the dominant species in the co-culture when comparing the initial species distribution in all three experimental scenarios (Table 2.2). *B. cepacia* has a slightly higher growth rate than *P. aeruginosa* (RB-8) which might 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. This may indicate that RB-8, despite the slower growth rate, is a persistent species in the biofilm. Banks & Bryers (1991) have reported similar observations with dual species biofilms where the two species have different growth rates. The faster growing species was dominant in the biofilm while the slower growing species remained established through the experiment. Komlos *et al.* (2005) reported that the population density in a dual species biofilm changed with nutrient concentrations and planktonic growth rate was not an indicator of population distribution. As shown in experiments with cell free extracts in this study, RB-8 and FS-3 do not produce substances that

influence the disinfection tolerance of the other strain. 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, perhaps making them more tolerant to chlorine. In contrast to *B.cepacia*, when *P. aeruginosa* was grown as a pure culture it produced more large clusters in the chemostat which may result in increased tolerance to disinfection.

These results indicate that increased resistance may be directly linked to more cells being contained in larger clusters and thus being shielded from disinfection. Also, an increased amount of extracellular polymeric substances surrounding clusters and cells stemming from the biofilm may be protective in a disinfection scenario. More organic substances in the sample react with the available chlorine making less of it available for disinfection as has been reported by deBeer *et al.* (1994) and Xu *et al.* (1995). The reaction of the biofilm matrix with the available chlorine also results in a retardation of diffusion into the biofilm. Xu *et al.* (1995) 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 in the CS, cells are actively growing which is known to make cells more susceptible to disinfection than cells in the stationary phase (Benjamin *et al.* 1986, Mittander & Littbrand 1980). Growth rates in the biofilm

highly depend on the location within the biofilm and the availability of nutrients. Biofilm layering may also influence the physiological states of the cells in the clusters and thus alter disinfection susceptibility (Huang *et al.* 1995).

Slower growth rates and nutrient limitation may be factors in making biofilms more tolerant to disinfection compared to planktonic samples and tubing reactor effluent samples (Walters *et al.* 2003; Rani *et al.* 2007). The attachment to a surface (silicone tubing in this study) may be protective to the biofilm since chlorine can attack the biofilm from only one side and chlorine is reacting with the EPS matrix before it can reach cells in lower layers of the biofilm.

Disinfection of *B. cepacia* and *P. aeruginosa* as a Single Species and in Co-culture

The disinfection susceptibility of the two strains RB-8 and FS-3 depends on whether each is grown as a single species culture or in co-culture. Single species in the CS compared to the co-culture in the CS were more likely to survive disinfection (Figure 2.4).

In contrast, the two species in detached biofilm clusters responded more similarly to disinfection when grown as a co-culture which leads to better survival of RB-8 and more efficient killing of FS-3 compared to the single species. Interestingly, the dual species biofilm reactor effluent had a high percentage of single cells and smaller clusters (> 80%) in comparison to the single species (Figure 2.1), but the tolerance to chlorine has increased (Figure 2.4). This outcome suggests that the disinfection efficacy largely depends 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 co-culture. Inter-species competition experiments showed that both strains do not produce substances that make the other species more susceptible to disinfection.

Co-culture 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, 30, and higher if applicable). This may indicate the presence of tolerant specialized survivor cells (persister cells) in the biofilm community that are known to occur in bacterial populations (Keren *et al.* 2004) and their development may have been triggered by the dual species growth. The co-cultured biofilm was also the thinnest at 23 µm (± 4 µm) but also the most resistant to disinfection, supporting the possibility for protective interactions.

Image analysis of sectioned biofilms showed that RB-8 biofilms were the thickest on average with 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 the biofilm is exposed to during growth, and the availability of nutrients (Stoodley *et al.* 1994, Simões *et al.* 2005, Costerton *et al.* 1995). Some strains and phenotypes of *P. aeruginosa* are well known for the production of increased amounts of extracellular substances (Govan & Deretic 1996). The environmental *P. aeruginosa* strain used in this study also produced large, glossy colonies with undefined borders that were visible after 24 hours while *B. cepacia* formed well defined small colonies that were only visible after

48 hours incubation. These observations may indicate 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. This might be due to 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. This may indicate that the majority of cells in the samples were easily disinfected within the first minute and a smaller subpopulation was more tolerant to chlorine.

The $k_{initial}$ values were an excellent indicator of chlorine tolerance of samples. The $k_{initial}$ values for RB-8 and FS-3 in co-culture were very similar which supports findings of the disinfection experiments with incrementally increasing chlorine doses. Figure 2.5 shows that species and sample types with high initial k values also resulted in high $\log_{10}$ reductions after 30 minutes 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 numbers of large clusters is high compared to samples with a lower number of large clusters.

When RB-8 and FS-3 were grown in co-culture, the relative number of larger clusters was reduced compared to the single species samples, but the tolerance of the TRE and biofilm samples was 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 multi-species cultures. The k values for the first minute of exposure to chlorine were a good indicator value for 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 mono-species cultures

may not sufficiently describe the disinfection tolerance observed in multi-species scenarios.

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Supplemental Materials

Table 2.4 Species comparison of cluster sizes pooled across the two sample types (chemostat and tubing reactor effluent samples). Mean differences in the proportions of clusters are shown.

species	single cell	2 to 5	6 to 10	11 - 100	101 - 1000
FS-3 - RB-8	-0.00	0.08 †	-0.05	-0.05	0.01
FS-3 - DUAL	-0.15 *	0.00	0.06 *	0.08	0.01
RB-8 - DUAL	-0.15 *	-0.08	0.11 **	0.12	0.01

Significance at 5% is shown with **, 10% significance is shown with *. † indicates that there was no species difference in cluster proportions when pooled across chemostat and tubing reactor effluent for FS-3 vs RB-8 but there was a significant difference in the cluster proportion in chemostat samples at a significance level of 10%.

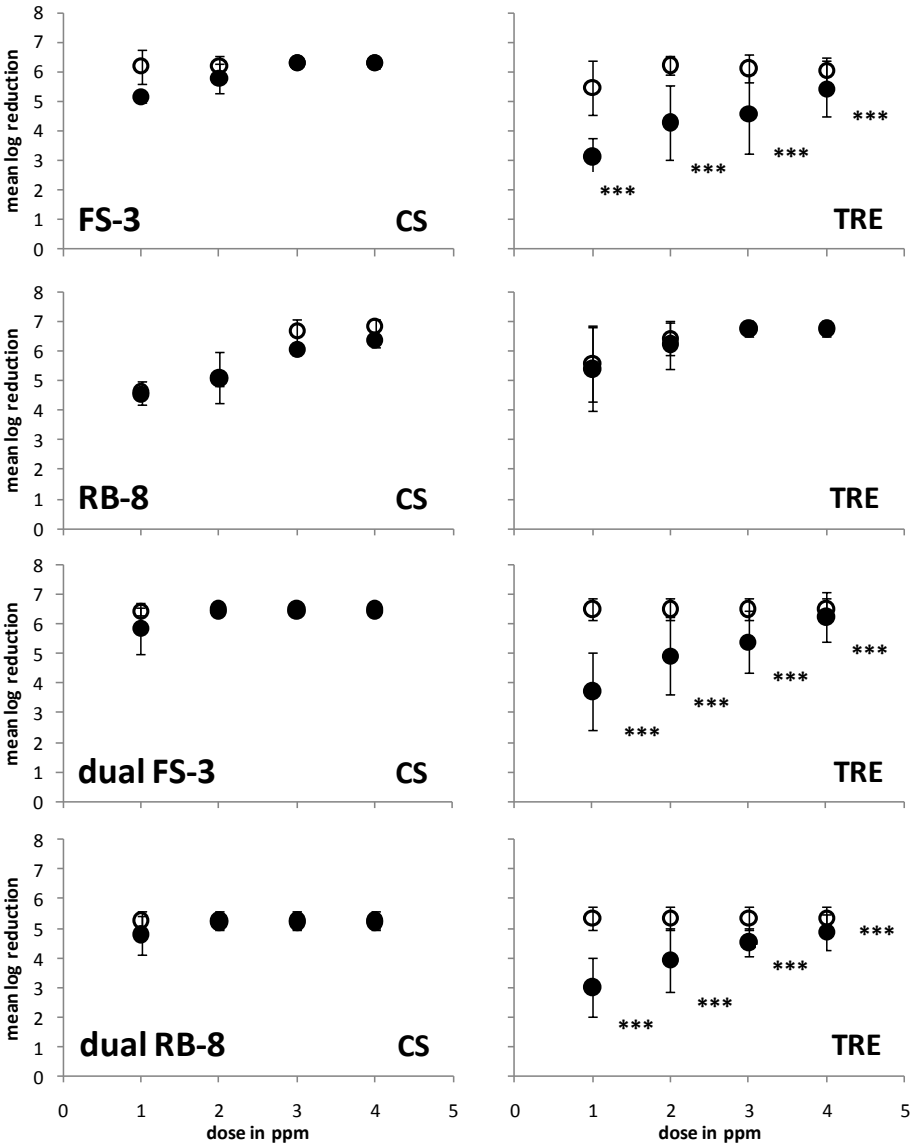

Figure 2.6 Log_{10} reductions of homogenized and not homogenized *B. cepacia* and *P. aeruginosa* as single species and in co-culture in the chemostat and the tubing reactor effluent. Open circles are homogenized samples and filled circles are not homogenized samples. Significance at 1% is indicated by***, significance at 5% is indicated by **, and significance at 10% is indicated by *.

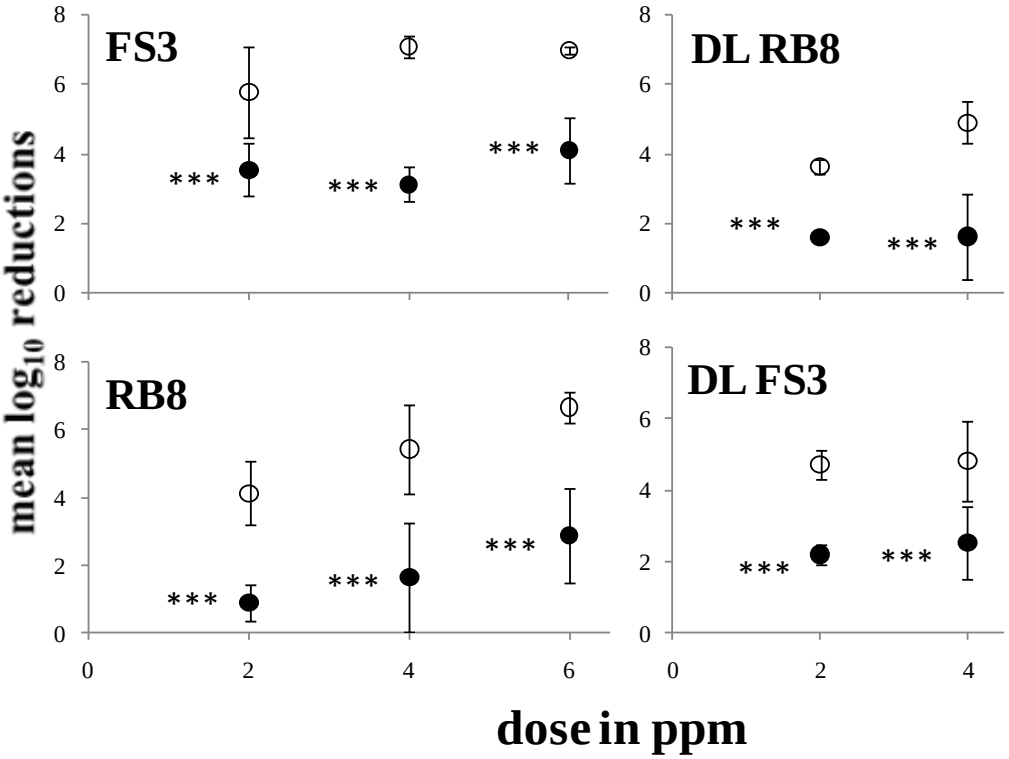

Figure 2.7 Log_{10} reductions of homogenized and not homogenized *B. cepacia* and *P. aeruginosa* as single species and in co-culture in the biofilm. Open circles are homogenized samples and filled circles are not homogenized samples. Significance at 1% is indicated by***, significance at 5% is indicated by **, and significance at 10% is indicated by *.

Table 2.5 Cell cluster size comparison of homogenized and non-homogenized samples, by species, for the two sample types (chemostat and tubing reactor effluent). Mean differences (non-homogenized minus homogenized) in the proportions of clusters are shown.

		Cluster type				
Species	Sample type	1 cell	2 to 5	6 to 10	11 - 100	100+
FS-3 - FS-3 Hom	CS	-0.01	0.01	0.01	0.00	-0.01
	TRE	-0.04 **	-0.09 **	-0.02 **	0.12 **	0.04 **
RB-8 - RB-8 Hom	CS	-0.02	-0.06	-0.01	0.06	0.03
	TRE	-0.02	-0.03	0.01	0.04	0.00
DUAL - DUAL Hom	CS	0.00	0.01	0.00	-0.01	0.00
	TRE	-0.01	-0.02 *	-0.02	0.05 **	0.00

Significance at 5% is shown with **, 10% significance is shown with *.

Table 2.6 k value sample type differences in the different species. k values in $L*mg^{-1}*min^{-1}$.

$k_{initial}$	Difference CS vs. TRE	k_{end}	Difference CS vs. TRE
FS3	3.20**	FS3	-
RB8	-5.04**	RB8	-
DL FS3	-	DL RB8	-
DL RB8	-	DL FS3	-

Significance at 5% is shown with **, 10% significance is shown with *.

Table 2.7 k value species differences for each sample type. k values in $L*mg^{-1}*min^{-1}$.

	Species			
	FS3 - RB8	FS3 - DL FS3	RB8 - DL RB8	DLFS3 - DLRB8
sample type				
CS $k_{initial}$	2.00*	-4.10**	-5.26**	-
TRE $k_{initial}$	-6.24**	-	4.11*	-
CS k_{end}	-	0.02**	-	-
TRE k_{end}	-	-	-	-

Significance at 5% is shown with **, 10% significance is shown with *.

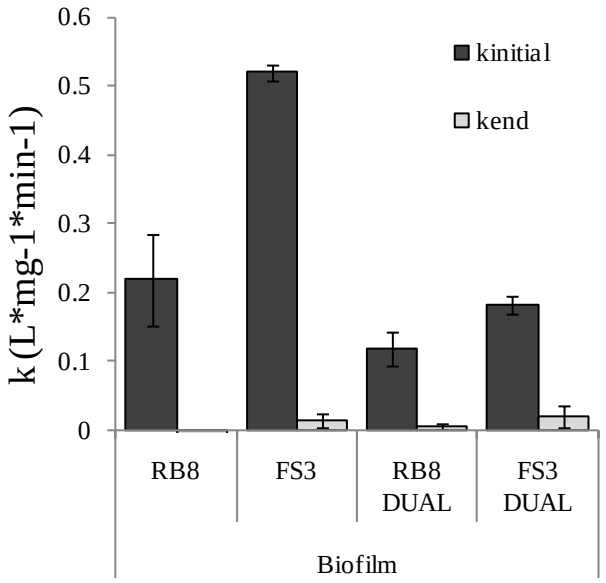

Figure 2.8 k values ($k_{initial}$ and k_{end}) of *B. cepacia* and *P. aeruginosa* as single species and in co-culture in the biofilm samples.

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CHAPTER 3

DIFFERENTIAL CHLORINE DIOXIDE DISINFECTION TOLERANCE OF DETACHED CLUSTERS, PLANKTONIC CELLS, AND BIOFILMS IN MONOCULTURES AND BINARY CULTURES

Contribution of Authors and Co-authors

Manuscript in Chapter 3

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Abstract

Standard disinfection techniques are commonly based on efficacy tests against planktonic cells or more recently, biofilms. While is has been noted that disinfectants are orders of magnitude less effective against cells in biofilms compared to planktonic cells, questions regarding the disinfection tolerance of detached biofilm clusters remain largely unanswered.

Burkholderia cepacia and *Pseudomonas aeruginosa* as model organisms were grown in chemostats and biofilm tubing reactors, with the tubing reactor also serving as a source of detached biofilm clusters. Since recent studies have reported differential efficacy of sanitizers against single species and binary biofilms, chlorine dioxide susceptibility was assessed for *B. cepacia* and *P. aeruginosa* in these 3 sample types not only as monocultures, but additionally as binary cultures.

Chlorine dioxide inactivated samples of chemostat and tubing reactor effluent with similar doses and no statistically significant difference between the $\log_{10}$ reductions of the two samples types was found in this study. As expected, biofilms were more tolerant and required chlorine doses ten times as high as chemostat and tubing reactor effluent samples.

The presence of a second species was advantageous in all sample types and resulted in lower $\log_{10}$ reductions when compared to the single species cultures suggesting an interaction of the species.

Introduction

When single bacteria become attached to surfaces and are exposed to suitable aqueous living conditions, they proliferate and form microcolonies that eventually develop into biofilms. These bacterial communities produce extracellular polymeric substances as a protective layer and are highly heterogeneous in their composition with regard to gene expression and nutrient requirements (Donlan & 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, Steed & Falkinham 2006; Donlan & Costerton 2002; Davies 2003). In addition, it is 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 which make them more tolerant (Walters *et al.* 2003; Rani *et al.* 2007).

In mixed species biofilm bacteria inhabit suitable niches in a particular microenvironment and may undergo symbiotic relationships between different bacterial species (Møller *et al.* 1996, Møller *et al.*1998). It has been suggested that cell to cell signaling in multispecies biofilms increases disinfection tolerance by boosting transcription of genes responsible for survival of oxidative stress (Loewen & Hengge-Aronis 1994), altering EPS composition and viscosity (Burmølle *et al.* 2006), or influencing biofilm architecture and 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 cell 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 (Simões *et al.* 2008; Simões *et al.* 2005, Davison *et al.* 2010). A wide range of cluster sizes detaches from biofilm from single cells to large clusters containing more than 1000 cells while the proportions are dependent on species composition and growth conditions of the biofilm (Wilson *et al.* 2004, Behnke *et al.*, submitted).

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 planktonic growth protein expression profiles 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 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 &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 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 (Ingols & Ridenour 1948, Ridenour & Ingols 1947). 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 & 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 thickness which suggests more effective killing for thinner biofilms.

The goal of this study is to determine the differential efficacy of chlorine dioxide against bacterial monocultures and co-cultures of *P. aeruginosa* RB-8 and *B. cepacia* FS-3. Cultures grown as planktonic cells, biofilms, and detached biofilm clusters harvested from the biofilm effluent are tested and log reductions are 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 us to believe that chlorine dioxide will effectively disinfect biofilm samples and rapidly inactivate planktonic cells and detached biofilm clusters at low concentrations.

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 that causes 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).

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, 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 Media

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 glucose, 0.018 g/L NH_4Cl, 3.93g/L phosphate buffer (2.71 g/L Na_2HPO_4; 1.22 g/L KH_2PO_4), and 2 ml/L 0.1 M $MgSO_4$ (Fisher Scientific, Pittsburgh, PA) at room temperature (22 $\pm$ 2°C). Maximum growth

rates of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 in the defined medium were 0.198 (± 0.02) hr^{-1} and 0.173 (± 0.02) hr^{-1}, respectively.

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.*, submitted). In the tests, spent medium (supernatant) of the FS-3 culture did not increase the susceptibility of a RB-8 chemostat culture to disinfection or vice versa. In addition, auto-aggregation and co-aggregation tests determined whether FS-3 and RB-8 have a tendency to interact with each other or themselves with help of cell-surface adhesions, but no auto- or co-aggregation was detected (Behnke *et al.*, submitted).

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 hours was run using a nutrient flow of 0.5 ml/min. The chemostat was filled with the defined medium mentioned above and then inoculated with 3 ml of the 18h overnight cultures (1.5 ml of each strain for dual culture inoculation). Batch mode (no flow) for 2 doubling times (12 hours) was followed by continuous flow for another 6 doubling times (36 hours) 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 hours. 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 and a residence time of 6.8 minutes which was much lower than the doubling time of the two strains and thus avoided the possibility for 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 hours, followed by 4 days of growth under flow conditions at room temperature (22 ± 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. 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 10ml of chilled buffer for 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 tweezers, 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 the RB-8, FS-3, and dual species biofilms were previously determined by measuring at least 15 randomly chosen cross-sections of the cryosectioned biofilm using a microscope and image analysis software (Behnke et al., submitted). The FS-3 biofilm measured 26 µm (±4 µm) while The RB-8 and dual species biofilm were 28 µm (±4 µm) and 23 µm (± 4 µm) thick, respectively. There was a significant difference between the dual species biofilm and the RB-8 biofilm (p-value = 0.002, according to an ANOVA with species as a factor in Minitab®).

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.

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 via direct measurement at 360 nm (molar absorption coefficient = 1250 L mol^{-1} cm^{-1}) using a Genesys spectrophotometer (Gauw *et al.* 1999). 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 0^{th} dilution.

Initial cell $\log_{10}$ densities of the planktonic cells and detached particles were standardized to 7.0 $\log_{10}$ (CFU/ml) by dilution with sterile buffer before exposure to chlorine dioxide. Attached biofilm was standardized by choosing the suitable length of tubing (1 to 1.5 cm) that resulted in approximately 7.0 $\log_{10}$ (CFU/ml) after homogenization in 10 ml of buffer. The $\log_{10}$ densities of attached biofilm are therefore also expressed in CFU/ml.

After addition of chlorine dioxide, samples were exposed for 30 minutes in a shaking incubator at room temperature along with untreated control samples. Neutralization of samples was done with sodium thiosulfate (Fisher Scientific, Pittsburgh, PA).

Bacterial Counts

Serial dilutions in sterile1x PBS buffer (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24g of KH_2PO_4 suspended in 1 L purified water, chemicals by Fisher Scientific, Pittsburg, PA) were carried out previous 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 hours at 30°C. Colony appearance allowed for visual discrimination of the two strains on R2A plates. 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 minutes, neutralized and plated to calculate inactivation rate constants. Inactivation curves were generated in Microsoft Excel by plotting the ln(CFU/ml) that survived the treatment versus 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 minutes. 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 CFU/ml and 'N' is the CFU/ml after time 't', and 'C' is the 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 compaired 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 calculation 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 & Hochberg 1995) 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 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, and detached biofilm clusters harvested from the biofilm effluent 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

Initial log densities (LD) of the two species as monocultures were compared to the two species grown together as a co-culture (Table 3.1). 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 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 hours (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 present in tubing reactor effluent and 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 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 continues to detach from the biofilm after 4 days.

Table 3.1 Starting mean $\log_{10}$ densities of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as a binary culture (co-culture of FS-3 and RB-8). One standard deviation is shown. CS = Chemostat, TRE = Tubing reactor effluent, BF = Biofilm, DL = dual (species in co-culture). StDev = one standard deviation. n = number of replicates.

Sample type	Species	Mean	StDev	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

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 3.1).

Chemostat samples of the FS-3 monoculture were more susceptible to chlorine dioxide disinfection compared to tubing reactor effluent and biofilm. The tubing reactor effluent was more tolerant, but there was no significant difference between the mean

$\log_{10}$ reductions of chemostat and tubing reactor effluent over all doses (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 displays 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 susceptible to the disinfectant at these low doses although complete killing was also achieved with 10 ppm (Figure 3.6, supplemental materials).

When comparing both species within one sample type there were no significant differences in the chemostat or tubing reactor effluent sample $\log_{10}$ reductions (p-values > 0.05). 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).

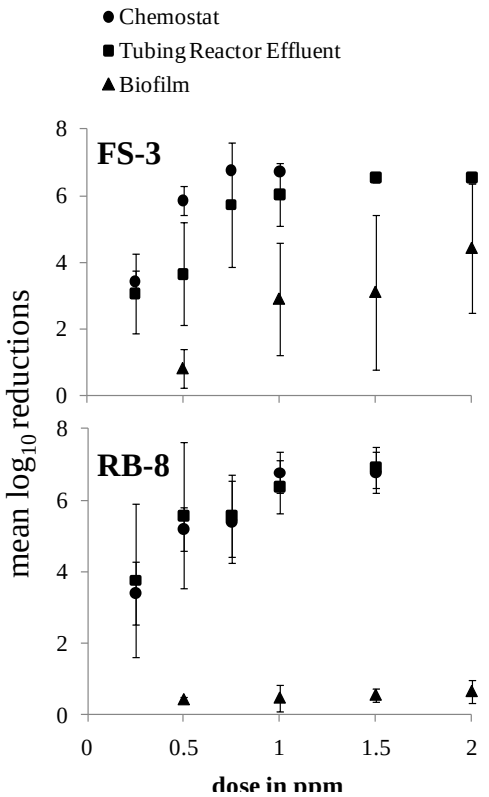

Figure 3.1 Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 monocultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one standard deviation ($n \geq 3$).

Disinfection of Binary Cultures

When FS-3 and RB-8 were co-inoculated into the chemostat or tubing reactor and grown together, CFUs can 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 $\log_{10}$ reductions 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 3.2, also see Table 3.1 for comparison). Tubing reactor effluent samples were more susceptible to chlorine dioxide than the chemostat samples when both species were grown together. However, the $\log_{10}$ reduction differences between the chemostat and detached clusters were not significant (p-value = 0.07). The biofilm $\log_{10}$ reductions were significantly lower that the chemostat and detached cluster $\log_{10}$ reductions for both species (p-values < 0.001).

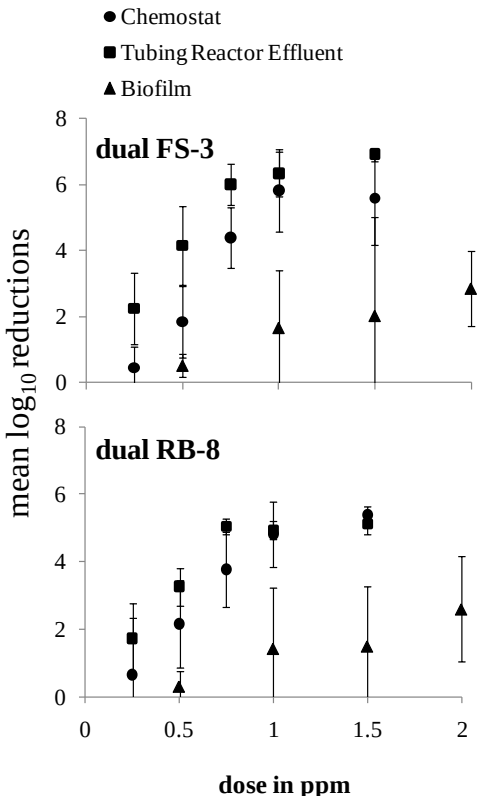

Figure 3.2 Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as binary cultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one standard deviation ($n \geq 3$).

Comparison Between Monocultures and Binary Cultures

Generally, FS-3 and RB-8 have lower $\log_{10}$ reductions when grown in co-culture samples meaning that single species cultures were more easily disinfected (Figure 3.3). 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 displays significantly lower $\log_{10}$ reductions in 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).

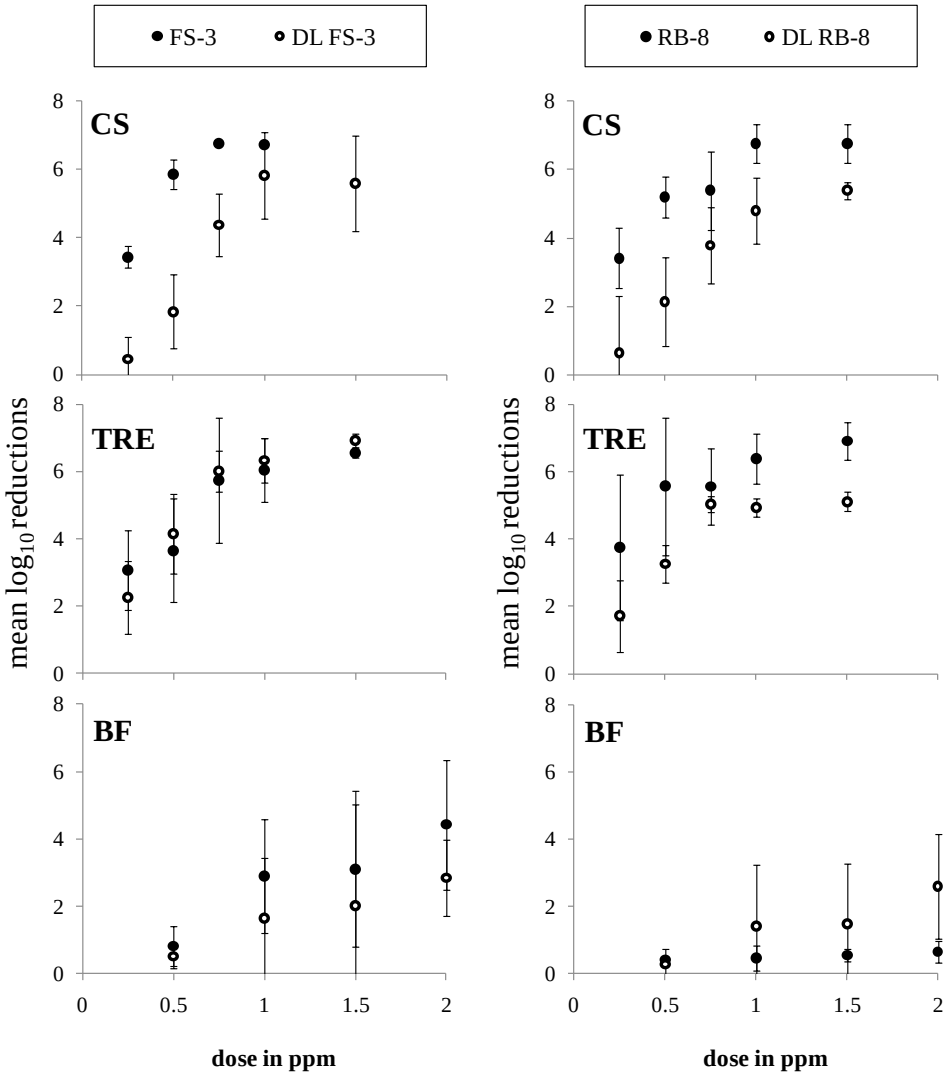

Figure 3.3 Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and binary cultures in comparison. Bars represent one standard deviation ($n \geq 3$).

Disinfection of Homogenized Aliquots

Aliquots of chemostat, tubing reactor effluent, and biofilm samples were shear homogenized for 1 minute prior to treatment with chlorine dioxide. Untreated controls were homogenized to compare initial $\log_{10}$ densities to not homogenized and untreated control samples. No significant differences were found between the homogenized and not homogenized initial $\log_{10}$ densities (p-values > 0.284) (Figure 3.8, supplemental materials).

Homogenization of chemostat samples slightly increased susceptibility in all species types (FS-3, RB-8 and co-culture) with significant susceptibility increases for RB-8 and dual RB-8 (p-values < 0.016). None of the tubing reactor effluent samples increased significantly in susceptibility across all doses after homogenization while FS-3 and dual FS-3 in tubing reactor effluent were significantly more 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 < 0.011) (Figure 3.8, supplemental materials).

Disinfection Rates (k Values)

Using broken line regression, the concentration over time (Ct) graphs (Figures 6.1 – 6.6, appendix) 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 minutes. The remaining disinfection reaction (after minute 1) is described by the second k value (k_{end}).

Most of the killing of cells occurs within the first 2 minutes after addition of chlorine dioxide. Disinfection rates ($k_{initial}$) were very similar in the chemostat and tubing reactor effluent samples (p-values > 0.934) indicating that similar $\log_{10}$ reductions occur within the same time period for all 4 species scenarios and both sample types (Figure 3.4). The dual species FS-3 and RB-8 result in 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). Biofilm $k_{initial}$ were significantly lower than $k_{initial}$ for all chemostat and tubing reactor effluent samples regardless of species (p-values < 0.0257) indicating that the $\log_{10}$ reductions within the first minute of disinfection were much lower for all tested biofilms.

k_{end} values indicate the disinfection rate from minute 2 to minute 30. Since most of the killing occurs within the first two minutes, 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 range from 10 $L*mg^{-1}*min^{-1}$ to 18 $L*mg^{-1}*min^{-1}$ (Figure 3.5). 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. 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.

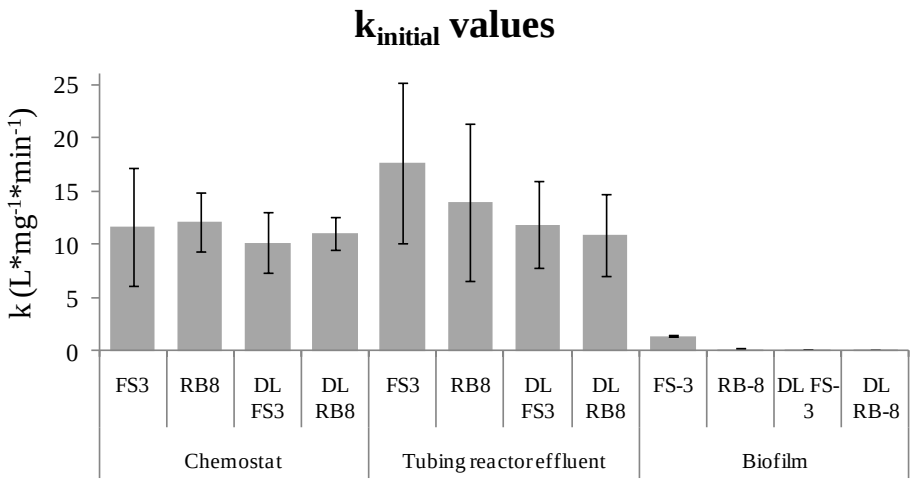

Figure 3.4 k values for the first minute ($k_{initial}$) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as a binary culture). Error bars represent one standard deviation ($n \geq 3$).

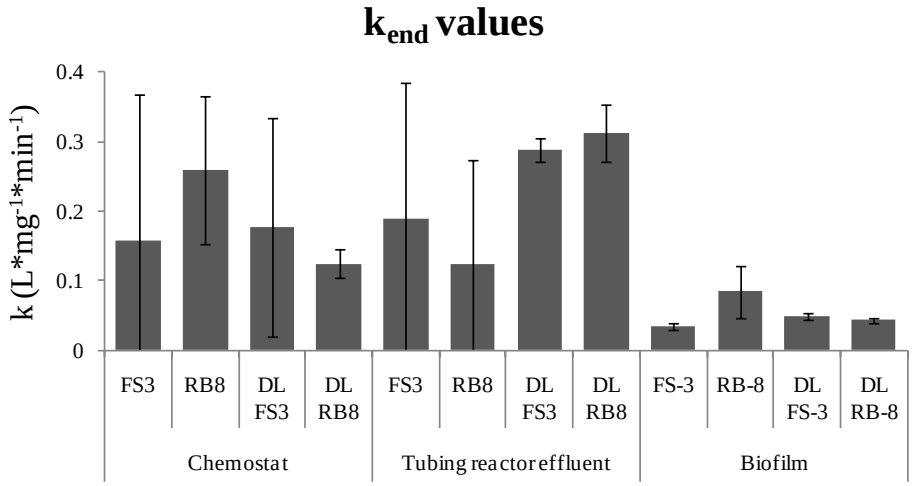

Figure 3.5 k values for minute 2 to minute 30 (k_{end}) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (FS-3 and RB-8 as monocultures and as a binary culture). Error bars represent one standard deviation ($n \geq 3$).

Disinfection of Homogenized
Control Samples (k Values)

Shear homogenization of aliquots of samples resulted significantly higher $k_{initial}$ in the biofilm samples for all 4 species types (p-values < 0.018). This indicated that homogenization makes biofilm cells more susceptible to disinfection (Figure3.9 and 3.10, supplemental materials).

Surprisingly, for homogenized chemostat and tubing reactor effluent samples, $k_{initial}$ did not increase, but instead decrease 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 = 0.015) with $k_{initial}$ being lower for homogenized samples.

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. k_{end} values increased for all chemostat samples after homogenization (p-values < 0.029) meaning that more inactivation occurred after 2 minutes than in the not homogenized samples. In the co-culture, dual FS-3 and dual RB-8 k_{end} increased significantly in the tubing reactor effluent samples after homogenization as well (p-values < 0.021). None of the biofilm samples or single species FS-3 and RB-8 tubing reactor effluent k_{end} increased significantly after homogenization (p-values > 0.107).

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 3.1). *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 continues to detach from the biofilm after 4 days. Banks and Bryers (1991) have observed that the faster growing species had a competitive advantage in the biofilm while the slower growing species remained established throughout the experiment. Population densities and species distribution are also influenced by nutrient concentrations and are not solely dependent on planktonic growth rates of the individual species (Komlos *et al.* 2005). Experiments have previously shown that RB-8 and FS-3 cell free chemostat extracts did not alter the disinfection susceptibility of the other strain by producing harmful substances that are released into the nutrient medium (Behnke *et al.*, submitted). However, the possibility for direct physical interactions or cell to cell signaling cannot be excluded especially in cells from attached or detached biofilms. Experiments with cell-free extracts were only run for chemostat cells and may not reflect processes occurring in the biofilm.

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 while the biofilm samples were significantly different from both other sample types which applied to FS-3 as well as RB-8. These results indicate that detached clusters are more similar to chemostat cells than biofilm cells which 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 one hour of sample collection so that the proposed transitional phenotype should still be expressed. One explanation could be that the phenotypical switch from biofilm to planktonic state may occur much faster than initially proposed.

Interestingly, RB-8 biofilms were very tolerant to chlorine dioxide ($\log_{10}$ reductions less than 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. This finding suggests that RB-8 biofilms may contain more extracellular polymeric substances that

react with chlorine dioxide and protect the cells. Jang *et al.* (2006) suggested that extracellular polymeric substances may reactively deplete chlorine dioxide and thus protect cells in deeper layers of the biofilm when low doses are applied. 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).

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 starting LDs for RB-8 were lower. Lindsay *et al.* (2002) have previously reported this behavior for binary cultures before and concluded that co-cultured bacteria influenced each other's disinfection susceptibilities and also their attachment characteristics. In this study the beneficial relationship between the two species was seen in biofilm samples as well as to chemostat cells and tubing reactor effluent samples.

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 (no significant difference). A multitude of mechanisms and conditions can affect the disinfection tolerance of bacteria so that further testing is required to identify factors involved here. An important aspect may be the growth phase of the cells in chemostat or detached

clusters that was shown to influence disinfection tolerance (Gilbert *et al.* 1987). Cells with higher metabolic activity due to the presence of sufficient nutrients and oxygen are commonly more susceptible to disinfectant challenges.

Comparison of Monocultures and Binary Cultures

When directly comparing the $\log_{10}$ reductions of the 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-3in co-culture were lower (or almost identical in the tubing reactor effluent samples) 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 more tolerant (or almost identical in tolerance in biofilm samples) 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 (Lindsay *et al.* 2002; Elvers *et al.* 2002; Leriche & Carpentier 1995). Cowan *et al.* (2000) additionally reported commensal relationships in binary biofilms that resulted in adaptive strategies and increased survival for the species 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 (Riedel *et al.* 2001, McKenney *et al.* 1995) 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 which presumably breaks up cell clusters and removes EPS material from cells. We have shown 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*, submitted).

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 (Figure 3.6 and 3.7, supplemental materials). 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 & Raquepas 1995; Chen & Stewart 1996; Stewart 2003). Homogenized biofilm cells may additionally be protected by displaying a

biofilm phenotype characterized by lower metabolic activity and expression of biofilm-specific genes (Sauer *et al.* 2004).

However, chemostat and tubing reactor effluent disinfection tolerance did not change as significantly as the biofilm tolerance after homogenization (p-values < 0.011). Differences between LRs of homogenized and unhomogenized chemostat samples were limited to RB-8 (p-value = 0.016) 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 = 0.09, 0.0001, 0.0001, 0.001).

Chlorine dioxide disinfection experiments with unhomogenized samples demonstrated that tubing reactor effluent cells are 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 tubing reactor effluent samples did not decrease significantly(across all doses) in tolerance after homogenization as was seen in the biofilm. If metabolic activity as a result of nutrient and oxygen availability is a key factor to bacterial susceptibility, cells contained in small clusters may be comparable to planktonic cells because nutrients and oxygen diffuse into these small clusters more easily and trigger active metabolism (Walters *et al.* 2003).

Disinfection Rates

Chlorine dioxide added to cell culture samples of the chemostat and the tubing reactor effluent decayed slowly over the course of 30 minutes and no residual remained (data not shown).

Most of the $\log_{10}$ reductions occurred within the first two minutes of the experiment while no significant reductions occurred after that. 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 > 0.934). This indicates that cells in these samples were inactivated at similar rates within the first minute of exposure to chlorine dioxide. Cells from these two sample types may be very similar with respect to metabolic activity as well as nutrient and oxygen availability (Walters *et al.* 2003).

Regardless of species, $k_{initial}$ of biofilm samples were significantly different from the chemostat and tubing reactor $k_{initial}$ (p-values < 0.026) which was also supported by the results of the disinfection experiments (Figures 3.1 and 3.2). 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 & Raquepas, 1995). Biofilm cells maybe also inactivated at a slower rate due low metabolic activity or dormancy which has been shown to be protective (Rani *et al.* 2007; Walters *et al.* 2003).

Disinfection Rates of Homogenized Samples

Disinfection rates of homogenized aliquots of samples reflect findings from the disinfection experiments with homogenized samples. The $k_{initial}$ significantly increase 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 result suggests that homogenization removed EPS constituents from cells and clusters and was brought into solution where it reacted with chlorine dioxide in the first minutes and thus slowed disinfection of the cells (Chen & Stewart 1996). After EPS material has been oxidized during this disinfection lag phase, the disinfection rate increased and most killing occurred after 2 minutes.

Conclusions

In this study, detached biofilm clusters have similar $\log_{10}$ reductions and disinfection rates to chemostat cells and are very different from biofilm cells. This finding suggests that detached clusters may switch to a planktonic life style shortly after the detachment event due to increased availability of nutrients and oxygen. Homogenized biofilms partially lose their increased tolerance to chlorine dioxide disinfection, but they remain more tolerant than chemostat and tubing reactor which may indicate that the biofilm-specific phenotype was still expressed (Sauer *et al.* 2004).

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). Multiple studies have found similar results with binary cultures (Lindsay *et al.* 2002; Elvers et al. 2002; Leriche &

Carpentier 1995), but mechanisms for this increased tolerance have not been sufficiently studied. Researchers have suggested a change in EPS matrix constituents due to the presence of a second species that can lead to viscosity and permeability changes (Burmølle *et al.* 2006, Skillman *et al.* 1999). 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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Supplemental Materials

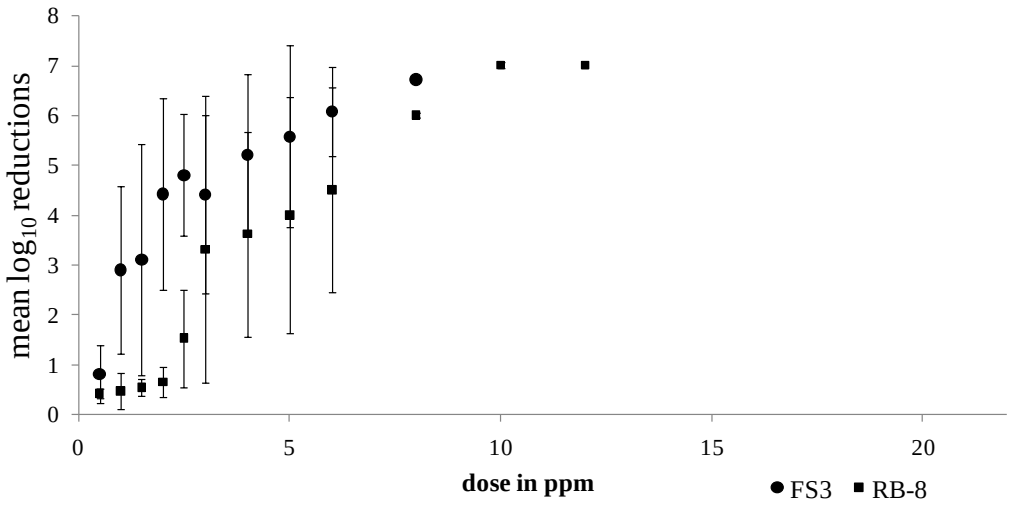

Figure 3.6 Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures in the biofilm. The bars represent one standard deviation (n ≥3).

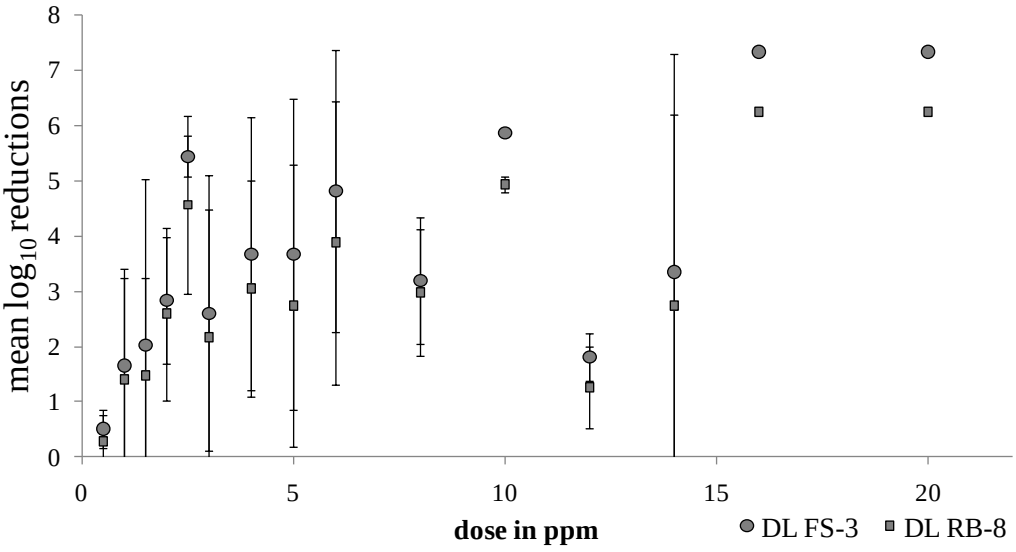

Figure 3.7 Mean $\log_{10}$ reductions of *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as a dual biofilm culture. The bars represent one standard deviation (n ≥3).

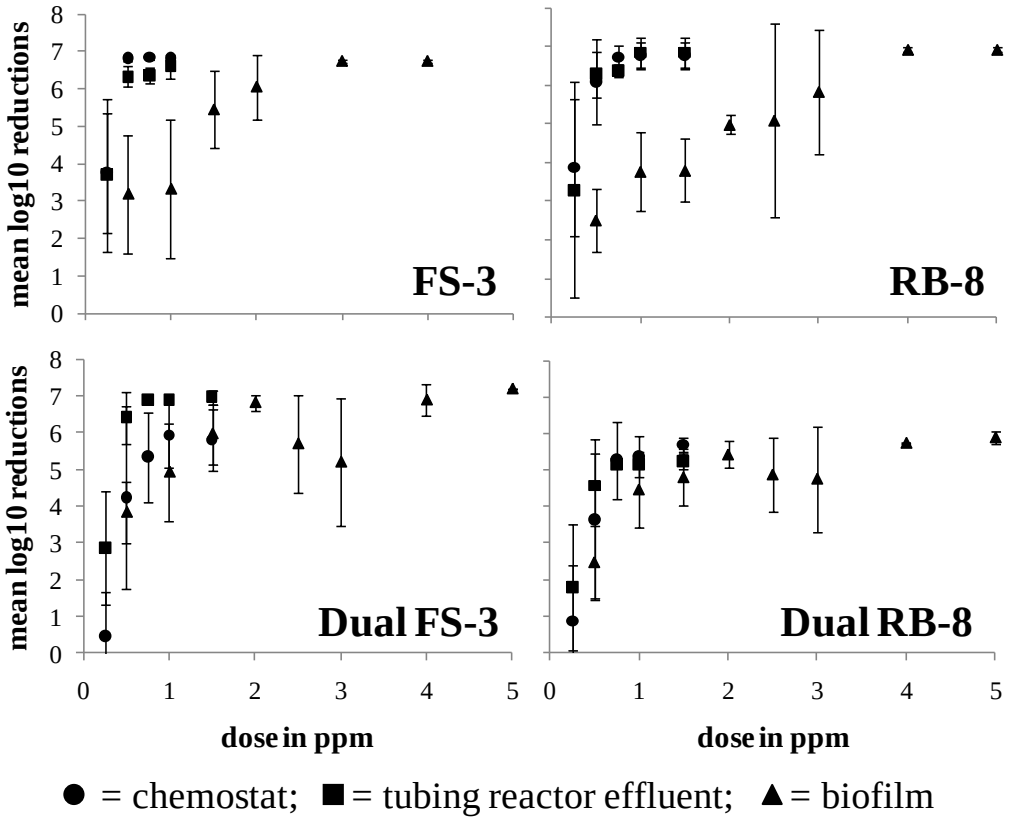

Figure 3.8 Mean $\log_{10}$ reductions of homogenized *B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and binary cultures in the chemostat, the tubing reactor effluent and the biofilm. The bars represent one standard deviation (n >3).

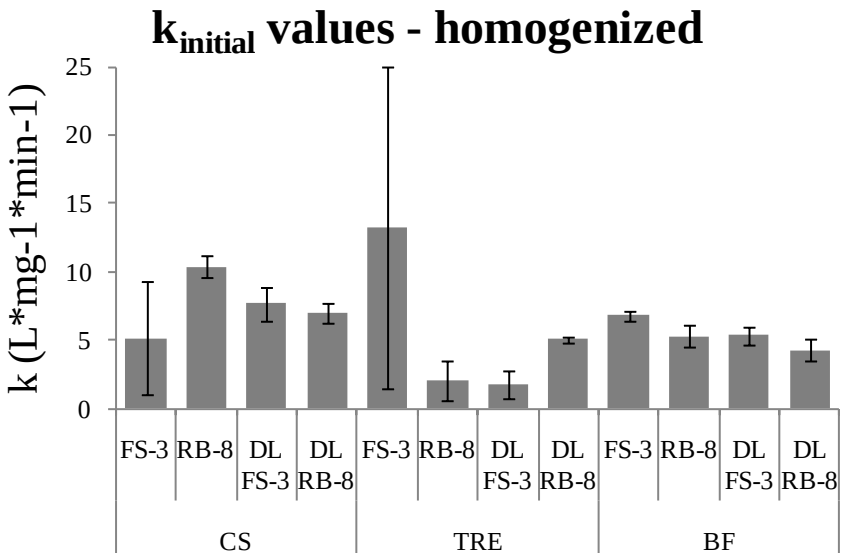

Figure 3.9 k values of homogenized samples for the first minute ($k_{initial}$) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as a binary culture). Error bars represent one standard deviation ($n \geq 3$).

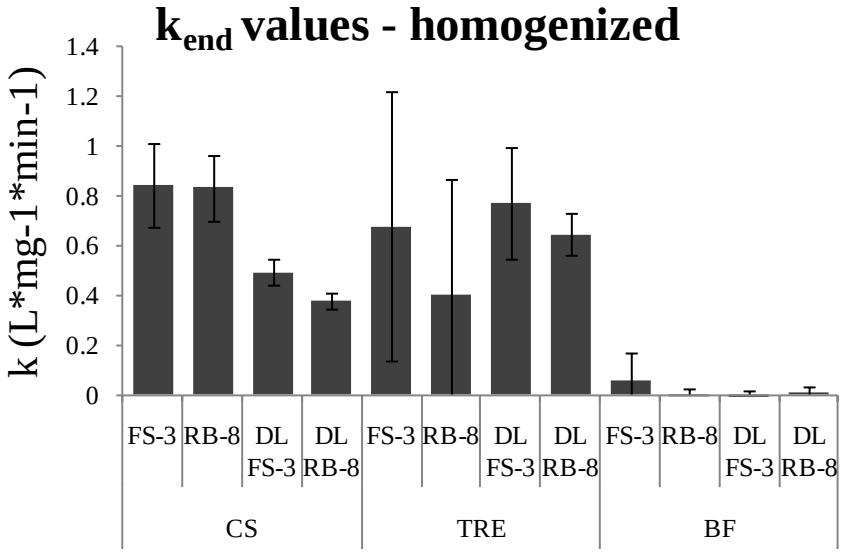

Figure 3.10 k values of homogenized samples for minute 2 to minute 30 (k_{end}) of disinfection after the addition of chlorine dioxide for the chemostat, the tubing reactor effluent, and the biofilm for all 4 species scenarios (*B. cepacia* FS-3 and *P. aeruginosa* RB-8 as monocultures and as a binary culture). Error bars represent one standard deviation ($n \geq 3$).

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CHAPTER 4

SUSCEPTIBILITY OF *BURKHOLDERIA CEPACIA* FS-3 AS PLANKTONIC CELLS, DETACHED CELLS AND CLUSTERS, AND BIOFILMS TO DISSOLVED OZONE

Abstract

Ozone as a gas or dissolved in water is an efficient antimicrobial with a wide range of activity against microorganisms. Significant log reductions can be achieved with low doses when ozone is applied to suspended bacteria but biofilm disinfection requires higher doses because extracellular polymeric substances deplete ozone through reaction, thus protecting cells. Biofilm particles, however, can detach from the biofilm surface and return to the bulk fluid as cell clusters bound by extracellular polymeric substances. These detached cell clusters are to date largely unstudied with respect to disinfection tolerance, but are of importance in a multitude of industrial, clinical, and environmental settings. Inadequate dosing of disinfectants such as ozone may lead to contamination events, disseminate bacteria, and even cause disease outbreaks.

In this study, *Burkholderia cepacia* as a model organism was grown in a chemostat and a biofilm tubing reactor, with the tubing reactor also serving as a source of detached biofilm clusters. These three sample types were tested with respect to their tolerance against dissolved ozone at a variety of concentrations.

Chemostat grown cells and detached cells and clusters were readily inactivated after the addition of a single dose of dissolved ozone below 1 ppm. The attached biofilm could not be inactivated with the ozone concentrations achieved with the ozone generator

used in this study. These findings suggest that *B. cepacia* FS-3 biofilms require higher single doses than applied here or continuous dosing of lower concentration to overcome reaction-diffusion limitations that deplete ozone before it can fully penetrate the biofilm.

Detached cells and clusters were similar to planktonic cells with regards to their ozone susceptibility which was contributed to the detachment of mostly single cells and small clusters and very few larger clusters which decreased the influence to reaction-diffusion limitations.

Introduction

Biofilm control has become an important issue in water quality management, food processing plants, industrial water lines and many other applications. Studies have recognized that cells in biofilms are much more tolerant to disinfection than planktonic free-floating cells due to a variety of reasons such as production of extracellular polymeric material by the cells, differential gene expression, and low metabolic activity (Steed & Falkinham 2006; Donlan & Costerton 2002, Davies 2003). Since biofilm cells display different phenotypes, they can differentiate into complex multicellular structures and optimize survival during treatment with antibiotics or disinfectants (Davies *et al.* 1998, Sauer *et al.* 2003). To control biofilms in industrial settings, oxidizing agents like chlorine, chlorine dioxide, or ozone are frequently used because they are inexpensive and easily accessible. A disadvantage of chlorine is the production of harmful halogenated disinfection byproducts, for example trihalomethanes and halogenated acetic acids. Chlorine dioxide, on the other hand, does not react with ammonia nitrogen, amines or other oxidizable organic matter, but is explosive under pressure and dangerous acids are

needed for its production. Ozone, however, can be produced on site and decomposes into O_2 while by-product formation depends on the water quality. It has been successfully used in water treatment facilities in Europe for several decades and also has been recognized by the food industry as an efficient and safe disinfectant. In the gaseous or aqueous phase, it is a useful disinfectant with a wide range of activity as a powerful antimicrobial agent against bacteria (Kim & Yousef 2008), fungi (Lezcano *et al.* 2000), protozoa (Rennecker *et al.* 1999), and viruses (Vaughn *et al.* 1987; Katzenelson & Biedermann 1976).

Ozone decays in water and forms free radicals that react with organic substances as well as cells and cell constituents. Scott and Leshner (1962) reported membrane damage in *Escherichia coli* after treatment with ozone while Hamelin *et al.* (1978) observed single stranded breaks in DNA of treated bacteria which resulted in extensive DNA damage followed by cell death. Ishizaki et al. (1987) also confirmed that ozone is able to diffuse into cells through the membrane and react with cell constituents. They proposed chromosomal DNA damage as one of the reasons for inactivation by ozone. Although ozone is very efficient at killing suspended cells at low concentrations, much higher concentrations can be necessary to kill sessile cells (Viera *et al.* 1999a, Viera *et al.* 1999b). Studies found that the disinfection efficacy of ozone on biofilms does not only depend on the applied concentration, but also on the exposure time (Viera *et al.* 1999a, Viera *et al.* 1999b). Before ozone can reach the cells it reacts with extracellular polymeric substances (EPS), thus protecting the biofilm (Tachikawa *et al.* 2009). Species composition of the biofilm is a crucial factor that determines ozone dose and exposure

time and a study found that environmental strains can be more tolerant to ozone treatment than regularly used, commercially available lab strains (Lezcano *et al.* 1999).

As part of the natural biofilm life cycle, cells and clusters can detach from biofilms growing in a liquid flow scenario (Simões *et al.* 2008; Simões *et al.* 2005). Compared to planktonically grown cells, detached cells and clusters show increased tolerance to antibiotics (Fux *et al.* 2004) or disinfection with chlorine (Steed & Falkinham 2006), but are less resistant than the sessile biofilm. These cell clusters may not be inactivated properly if insufficient amounts of disinfectant are applied which can also depend on particle size (Winward *et al.* 2008). Surviving cells in clusters may initiate re-growth of the biofilm downstream of the disinfection site which can present a risk to human health in water treatment and food processing and is also relevant in other industrial settings.

Detachment of cell clusters from biofilms is an important factor in the distribution of bacterial contamination in both public health and industrial settings, but the disinfection efficacies of commonly used biocides on detached clusters have not been sufficiently studied.

The goal of this study was the evaluation of the inactivation capabilities of ozone dissolved in water on suspended cells and sessile cells, compared to cells and clusters that naturally detach from biofilm. Monocultures of *Burkholderia cepacia* FS-3 were grown in standardized reactors and treated with a single dose (no continuous dosing) of ozonated water.

To represent the three physiological states of planktonic, sessile, and detached, cultures of *B. cepacia* FS-3 were grown in 3 different experimental scenarios: grown as planktonic cultures in a continuously stirred chemostat, grown as biofilms attached to the inner walls of silicone tubing, and as detached cells and cell clusters from the effluent of the tubing reactor. Before disinfection, the size distribution and occurrence of clusters detaching from *Burkholderia cepacia* (FS-3) biofilms grown in tubing reactors was characterized and compared to the cluster sizes found in the chemostat. A low nutrient medium was supplied to the chemostat culture and the biofilm tubing reactor to simulate low nutrient environments.

Materials and Methods

To compare the chlorine susceptibilities of planktonic cells to cells detached from biofilm and attached biofilm, environmental isolates of *B. cepacia* FS-3 (FS-3) were grown as monocultures in chemostats and biofilm tubing reactors. Cluster sizes were analyzed for chemostat samples and tubing reactor effluent, and disinfection susceptibilities were assessed for all sample types (chemostat, tubing reactor effluent, and attached biofilm, as well as the homogenized control samples).

Bacterial Strain and Nutrient Media

Cultures of FS-3 were grown at room temperature (22 ± 2°C). Inoculation cultures were grown for 18 h on a shaker in a low nutrient sterile defined medium consisting of 0.1 g/L glucose, 0.018 g/L NH_4Cl, 3.93g/L phosphate buffer (2.71 g/L Na_2HPO_4; 1.22 g/L KH_2PO_4), and 2 ml/L 0.1 M $MgSO_4$ (Fisher Scientific, Pittsburgh,

PA). The growth rate of FS-3 in the low nutrient medium was 0.198 (± 0.02) hr^{-1}. In addition, FS-3 was tested for auto-aggregation of thoroughly washed cells via cell surface adhesions using the method by Rickard *et al.* (2000). Washed, concentrated cell suspensions of FS-3 did not auto-aggregate under the experimental conditions.

Chemostat Cells

Planktonic cultures were grown in the defined medium mentioned above. The chemostat (BioSurface Technologies Corp., Bozeman, Mont.) had a volume of 170 ml and the residence time was set to be slightly over 6 hours using a nutrient flow of 0.5 ml/min. The chemostat was filled with medium and then inoculated with 3 ml of the 18 h overnight culture. Batch mode (no flow) for 2 doubling times (12 hours) was followed by continuous flow for another 6 doubling times (36 hours) 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 hours. 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 reactor consisting of 45 cm of silicone tubing (ColeParmer Masterflex size 16 peroxide-cured silicone tubing) with an inner diameter of 0.31 cm resulting in a total reactor volume of 3.4 ml and a residence time of 6.8 min for a flow rate of 0.5 ml/min. The autoclaved, sterile reactor was filled with the medium described before and inoculated with 2 ml of overnight culture, followed by an attachment period of 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 FS-3, it was assumed that planktonic cells were washed out of the reactor and cells and clusters found in the effluent were the result of detachment.

Sampling of Detached Clusters and Cells. Immediately before collecting 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 before cutting the tubing into 1.5 cm pieces. The pieces were individually submerged into 10 ml cooled buffer for quantification or treatment. For enumerating cells, biofilm was loosened 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 with a shear homogenizer (IKA Labortechnik, Staufen, Germany) at 20,500 rpm for another minute.

To measure the thickness of the biofilm, the tubing was cut into smaller sections and then cut lengthwise using a clean, sterile razorblade. The exposed biofilm was subsequently covered with OCT embedding medium (Tissue-Tek, Sakura Finetech U.S.A., Inc., Torrance, CA) and frozen on dry ice. Using sterile forceps, the silicone tubing was carefully removed and the biofilm sectioned into 5 µm slices in a cryostat (Leica CM1850) and viewed under transmitted light and a 20x or 4x objective (Nikon). Thickness was calculated using the MetaMorph® (Universal Imaging Corporation,

Downingtown, PA) software. After four days of growth at continuous flow, the FS-3 biofilm had thickness of 26 µm (±4 µm).

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, IKA Labortechnik, Staufen, Germany) at 20,500 rpm for 1 min. The homogenizer was sterilized between samples by flaming with ethanol.

Disinfection Experiments

A commercial ozone generator (ClearWater Tech LLC – Microzone 500) was used for the experiments and was able to generate about 500 mg O_3/hr. The air containing ozone gas (O_3) was bubbled into chilled nanopure water (half –life of O_3 in water is longer at lower temperatures) using a very low air flow rate to maximize the concentration of O_3 in the water. A 1 liter glass column and two gas diffusion rods with small pores were used to maximize the contact time and surface between air bubbles and water. The water was gently stirred on an electric stir plate. Only 10 minutes of air-O_3 flow were necessary to reach the final concentrations. Measurements were done with the HACH® indigo method at 600 nm and the HACH® DR2000 spectrophotometer. The range of the test kit is 0 – 1.75 mg/L. The blue indigo dye in the test ampoules is bleached when O_3 is present and stabilized by a low pH. The absorbance reading was compared to a blank control with nanopure water. We were able to obtain concentrations between 0.25 and 1.25 ppm of dissolved ozone.

Due to the low concentration of O_3 in solution, ozonated water was directly added to culture samples in different ratios along with controls that were mixed with nanopure water. The colony forming units (CFU) of the attached biofilm were standardized by immersing a cut piece of tubing (1.5 cm length) into 10 ml of sterile buffer that resulted in approximately 7 $\log_{10}$ (CFU/ml) after homogenization in the buffer. Therefore, the CFU of the attached biofilm are also expressed in CFU/ml. Samples were exposed to ozone for 30 minutes in a shaking incubator at room temperature along with untreated control samples. Neutralization was done with sodium thiosulfate (Fisher Scientific, Pittsburgh, PA).

Bacterial Quantification

Samples were appropriately diluted in sterile1x PBS buffer (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24g of KH_2PO_4 suspended in 1 L purified water, chemicals by Fisher Scientific, Pittsburg, PA) and plated on R2A plates (Difco™ R2A Agar, BectonDickinson, Franklin Lakes, NJ) using the drop plate method (Herigstad et al. 2001). The incubation period was 48 hours at 30°C. Total inactivation was defined as the concentration that resulted in the absence of any 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).

Microscopy and Image Analysis
for Cluster Size Determination

LIVE/DEAD® BacLight™ stain (Invitrogen – Molecular Probes, Carlsbad, CA) was added to samples from chemostats and tube reactor effluents and incubated for 15 min. Then, the samples were filtered onto a black polycarbonate membrane (Poretics®, 0.22 µm, 25 mm). Fluorescent samples were visualized using a fluorescent microscope (Nikon Eclipse E800, software: MetaVue® for Fluorescence (Universal Imaging Corporation, v 7.4), and a 100x oil immersion lens (Nikon). Image analysis was done with the MetaMorph® (Universal Imaging Corporation, Downingtown, PA) software to determine the number of single cells, number of clusters, and the number of cells per cluster. Thirty random fields of view (6445 μm^2) were recorded using MetaVue® software and analyzed with the MetaMorph® software. 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. A calibration was done to associate the size of the area with the number of bacteria in the cluster (Wilson *et al.* 2004). This also takes into account the amount of extracellular polymeric substances produced by the bacteria.

Statistical Analysis

The density of organisms were reported as CFU/ml, and then log 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 was applied, and LD_{dose} is the LD of organisms which survived disinfection at some specified dose.

Comparison of CS, TRE and BF Samples: An ANOVA with dose as covariate and reactor as a fixed effect was run. The date of the experiment was considered as a random factor. 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).

Cluster Analysis: The cell cluster analysis was performed by first dividing the areas for each type of cluster by the total area of all clusters for each species. For each of the cluster types (single, 2 - 5, 6 - 10, 11 - 100, and 100+ cells), these proportions were analyzed separately using an ANOVA with reactor (chemostat and detached), as a fixed effect. For the 6 - 10 and 11 - 100 cell clusters, a weighted least squares analysis was performed, since the variance of the proportions in these cases increased as the mean proportion increased.

Homogenized Cluster Analysis: To determine the effect of homogenization on the cluster distributions, the cluster type proportions for the homogenized cells were subtracted from the proportions for the non-homogenized cells for each reactor. These differences were analyzed separately for each species using an ANOVA with 'Cluster type' (single, 2 - 5, 6 - 10, 11 - 100, and 100+ cells as levels) and reactor (chemostat and detached) as fixed effects, and experimental date as a random effect.

Results

Cluster Size Distribution of FS-3 in Chemostat and Tubing Reactor Effluent

Microscopic analysis FS-3 (*B. cepacia*) showed that chemostat grown cells do not always exist as single cells (Figure 4.1). Twenty-one percent of all cells in the chemostat samples were present as single cells, 59% of cells were contained in small clusters of 2 to 5 cells, while the remaining 20% consisted of clusters between 6 and 100 cells per cluster. The tubing reactor effluent contained a higher percentage of clusters containing more than 6 cells (40%) and less small clusters with 2-5 cells. While the chemostat samples did not contain clusters with more than 100 cells, the tubing reactor effluent had 5% of these large clusters. Although there were differences between the two reactor samples, none were statistically significant (p-value > 0.05) due to high variability in the cluster size proportions.

Auto-aggregation did not appear to be responsible for cluster formation as determined by experiments done after Rickard *et al.* (2000).

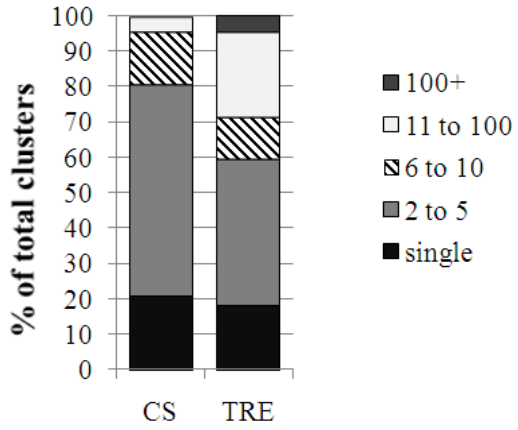

Figure 4.1 Cluster size distribution for *Burkholderia cepacia* FS-3 in the chemostat and the tubing reactor effluent.

Initial Cell Numbers for
Disinfection Studies

Both reactors (chemostat and tubing reactor) were inoculated with overnight cultures containing 8.0 $\log_{10}$(CFU/ml). Initial $\log_{10}$ densities for the ozone disinfection experiments varied with the dilution of the samples with ozonated water. Large volumes had to be added to the culture samples in order to achieve higher concentrations of O_3 in the samples. However, controls with identical dilution factors were run in parallel to calculate $\log_{10}$ reductions. The means of the initial $\log_{10}$ densities are shown in Table 4.1.

Table 4.1 Log_{10} densities of untreated controls (n=6) with one standard deviation.

Reactor	**log10 CFU/ml**
Chemostat	6.33 (±0.34)
Tube Reactor Effluent	6.49 (±0.37)
Biofilm	7.13 (±0.14)

Disinfection Susceptibilities of Planktonically Grown Cells Compared to Detached Biofilm Particles and Attached Biofilm Samples

Chemostat cells, detached biofilm particles, and attached biofilm were treated with a single dose of ozone dissolved in nanopure water, exposed for 30 minutes, and finally neutralized with sodium thiosulfate. Log_{10} reductions (LRs) of the chemostat samples show a dose-dependent linear response to the ozone treatment with total inactivation of the culture just below 1 ppm (Figure 4.2). Tubing reactor effluent samples are slightly more tolerant to exposure to ozonated water with total inactivation around1.20 ppm (Figure 4.2). Chemostat and tubing reactor effluent sample LRs are not significantly different from each other due to the high variability. However, the LRs of biofilm were significantly lower than the LRs of chemostat and tubing reactor effluent samples. One log_{10} was inactivated in all biofilm samples up to concentrations of 1.20 ppm (Figure 4.2).

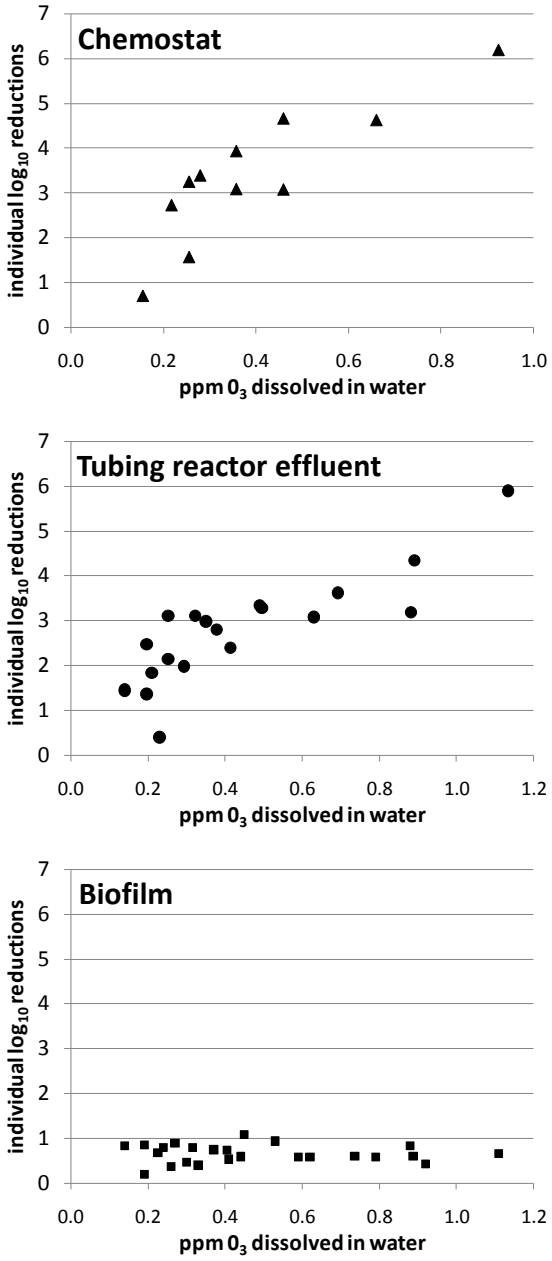

Figure 4.2 Individual $\log_{10}$ reductions of *B. cepacia* FS-3 in the chemostat, the tubing reactor effluent, and the biofilm.

Homogenization of Cells and Clusters

Shear homogenization at 20,500 rpm was unable to disrupt smaller cell clusters but somewhat reduced the number of larger clusters. Homogenized FS-3 from the TRE had significantly higher proportions of smaller cluster sizes (single cells, 2-5, 6-10) and higher proportions of larger clusters (11-100, 100+ cells) than when not homogenized (p-value < 0.01) while single FS-3 CS samples did not lead to significant change in cluster proportions (p-value > 0.05) (Table 2.5, Chapter 2, supplemental materials).

Disinfection of Homogenized Samples

Control samples of chemostat, tubing reactor effluent and biofilm samples were shear homogenized before being treated with the same doses of ozonated water. LRs of the homogenized chemostat showed a slight but not significant (p-value > 0.05) increase in susceptibility with complete inactivation below 0.8 ppm of dissolved O_3 (Figure 4.3). The homogenized tubing reactor effluent was also more susceptible with total inactivation below 1.0 ppm but this difference is also not statistically significant (p > 0.05) (Figure 4.3). Biofilm samples that were manually removed, vortexed and shear homogenized were significantly (p-value < 0.001) more susceptible to O_3 treatment than the unhomogenized samples. Interestingly, the LRs of the homogenized biofilm range from 3 to 7 logs, even at very low concentrations. Total inactivation did occur below 1.0 ppm (Figure 4.3).

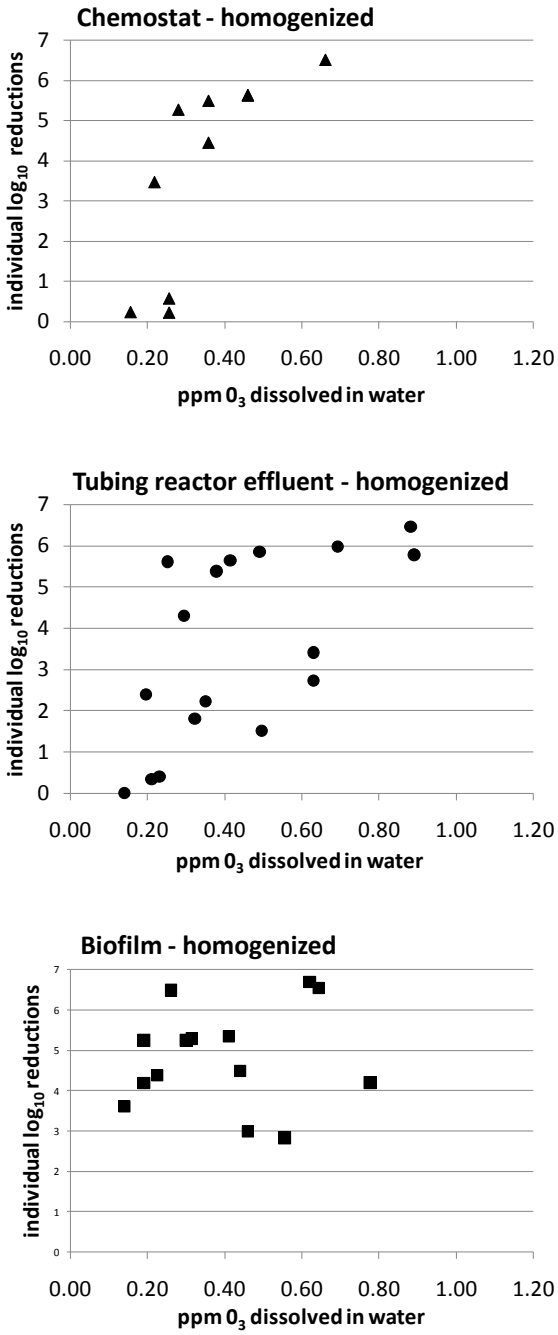

Figure 4.3 Individual $\log_{10}$ reductions of homogenized *B. cepacia* FS-3 in the chemostat, the tubing reactor effluent, and the biofilm.

Discussion

Ozone is a strong oxidizing antimicrobial, successfully used in water treatment, food processing and other industrial processes against suspended cells and biofilms. The goal of this study was the assessment of the disinfection tolerance of biofilm-detached clusters of *B. cepacia* FS-3 to ozone treatment in direct comparison with planktonic and biofilm samples. Initial cluster size analysis demonstrated that the biofilm reactor effluent contained a higher percentage of large clusters than the chemostat samples as determined by image analysis. The tubing reactor effluent also contained clusters with more than 100 cells which were not detected in chemostat samples. Although none of these differences were statistically significant, they indicate that both sample types contain a wide range of clusters sizes with a high variability. Auto-aggregation experiments were performed on washed FS-3 cells to test the cells' ability to attach to one another via adhesions and form aggregates in suspension (amended method by Rickard *et al.* 2000). The results of this test were negative with no indication for auto-aggregation in FS-3, suggesting that this process of aggregation in the chemostat may be mediated by the production of EPS in the culture and thus making cells "stickier" especially in low nutrient solutions due to enhanced capsule formation (Singh & Vincent, 1987).

Disinfection experiments showed that chemostat cells were slightly more susceptible in comparison to detached cells and clusters but statistical analysis did not find significant differences between the $\log_{10}$ reductions (LRs).

Biofilm samples were most tolerant with a steady 1 log reduction independent of dose up to 1.2 ppm. This suggests that the applied ozone may not be able to penetrate the

entire depth of the biofilm since it was reacting with EPS constituents as it diffused into the biofilm (Tachikawa *et al.* 2009). Additionally, O_3 is only able to penetrate into the attached biofilm from one direction (if we assume a flat homogenous biofilm surface) while O_3 attacks free-floating cells and clusters from every direction. The dose-independent one $\log_{10}$ reduction of biofilm cells suggests that a very active layer of cells on the surface of the biofilm is easily inactivated (Rani *et al.* 2007; Vieira *et al.* 1993), even with low concentrations of ozone while deeper layers are protected by EPS. Concentrations higher than 1.2 ppm of O_3 were not achieved with the experimental setup, but a dose-dependent response of the biofilm to O_3 may occur at higher doses where reaction-diffusion limitations are overcome (Stewart & Raquepas 1995; Chen & Stewart 1996 for research done with chlorine).

Concentration was an important factor in the disinfection of chemostat and tubing reactor samples, but contact time has also been identified as a crucial determinant of disinfection efficacy (Viera *et al.* 1999b). While some studies determine a bacterial inactivation rate at constant concentrations of O_3 (Viera *et al.* 1999a; Katzenelson & Biederman 1976; Rennecker *et al.* 1999), this study focused on applying a single dose at the start of the experiment and then allowing the O_3 to decay. Since O_3 reacts with water, the applied dose quickly decreases even in cell-free samples (Tachikawa *et al.* 2009).

Here, we showed that single dose treatment is very effective against suspended cells and clusters from chemostat and biofilm effluent but is unable to eradicate a biofilm population, most likely due to diffusional resistance and simultaneous reaction with EPS

constituents. Continuous dosing or bubbling of O_3 gas directly onto the biofilm may remove EPS from the biofilm and make it susceptible to disinfection.

Homogenized samples of the tubing reactor effluent contain a higher percentage of smaller clusters and a lower percentage of larger clusters which indicates that larger clusters have been disrupted during homogenization and presumably EPS has been disrupted and / or removed from cells as well. This disruption of aggregates and EPS renders cells more susceptible to disinfection as seen with chlorine disinfection (see Chapter 2) but does not affect O_3 disinfection significantly. When treated with low O_3 concentrations, LRs of homogenized chemostat and tubing reactor effluent samples were very small suggesting that O_3 reacts with EPS that has been brought into solution via homogenization. Homogenized biofilm samples, however, display significant LRs in the range between 3 and 7 logs at concentrations from 0.1 to 0.8 ppm. This indicates that biofilm cells that were removed from the surface, vortexted, and homogenized, are very susceptible even to low concentrations of O_3. The biofilm removal procedure including mechanical removal with tweezers and pulse vortexing may cause cells to be more susceptible to O_3 than homogenization alone.

This study suggests that O_3 disinfection may be dependent on cluster size in chemostat and tubing reactor effluent of unhomogenized samples, although cluster size distribution and $\log_{10}$ reductions of the two reactors were not significantly different. Further cluster size analysis and disinfection studies with different bacterial strains are necessary to confirm this result. Growth as a biofilm was an advantage to FS-3 when compared to samples with cells and clusters in suspension.

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CHAPTER 5

OVERALL CONCLUSIONS

The goal of this study was the evaluation of the disinfection susceptibility of particles and cells detached from biofilm. Previous studies found that detached particles display an intermediate susceptibility to antibiotics (Fux *et al.* 2004) and chlorine disinfection (Steed & Falkinham 2006) when compared to planktonic cells and attached biofilms. Steed and Falkinham (2006) collected detached cells by removing biofilm from beads which can be considered as disrupted biofilm rather than actively detaching cells and clusters. Detached biofilm particles may behave more like biofilms right after detachment although they are not as tolerant to disinfection as sessile biofilms (Rollet *et al.* 2009).

In this work, the primary objective was to examine, quantify, and test clusters that naturally detached from mature biofilms rather than using mechanically disrupted biofilm. Winward *et al.* (2008) and Xu *et al.* (1995) showed that disinfection of cells in particles is strongly correlated with the size of the clusters with bigger clusters being more tolerant to a biocide challenge. To assess the influence of cluster sizes and occurrences, biofilm effluent was analyzed using microscopy and image analysis. Since researchers suggest that mixed species or even dual species biofilms are more tolerant to disinfection challenges due to synergistic relationships between species (Burmølle *et al.* 2006), there is a possibility that the commensal relationships are still present in biofilm clusters that detached from these mixed-species biofilms. Thus, this work did not only test the disinfection susceptibility of detached particles on monospecies

biofilms, but also binary biofilms. These detached particles were compared with chemostat cells and attached biofilm cells with respect to the disinfection susceptibilities to 3 different commonly used oxidizing chemicals.

Chapter 2 describes the chlorine susceptibility testing of detached biofilm particles from *B. cepacia* FS-3 and *P. aeruginosa* RB-8 biofilms and the binary culture of the two strains. As expected, biofilms were most tolerant to disinfection requiring about 10 times the chlorine concentration necessary to kill planktonic cells. For detached particles and chemostat cells, a strong correlation between cluster sizes and disinfection efficacy was found. *B. cepacia* FS-3 cells were more tolerant as detached clusters than planktonic cells which coincided with the presence of a higher percentage of larger clusters in the tubing reactor effluent. Cluster analysis of *P. aeruginosa* RB-8 chemostat samples and detached particles showed that a higher percentage of larger clusters was actually present in the chemostat samples which also resulted in an increased tolerance of the chemostat sample compared with the detached particles. However, when in co-culture both species display more similar disinfection patterns with chemostat grown cells being most susceptible. Although detached clusters of binary biofilms contain more small clusters and less large clusters, they are very tolerant to disinfection compared to the single species equivalent which suggests synergism of FS-3 and RB-8 in the biofilm and detached particles.

Stewart et al. (2000) introduced resistance factors as a measure for the reduced susceptibility of biofilms that is calculated by dividing the $\log_{10}$ reduction of planktonic cells by the $\log_{10}$ reduction of biofilm cells at a given dose. These resistance factors allow

for a comparison between different organisms and different disinfectants and describe the tolerance differences between cells in biofilms and planktonic cultures. Here, resistance factors (hereafter called tolerance factors) were calculated for biofilms as well as detached clusters compared to planktonic cells.

Figure 5.1 shows how the chlorine tolerance of biofilms and detached clusters compares to the tolerance of chemostat cells. All biofilm samples were more tolerant than chemostat cells but the differences at 2 and 4 ppm vary with species. The closer the tolerance factor is to 1, the more similar the disinfection tolerances are. Figure 5.1 indicates that FS-3 biofilm $\log_{10}$ reductions are not much different from the $\log_{10}$ reductions of FS-3 cells from chemostats for 2 and 4 ppm. In contrast, RB-8 biofilm tolerance was up to 6 times higher than the RB-8 tolerance for chemostat cells showing that RB-8 biofilms are very tolerant to the disinfection challenge at low doses. As seen in Figure 2.3 (Chapter 2), the RB-8 biofilm can be completely inactivated with approximately the same concentrations of chlorine as the FS-3 biofilm, so that the initial tolerance to low doses was not recorded at higher doses. A reason for this observation may be an initial reaction-diffusion limitation. Dual species FS-3 and RB-8 were 2 to 4 times more tolerant as biofilms compared to chemostat cells.

The tolerances did not differ greatly when comparing chemostat cells and detached clusters and the factors are close to 1 or even lower for the single species RB-8, confirming that single RB-8 chemostat cells were more tolerant than detached clusters.

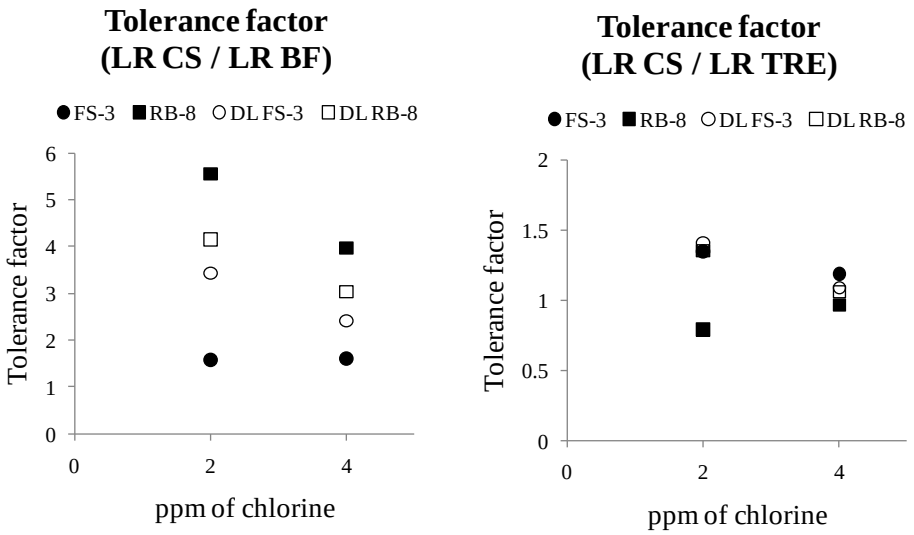

Figure 5.1 Tolerance factors for chemostat (CS) samples over biofilm (BF) samples and chemostat samples over detached cluster (TRE) samples for single species and dual (DL) species $\log_{10}$ reductions at 2 and 4 ppm of chlorine.

Homogenization of biofilms resulted in significantly higher $\log_{10}$ reductions than seen with intact biofilms. Figure 5.2 demonstrates that the tolerance of the biofilm cells after homogenization is comparable, although slightly higher than the tolerance of chemostat cells. This finding indicates that physical protection factors play a large role in the tolerance of biofilm cells and phenotypical or physiological changes are not as important.

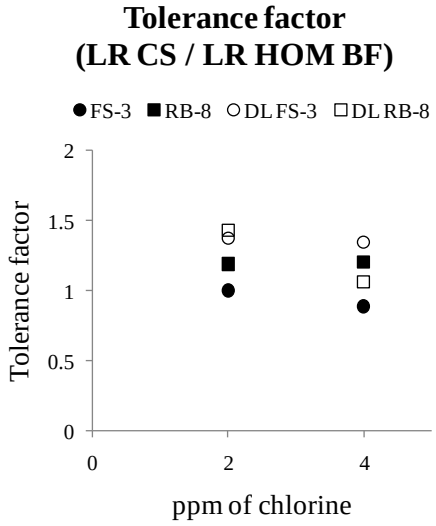

Figure 5.2 Tolerance factors for chemostat (CS) samples over homogenized (HOM) biofilm (BF) samples for single species and dual (DL) species $\log_{10}$ reductions at 2 and 4 ppm of chlorine.

Chlorine dioxide was tested as a disinfectant against detached particles, planktonic cells, and biofilm cells in the same single species and binary species cultures (Chapter 3). Again, biofilms were most tolerant to the disinfectant but the differences between chemostat cells and detached particles were not significant for any species. A correlation between cluster sizes and the disinfection susceptibility could not be found for any reactor or species. A synergistic relationship between FS-3 and RB-8 was observed in this set of experiments where binary cultures (chemostat cells, detached particles, and biofilms) were more tolerant than the monocultures.

Figure 5.3 compares the tolerance factors for biofilms and detached clusters with chemostat cells. When chlorine dioxide was used for disinfection, the tolerance of biofilm cells was up to 14 times higher than the tolerance of chemostat cells especially for the

single species RB-8 which has very low $\log_{10}$ reductions the low concentrations. Both dual species and the single species FS-3 biofilms were 2 to 8 times more tolerant than the chemostat cells. The biofilm tolerances observed after chlorine dioxide treatment were higher than the tolerances after chlorine treatment. Since tolerance factors were not calculated for higher doses, Figure 5.3 only identified initial differences. The high initial tolerances suggest that reaction-diffusion limitations may have existed at very low concentrations (0.5 and 1 ppm) and were overcome quickly, making physiological factors the most dominant factor for tolerance.

Detached cluster tolerances were almost identical to the tolerances of chemostat cells especially at 1 ppm of chlorine dioxide. These tolerance factors are similar to the factors observed in chlorine treatment when looking at chemostat cells and detached clusters and suggest no additional tolerance mechanisms in detached clusters.

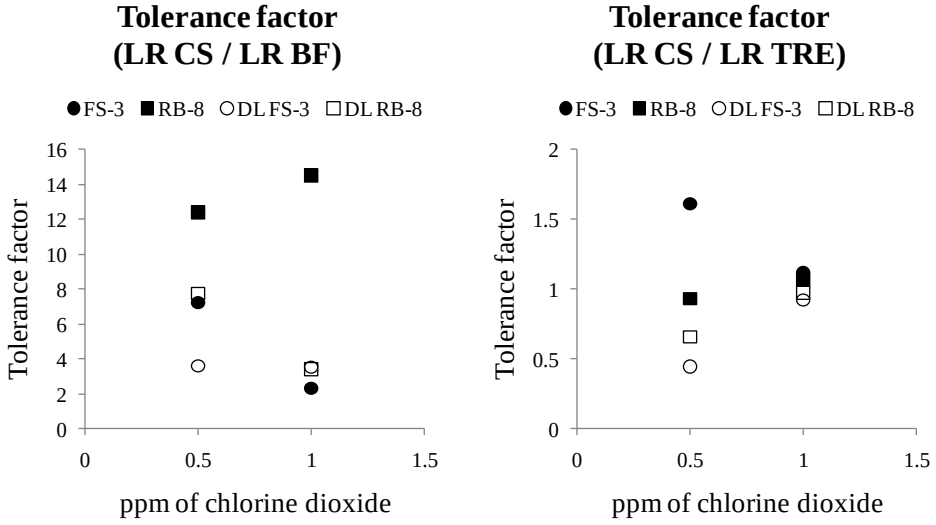

Figure 5.3 Tolerance factors for chemostat (CS) samples over biofilm (BF) samples and chemostat samples over detached cluster (TRE) samples for single species and dual (DL) species $\log_{10}$ reductions at 0.5 and 1.0 ppm of chlorine dioxide.

After homogenization the tolerances of biofilm cells were significantly reduced. The tolerance of the dual species FS-3 and RB-8 biofilms were close to 1, but the single species FS-3 and RB-8 biofilms were about twice as tolerant as the single species FS-3 and RB-8 chemostat cells. This finding may indicate that the spatial proximity of the two species in the dual biofilm was a crucial factor to resistance and was lost after homogenization.

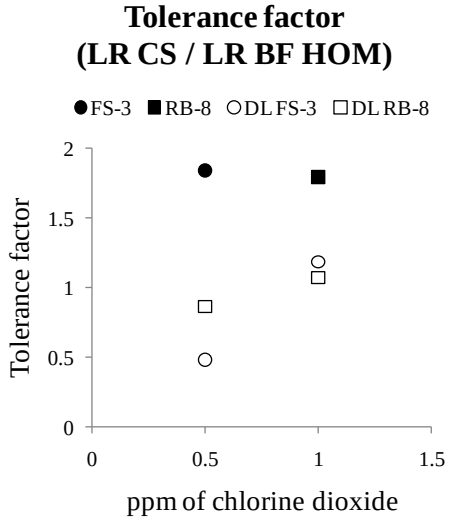

Figure 5.4 Tolerance factors for chemostat (CS) samples over homogenized (HOM) biofilm (BF) samples for single species and dual (DL) species $\log_{10}$ reductions at 0.5 and 1.0 ppm of chlorine dioxide.

Chapter 4 presented a short study on the disinfection tolerance of FS-3 cultures to dissolved ozone. Ozonation quickly inactivated chemostat and detached particle samples with no significantly different $\log_{10}$ reduction differences. Biofilms, however, were not affected by the ozone concentrations produced during the experiments which suggests

that ozone was depleted or reacted away with EPS constituents before it could cause significant $\log_{10}$ reductions in FS-3 biofilms.

Chlorine and chlorine dioxide are commonly used chlorine-based oxidizing disinfectants with differential disinfection capabilities that are recorded in this work. Chlorine is a very fast acting oxidizer that reacts with all substances that can release electrons and it required the highest treatment doses. Most of the killing with chlorine occurred within the first minute after addition of a specific dose followed by slower disinfection kinetics due to a persistent bacterial subpopulation and decrease of chlorine concentrations within the sample.

Chlorine dioxide required about a quarter of the dose and is a slower oxidizer compared to chlorine. Chlorine dioxide is more selective towards organic substances and does not react with all substances that can be oxidized, thus decaying more slowly.

Ozone is also an oxidizing compound and very successful at disinfection of chemostat and detached particle samples, but was unable to reach $\log_{10}$ reductions over 1.0 in the FS-3 biofilms. Doses in chlorine and chlorine dioxide experiments that were able to completely inactivate chemostat and detached particle samples always caused $\log_{10}$ reductions over 1.0 in the biofilms which may make ozone an unsuitable disinfectant for biofilms at low concentrations.

For the disinfectants, most of the killing occurs in the first minute or two when biocide concentrations are high, followed by a phase characterized by slow disinfection. This finding suggests that there may be a more tolerant sub-population of persister cells and additionally, disinfectant concentrations may be lower.

These findings are important where disinfection of water is required for health and water quality purposes such as drinking water applications and other medical and industrial settings. As soon as biofilms form on surfaces, biofilm control plays an important role but also disinfection of detaching particles has to be considered. This research shows that the low nutrient biofilms shed mostly smaller cell clusters that are, dependent on species, as easily inactivated as planktonic cells when disinfecting with chlorine dioxide, but when disinfected with chlorine require more disinfectant than planktonic cells.

Dependent on bacterial species and nutrient conditions, detachment may shift to a higher percentage of larger clusters than recorded in this study and make disinfection more difficult due to diffusion-reaction limitations. This suggests that monitoring for particle sizes in water and effluent samples using microscopy is a crucial tool in avoiding contamination problems.

Future Directions

Visualization of Different Species in Biofilm and Detached Clusters

As an addition to this research, *Burkholderia cepacia* FS-3 and *Pseudomonas aeruginosa* RB-8 in co-culture can be differentially visualized using either fluorescent labels or FISH (fluorescent in situ hybridization) probes. A probe or label specific to one species can be developed and the second species could be visualized with a general bacterial probe or stain. However, FS-3 and RB-8 are two closely related strains which may lead to more extensive method development with respect to finding a label or probe

that only labels one species. Once successful, this technique will allow for the investigation of species distribution in the biofilm and detached particles. FS-3 and RB-8 may be distributed homogenously in the biofilm and detached particles, or alternatively, they may occupy distinct niches in the biofilms depending on their nutrient demands and synergistic relationship. Since the ratios of FS-3 and RB-8 in biofilm and detached particles were very similar (Chapter 2, Table 2), an even distribution of the species within the biofilm is likely.

Cluster Size - Disinfection Efficacy Correlation

To assess whether larger clusters are more tolerant to disinfection than smaller detached clusters, experiments can be designed that treat samples with small or, alternatively, with large clusters. In this study, a separation of cluster sizes into small clusters and large clusters was not possible without potentially disrupting particles. By filtration or centrifugation cell clusters may be deformed, disrupted into smaller clusters or even aggregated further. Thus, it may be feasible to grow biofilms that shed larger clusters such as biofilms growing in rich nutrient conditions under low shear conditions. A concern in such a scenario could be the interference of nutrients in the medium with chlorine leading to the formation of chloramines, for example, if excess nitrogen is present in the sample. Alternatively, organisms that are known to produce large amounts of extracellular polymeric substances (EPS) can be used to form thick biofilms which may result in larger detached clusters.

Testing of Other Species

This study was performed with the two environmental strains FS-3 and RB-8 and also with a co-culture of both species. To confirm trends found in this study, other bacterial strains can be tested under the same or similar conditions tested here. Relevant strains can be identified depending on the setting.

In the medical field it may be feasible to identify pathogens involved in contamination events such as bacteria associated with catheter colonization. These bacteria may be gram negative organisms but can also be extended to gram positive organisms.

To apply this research directly to drinking water issues, a natural mixed-species biofilm inoculated with bacteria from drinking water samples may be spiked with a relevant organism such as a pathogen recovered from drinking water disease outbreaks. Retention and uptake of this organism can be monitored using a fluorescently labeled strain. Most importantly, monitoring of detachment events can provide information about the fate of the organism with respect to proliferation in the biofilm and detachment from the biofilm. The detached drinking water biofilm particles can be subjected to disinfection and a statement about the risk of pathogens in drinking water biofilms can be made with respect to detachment events.

Testing Other Disinfectants

This study tested the effect of 3 oxidizing disinfectants against detached particles from biofilm compared to planktonic cells and biofilms. The two chlorine-based disinfectants chlorine and chlorine dioxide performed differently with respect to required

treatment doses for total inactivation and disinfection kinetics. Further research can test the efficacy of chloramines to assess treatment doses and disinfection kinetics along with its efficacy against detached clusters compared to planktonic cells for this third chlorine-based biocide. Next to ozone, chlorine, and chlorine dioxide, chloramines are commonly used in drinking water disinfection (Richardson *et al.* 2007).

Alternatively, other non-oxidizing compounds with different modes of action can be tested on detached particles. Quaternary ammonium compounds are, for example, often used in industrial water systems or household cleaning products (Gilbert & McBain 2003) and have been shown to be effective disinfectants (McBain *et al.* 2004).

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APPENDIX A

CONCENTRATION X TIME (CT) GRAPHS

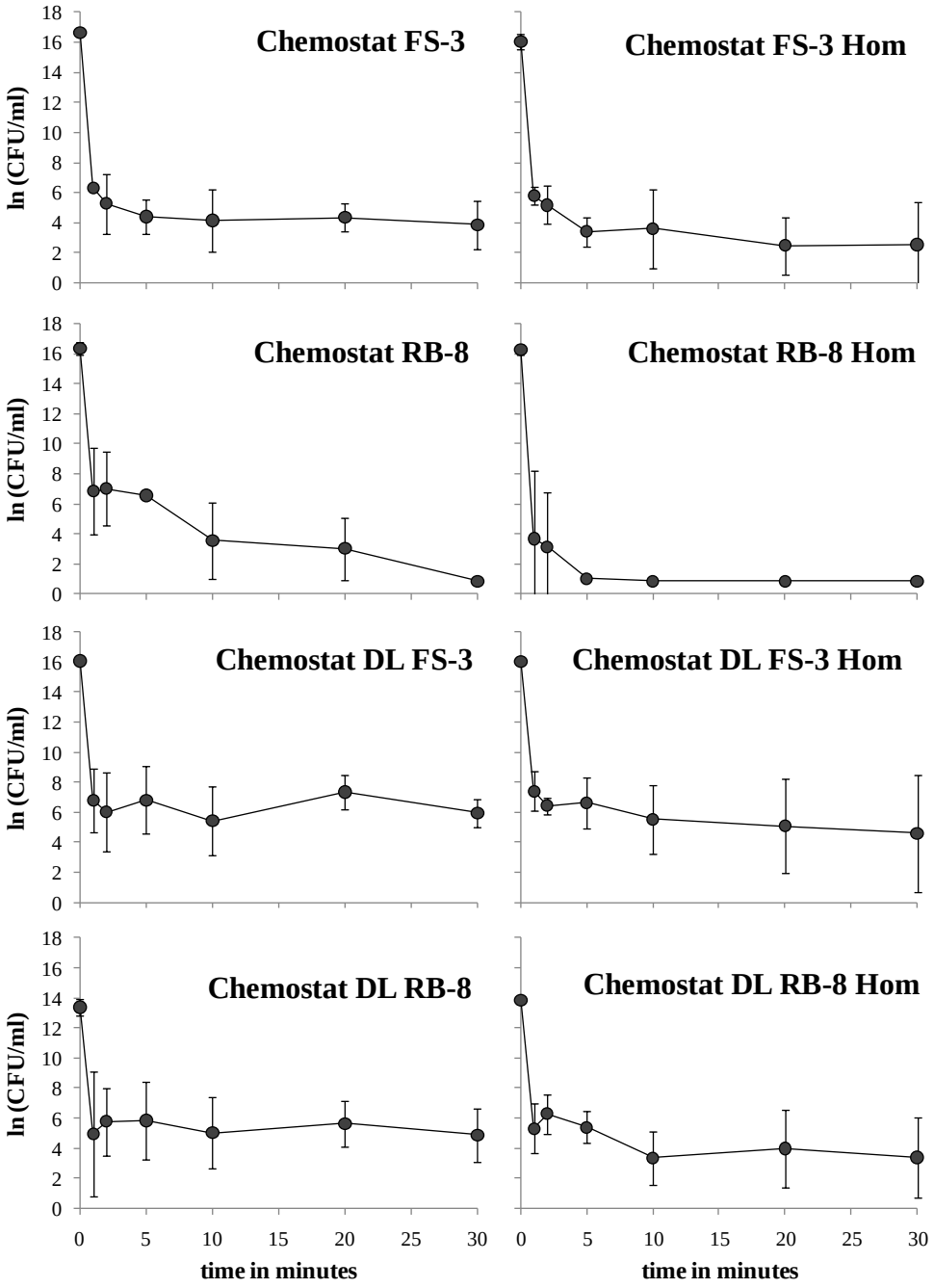

Figure 6.1 Concentration x time (Ct) curves for chemostat samples treated with chlorine. Bars and whiskers represent one standard deviation; n ≥3, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

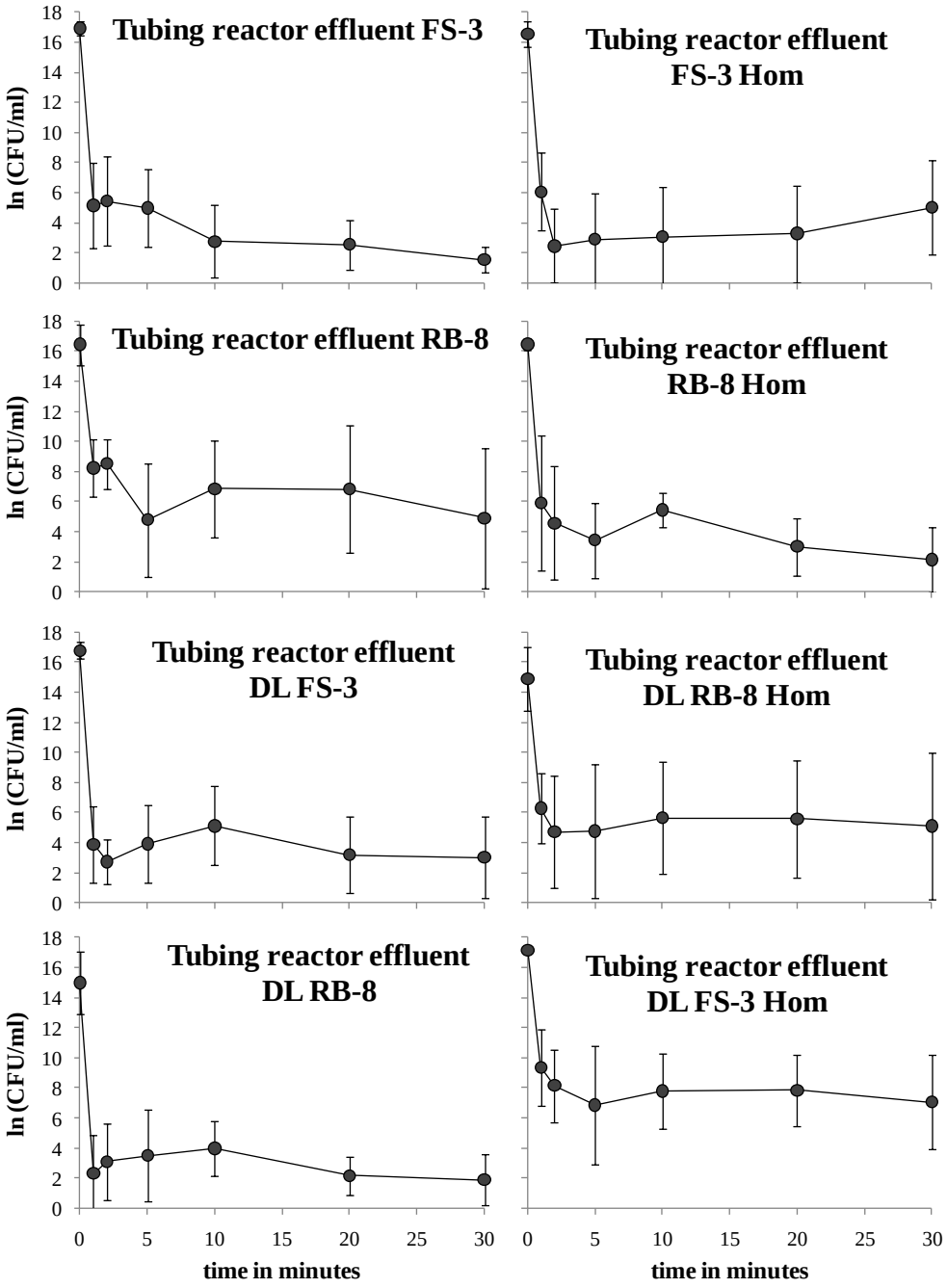

Figure 6.2 Concentration x time (Ct) curves for tubing reactor effluent samples treated with chlorine. Bars and whiskers represent one standard deviation; n ≥3, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

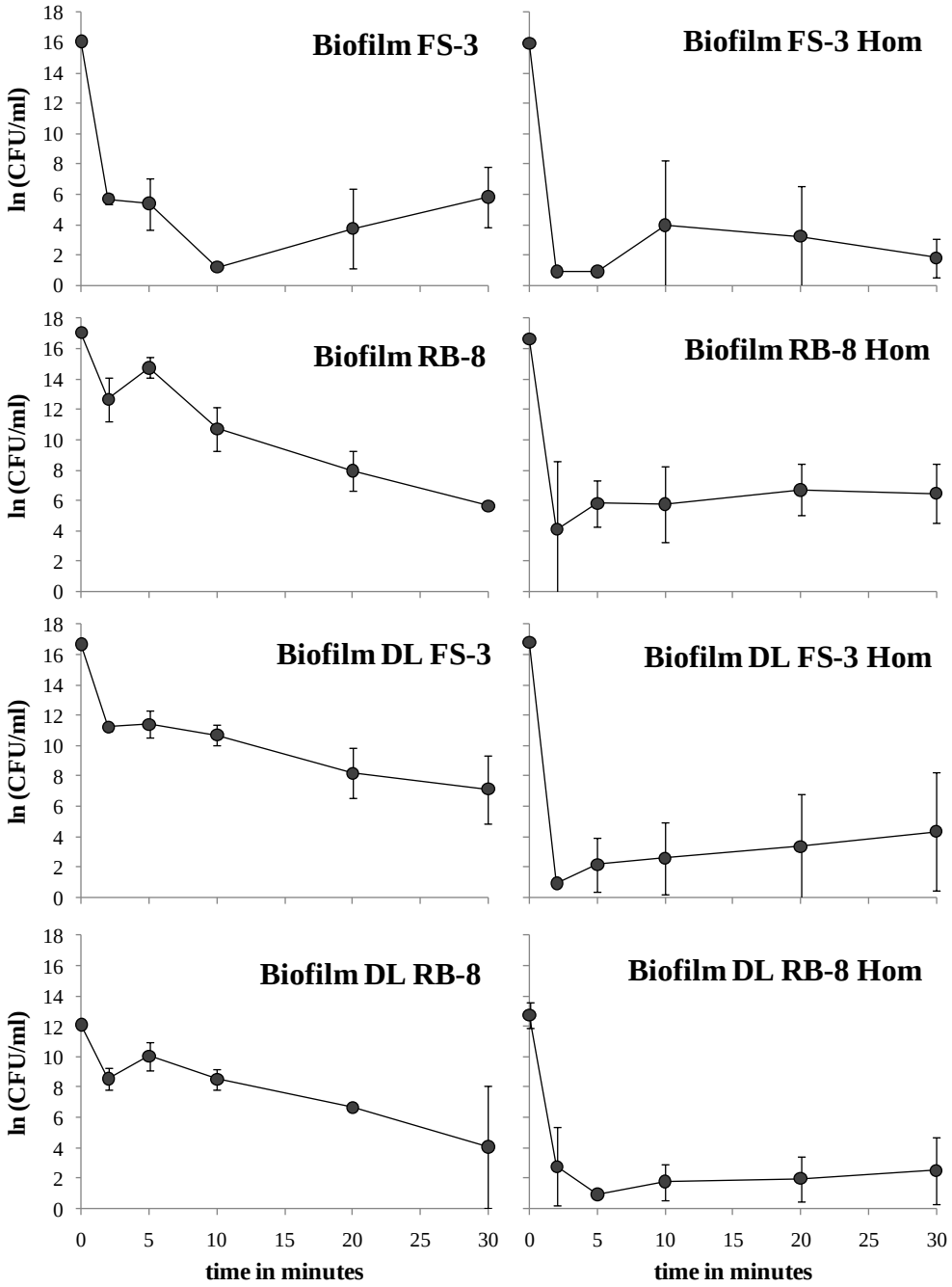

Figure 6.3 Concentration x time (Ct) curves for biofilm samples treated with chlorine. Bars and whiskers represent one standard deviation; n ≥2, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

Table 6.1 Subjective sub-lethal doses of chlorine chosen for CT curves. FS-3 = *B. cepacia*; RB-8 = *P. aeruginosa*, DL = dual (grown in co-culture).

	species	unhomogenized	homogenized
Chemostat	FS-3	2.00	0.50
	RB-8	3.00	2.00
	DL FS-3	1.00	0.50
	DL RB-8	1.00	0.50
Tubing reactor effluent	FS-3	6.00	1.00
	RB-8	1.00	0.50
	DL FS-3	3.00	0.50
	DL RB-8	3.00	0.50
Biofilm	FS-3	20.00	2.00
	RB-8	20.00	6.00
	DL FS-3	30.00	2.00
	DL RB-8	30.00	2.00

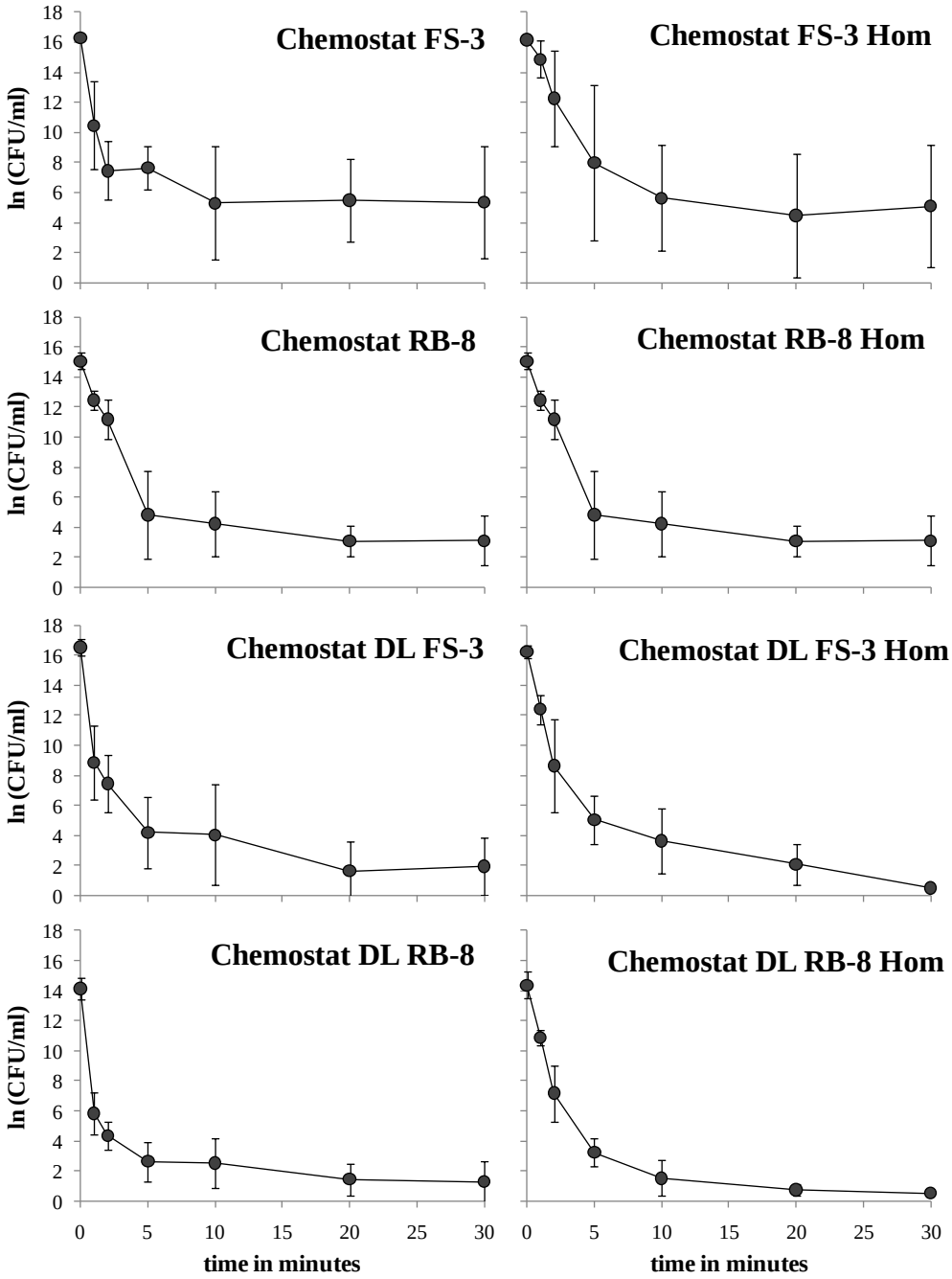

Figure 6.4 Concentration x time (Ct) curves for chemostat samples treated with chlorine dioxide. Bars and whiskers represent one standard deviation; n ≥2, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

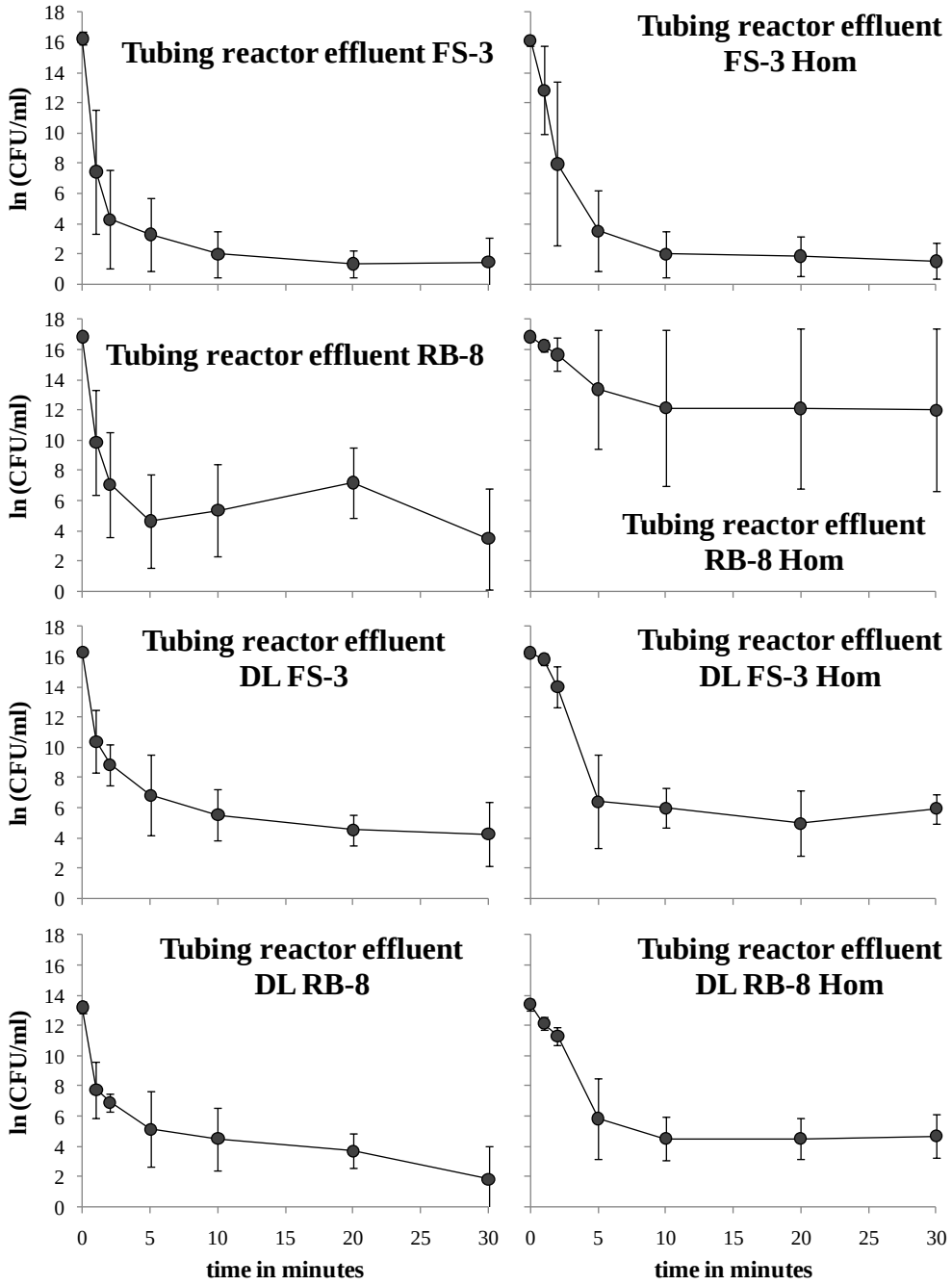

Figure 6.5 Concentration x time (Ct) curves for tubing reactor effluent samples treated with chlorine dioxide. Bars and whiskers represent one standard deviation; n ≥2, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

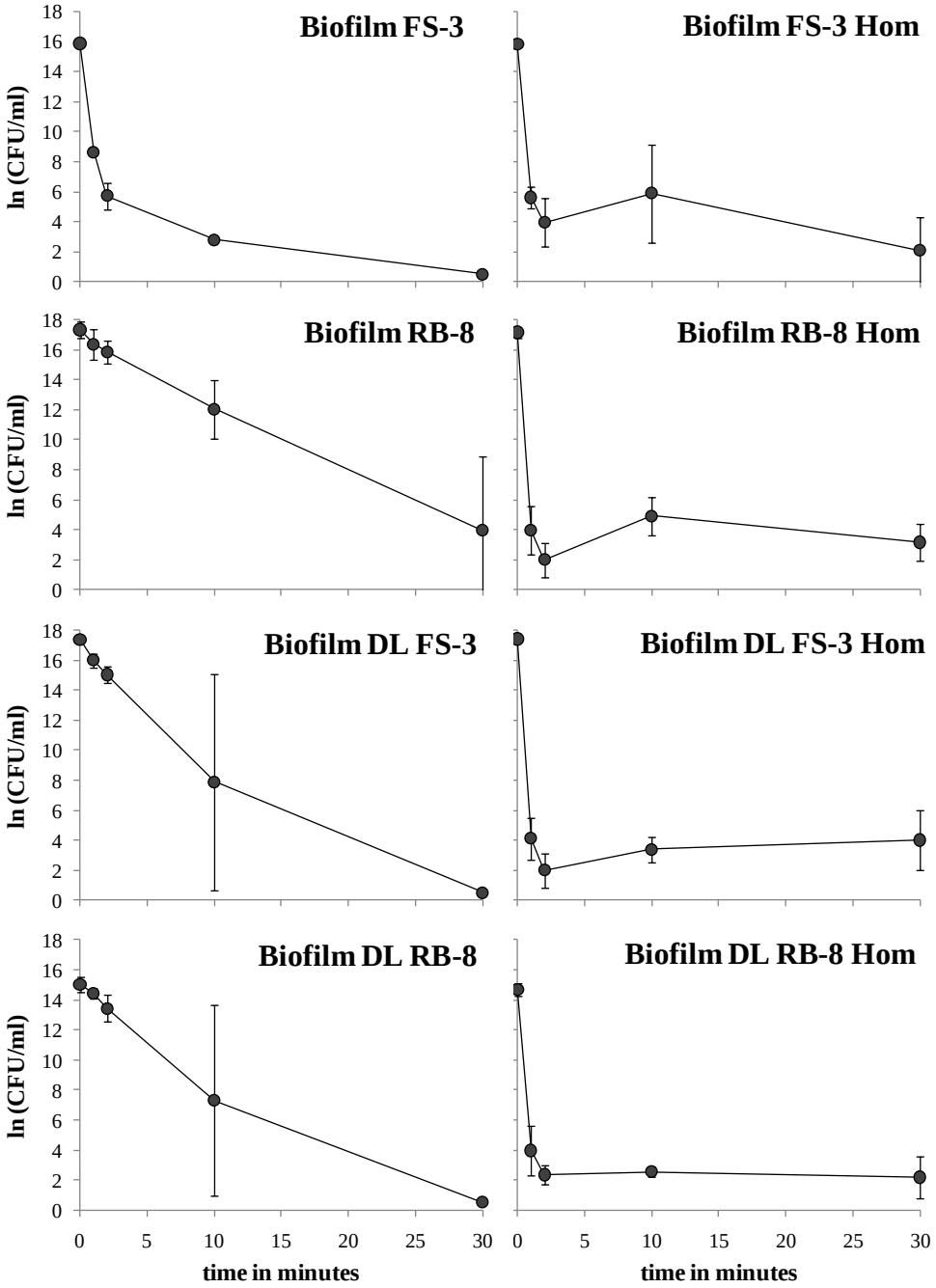

Figure 6.6 Concentration x time (Ct) curves for biofilm samples treated with chlorine dioxide. Bars and whiskers represent one standard deviation; n ≥2, FS-3 = *Burkholderia cepacia*, RB-8 = *Pseudomonas aeruginosa*, DL = dual (species in co-culture)

Table 6.2 Subjective sub-lethal doses of chlorine dioxide chosen for CT curves. FS-3 = *B. cepacia*; RB-8 = *P. aeruginosa*, DL = dual (grown in co-culture).

	species	unhomogenized	homogenized
Chemostat	FS-3	0.50	0.25
	RB-8	0.50	0.25
	DL FS-3	0.75	0.50
	DL RB-8	0.75	0.50
Tubing reactor effluent	FS-3	0.50	0.25
	RB-8	0.50	0.25
	DL FS-3	0.50	0.25
	DL RB-8	0.50	0.25
Biofilm	FS-3	5.00	1.50
	RB-8	5.00	2.50
	DL FS-3	10.00	2.50
	DL RB-8	10.00	2.50