Disinfectant Susceptibility of Mycobacterium avium

by

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Abstract

Mycobacterium avium, an opportunistic human pathogen, infects between 25 and 50% of advanced-stage acquired immuno-deficiency syndrome (AIDS) patients in the United States. M. avium has been isolated from many environmental sources including: natural waters, soils, and aerosols. M. avium has also been recovered from within municipal and hospital drinking water systems. Rhesus macaques (Macaca mulatta) infected with the simian HIV analog, SIV, have been shown to acquire M. avium infections from potable water.

Reduced-aggregate fractions (cell suspensions free of large aggregates) of Mycobacterium avium were exposed to chlorine, monochloramine, chlorine dioxide, and ozone and kinetics of disinfection measured. Chlorine disinfection kinetics was also measured in M. avium cultures grown in biofilms.

M. avium exhibited a high resistance to chlorine compared to E. coli. M. avium CT99.9% (disinfectant concentration × time to 3 logs cell inactivation) values were between 571- and 2318 -times those of E. coli. Clinical isolates of M. avium showed a 0.24 and 2.5-fold increase in resistance to chlorine compared to their pulsed-field-gel-electrophoresis- (PFGE) matched environmental isolates.

M. avium strains exhibited a mixed response to exposure to monochloramine. The CT99.9% values of three strains (2 clinical, 1 environmental) were between 6.3- and 23.5- times that of E. coli. Two strains (1 clinical, 1 environmental) exhibited CT99.9% values approximately the same as E. coli, a difference from all the other disinfectants which were much less effective on M. avium than on E. coli.

M. avium strains exhibited a high resistance to chlorine dioxide when compared to E. coli. M. avium CT99.9% values of between 133- and 706- times higher that that of E. coli. In the paired isolates tested, the clinical isolate was 5.3 times more resistant than the matched environmental isolate.

M. avium exhibited a high resistance to ozone when compared to E. coli. M. avium strains exhibited a CT99.9% value of between 52 and 90 times higher that that of E. coli. In the paired isolates tested, the clinical isolate was nearly identical as judged by CT99.9% values. M. avium strain 5002 exhibited an unusual disinfection kinetics curve. Disinfection rate increased by a non-logarithmic factor, indicating that inactivation efficiency was increasing over time.

M. avium strain 1060 showed between a 17% decrease to a 265% increase in CT99.9% value when grown as biofilms as opposed to suspension. Due to the large variance in biofilm density and and CT99.9% values, any conclusions based on these experiments should be considered tentative at best.

M. avium resistance to chlorine and chlorine dioxide approaches that of the protozoan cysts of Giardia muris and Entamoeba hystolytica. M. avium is much less resistant, relatively, to monochloramine possessing values similar to E. coli. Ozone resistance of M. avium is two orders of magnitude greater than E. coli and one order of magnitude of less than G. muris cysts.

A critical concentration threshold level for chlorine dioxide was found. That is, there was no linear relationship between concentration of chlorine dioxide and cell inactivation. Initial experiments using a range of concentrations from 0.1 ppm to 0.5 ppm chlorine dioxide showed a biphasic curve with the inflection point (indicating the critical concentration) between 0.3 and 0.4 ppm chlorine dioxide.
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Introduction

Clinical Relevance of Mycobacterium avium

Mycobacterium avium is an opportunistic human pathogen that infects between 25 and 50% of advanced-stage acquired immuno-deficiency syndrome (AIDS) patients in the United States (28). M. avium infection is found in advanced-stage AIDS patients of all human immuno-deficiency virus (HIV) transmission categories (35), likely indicating a common environmental source for infection, rather than transmission by person to person contact. Infection with M. avium HIV-infected patients is primarily disseminated, however, diarrhea is common and rare pulmonary infections are also observed (14). M. avium causes pulmonary infections in immuno-competent patients with pre-existing conditions such as pneumoconiosis (3). M. avium also causes cervical lymphadenitis in children (14).

M. avium has been isolated from many environmental sources including: natural waters (34), soils (38), and aerosols (36). M. avium has also been recovered from within municipal and hospital drinking water systems (17, 19, 21, 30, 35). M. avium isolates recovered from drinking water systems have been shown to share identical pulsed-field gel electrophoresis DNA fingerprints with isolates recovered from patients exposed to such sources. (35). Additionally, rhesus macaques (Macaca mulatta) infected with the simian HIV homolog, SIV, have been shown to acquire M. avium infections from potable water (26).

Factors Contributing to the Presence of M. avium in Drinking Water

One reason for the presence of M. avium in drinking water could be resistance to municipal water disinfection agents (e.g., chlorine). Previous studies examining mycobacteria other than M.
avium including Mycobacterium fortuitum and Mycobacterium chelonii (6), and Mycobacterium marinum (29) have shown that these bacteria are significantly more resistant to contact chemical disinfectants than are typical non-spore forming bacteria. \( CT_{99.9\%} \) values, that is, the concentration of disinfectant [C] in parts per million, times the time required to effect three logs of cell inactivation \( T_{99.9\%} \) for those mycobacteria tested were at least 900 times greater than for Escherichia coli (2, 6, 29).

The presence of M. avium in natural waters may serve as a consistent challenge to water disinfection systems, because M. avium may pass through a water treatment plant when disinfectant residuals have temporarily decreased due to increased chlorine demand in raw water. Mycobacteria have been shown to survive in water for extended periods of up to 12 months (9), are resistant to heavy metals (13), and they grow over a wide temperature range \( [i.e., 15.5^\circ C to 45^\circ C (14, 18)] \). Those characteristics may allow persistent colonization of distribution systems in spite of changing environmental conditions.

Mycobacteria have also been identified in biofilms in water systems (12). Biofilms are layers of bacteria and associated polymers, attached to a substrate. In the case of drinking water systems, biofilm substrates consist of the interior of the distribution system, including pipes and meters. Bacteria in biofilms have been shown to have between 150 and 3000 times the resistance to municipal water disinfectants than free-living or planktonic bacteria (25).

**Potable Water Disinfection Treatment**

Potable water is disinfected with one or a combination of four classes of chemicals: chlorine and hypochlorites (including molecular chlorine, Cl₂, the hypochlorite ion, OCl⁻, and hypochlorous acid, HOCl), chloramines (including monochloramine (ClNH₂), and dichloramine (Cl₂NH)), chlorine dioxide (ClO₂), and ozone (O₃). All are powerful oxidants, however they vary greatly in their
microbial disinfection efficiency. Figure 1 shows comparative disinfection efficiency for drinking water disinfectants per unit weight in solution. The values for E. coli exhibit a 4.5 log range depending on the disinfectant used.

<table>
<thead>
<tr>
<th>消毒剂</th>
<th>毒杀效率</th>
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<tr>
<td>O₃</td>
<td>ClO₂</td>
<td>HOCl</td>
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<tr>
<td>Ozone</td>
<td>Chlorine Dioxide</td>
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**Figure 1. Comparative efficiencies of disinfection agents. Data taken from (7)**

Of the chemicals listed, chlorine and hypochlorites are by far the most widely used disinfectants in the United States, both for water and wastewater treatment (37). Since 1970, the use of other disinfectants has increased. For example, ozone and chlorine dioxide are used as primary disinfectants, and chloramines as secondary or residual disinfectants. Primary disinfectants are used to initially disinfect raw water, it is typically added to water in a high concentration for a specific and short time. Secondary disinfectants are those that are added to regulate microbial contamination in water after the primary disinfection. Secondary disinfectants are added just before release of water into the distribution system, and are intended to regulate disinfection levels.

**Mechanism of Oxidative Cell Inactivation**

Oxidative cell inactivation seems to require contact with cellular components, based on the observation that cell and virus inactivation follow first order kinetics (37). In cells, the first site of oxidative damage in cells is the cell membrane. Chlorine has been shown to react with membrane-associated proteins (4). Ozone has the additional ability to react with glycolipids in the cell.
membrane (14). Chlorine or ozone exposure result in a loss of membrane integrity and lead to lysis of the cell (14). All the oxidative disinfectants have also been shown to alter nucleic acids, and in the case of DNA, to produce mutagenic effects (14, 37, 15). Recent research using myeloperoxidase-generated haloperoxides, have further identified the following targets of chlorine oxidants in the bacterial cell: the electron transport train, iron-sulfur centers, succinate oxidase, sulfhydryl containing enzymes, and unsaturated fatty acids (8, 23).

**Chlorine**

In aqueous solutions, chlorine exists in equilibrium between molecular chlorine, hypochlorous acid and the hypochlorite ion. This equilibrium is influenced by temperature and greatly influenced by pH. Below a pH of 1, molecular chlorine predominates. At pH values between 1 and 7.5, hypochlorous acid is the predominant species. Above pH 7.5, the hypochlorite ion is the dominant species. The pH-based equilibrium between the three species is relevant to disinfection studies, because the three vary in their disinfection efficiencies. Since molecular chlorine exists in such a small percentage (0.0063% @ pH6.5) at commonly encountered pH values (37), its disinfection impact is negligible at or near neutral pH. Hypochlorous acid is the most powerful disinfectant of the three reactive species. The hypochlorite ion is nearly as powerful an oxidant as is hypochlorous acid, however, since it is charged, diffusion through outer cell layers is much less likely, when compared to a non-charged disinfectant. Thus the hypochlorite ion has a lower disinfection efficiency than hypochlorous acid (7). Disinfection activity of hypochlorous acid is mediated in two ways: oxidation and chlorination. Oxidative stress is thought to be the main mechanism of cell inactivation, producing changes in both the cell membrane and in nucleic acids (33). Chlorination involves the transfer of a chlorine group from hypochlorous acid to an electrophillic center in a molecule (e.g. nitrogen containing groups).
Chlorine is not stable in aqueous solution. Factors serving to reduce the reactive life of chlorine include: organic matter, temperature, light, and aeration. Consequently, in order to maintain constant amount (residual) of chlorine in solution, loss must be measured and concentration maintained by addition of chlorine. In chlorine-based disinfection experiments, it is necessary to saturate solutions and glassware that come in contact with chlorine to render them ‘chlorine-demand-free’. This saturation prevents loss of reactive chlorine to organic and inorganic chlorine sinks that may be present in solution.

**Chloramine**

Inorganic chloramines (mono- and di-chloramines) are formed from the reaction of hypochlorous acid and ammonia in aqueous solution (37). Dichloramines are more effective disinfectants than are monochloramines, however they are not used in drinking water because they impart bad taste and odor to the water. Chloramines have less oxidative power than hypochlorous acid and are typically less effective a disinfectant (7). Some studies of monochloramines show it to be up to 100-times less effective as a disinfectant (16, 37). However, monochloramines are much more stable in solution than are hypochlorites (37). This has led to their use as a secondary disinfectant for municipal water distribution systems. Recently, LeChevallier et al. (25) have shown that monochloramines are more effective in disinfecting biofilms than are hypochlorites. This suggests that monochloramines may be able to penetrate into biofilms and hydrophobic substances more effectively than chlorine.

**Chlorine Dioxide**

Chlorine dioxide is a highly soluble gas that is, at high concentrations, explosive. This restricts the transport of the gas and thus it must be generated on site. Chlorine dioxide was mainly
used as a paper and flour-bleaching product until the 1970’s when it became cost-effective to use it in water and wastewater disinfection (37).

Chlorine dioxide has several advantages as a drinking water disinfectant. It is slightly more effective as a disinfectant than is hypochlorite due to its slightly higher oxidative potential (7). Chlorine dioxide is also effective as a disinfectant over a wider range of pH values than chlorine. This derives from the fact that chlorine dioxide has much less chemical interaction with water than chlorine.

Ozone

Ozone, an allotropic form of oxygen, has been used in Europe as a drinking water disinfectant for nearly 100 years (14). Recently, it has seen revived interest as a drinking water disinfectant in the United States, due to concerns over potential carcinogenic properties of chlorinated compounds in water (37). Ozone is an extremely powerful oxidant with an oxidation-reduction potential nearly 40% higher than that of hypochlorous acid (14). As a disinfectant, ozone is very effective (37). However, because of its reactivity, ozone is short-lived. Like chlorine dioxide, ozone must be generated on site, due to its short half-life. Since a persistent residual cannot be maintained for ozone, it is used solely as a primary disinfectant, with other disinfectants providing the post-treatment residual.

Special Characteristics of Mycobacterium avium Relevant to Disinfection

Members of the genus Mycobacterium have been shown to be relatively impermeable to solutes (3). The permeability of the cell walls of Mycobacterium avium was on the order of 3 orders of magnitude less than E. coli, and 1 order of magnitude less than Pseudomonas aeruginosa. Low
mycobacterial permeability was found for either hydrophobic or hydrophilic molecules (3). Since contact of chemical disinfectants with critical cell components is necessary for cell inactivation, the cell wall of mycobacteria may provide a significant diffusion barrier to effective contact. Hydrophobicity of the lipid-rich membrane in mycobacteria may also act to prevent disinfectant access to the cell.

*M. avium* is an acid-fast pleomorphic rod with a generation time of 12 – 24 hours. Long generation times may permit the bacterium to induce mechanisms to repair cell damage. *M. avium* has been found to express a dehalogenation enzyme when under thermal stress (27). Additionally, pathogenic mycobacteria like *M. avium* that are intracellular pathogens, may be resistant to hypochlorous acid produced by myeloperoxidase.

Mycobacteria typically form large cell aggregates (e.g. >25 cells / aggregates) in culture (20, 32). Large aggregates may provide an additional permeability barrier to chemical disinfectant contact (24). This may also be evidenced by the increased pathogenisity of *Mycobacterium bovis* when in aggregates as opposed to dispersed (5). Aggregation also prevents diffusion of nutrients, including oxygen, to cells in the center of the aggregate. This may induce a cell stress response due to decreased oxygen tension. Bacteria under cell stress have shown increased resistance to chemical disinfectants (24).

Aggregates also present a problem in enumeration of viable cells. For example colonies can be generated by an aggregate that consists of many viable cells. Thus if an aggregate is plated, colony counts may not can under represent the number of viable cells. Spreading can disperse aggregates (39). Glass spreading rods can generate shear forces sufficient to disperse aggregates. Because spreading may not be uniform, mycobacterial colony counts can vary.
Hypothesis

I propose to test the following hypotheses:

- *M. avium* cells will be relatively resistant to chlorine when compared to *E. coli*.
- *M. avium* cells will be relatively resistant to monochloramine when compared to *E. coli*.
- *M. avium* cells will be relatively resistant to chlorine dioxide when compared to *E. coli*.
- *M. avium* cells will be relatively resistant to ozone when compared to *E. coli*.
- *M. avium* cells in biofilms will have increased resistance to chlorine compared to cells in suspension.
Objectives

1. Measure the susceptibility and disinfection kinetics for *M. avium* exposed to chlorine.
2. Measure the susceptibility and disinfection kinetics for *M. avium* exposed to monochloramine.
3. Measure the susceptibility and disinfection kinetics for *M. avium* exposed to chlorine dioxide.
4. Measure the susceptibility and disinfection kinetics for *M. avium* exposed to ozone.
5. Measure the susceptibility for *M. avium* biofilms when exposed to chlorine.
6. Use *E. coli* as a benchmark to ensure disinfection conditions and results correspond to published data.
Materials and Methods

Bacterial strains.

*Mycobacterium smegmatis* strain VT307 and *Escherichia coli* strain C were obtained from the Virginia Tech culture collection. was used to assess the effect of prior, low level chlorine exposure on survival upon subsequent chloride challenge. *M. avium* strains 1508 and 5502 were isolated from hospital water systems (35) and strains 1060 and 5002 were isolated from *M. avium* infected AIDS patients (35). *M. avium* strains 1508 and 1060 shared the same pulsed field gel electrophoresis (PFGE) restriction fragment pattern and strains 5002 and 5502 identical, yet distinct, PFGE restriction fragment pattern (35).

Growth of Strains.

Cells of *M. avium* were inoculated into 10ml of Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, MD) containing 10% (v/v) oleic acid-albumen enrichment (OAA) (35) and 0.5 % (v/v) glycerol. Cultures were grown in glass screw-capped tubes (150 mm x 16mm) and were incubated at 37°C for 7 days on a rotator at 6 RPM. *E. coli* C was grown in Nutrient Broth (Difco Laboratories, Detroit, MI) in a water bath at 37°C with shaking at 55 RPM for 24 hours. Cultures were then washed three times by centrifugation (8000 x g for 15 min at 20°C) and resuspended in chlorine-demand-free phosphate buffer (CD FPB) (1).
**Preparation of the Reduced-Aggregate Fraction (RAF)**

To examine the disinfection susceptibility of *M. avium*, a cell suspension consisting of predominately single cells was required to reduce experimental error in counting viable cells in clumps and to ensure equal exposure of all cells to disinfectant. Centrifugation was used to separate small cells and aggregates (i.e., 5-25 cells) from larger aggregates (i.e., >25 cells). Washed cultures of *M. avium* and *E. coli* C were centrifuged at 1300 x g for 5 min. at 10 - 20°C and the supernatant collected. That supernatant suspension was called a reduced-aggregate fraction (RAF)

**Characterization of the RAF**

The RAF was examined by phase contrast microscopy using a Petroff-Hauser bacteria counting chamber (Hauser Scientific. Blue Bell, PA). The number of single cells (1 to 5 cells), small aggregates (5 to 25 cells) and large aggregates (>25 cells) in 20 squares (0.02 x 0.02mm) was counted and the percentage of each fraction calculated, based on total number of cells in all aggregates.

**Measurement of Chlorine, Chloramine, Chlorine Dioxide and Ozone Concentration**

Chlorine, chlorine dioxide, and ozone residuals were measured using N,N-diethyl-p phenylenediamine (DPD) free-chlorine measurement kits (Hach Co., Loveland, CO). Monochloramine was measured using the DPD total chlorine test (Hach Co., Loveland, CO). Dichloramine and nitrogen trichloride were measured by methods described in Standard Methods for the Examination of Water and Wastewater (1). Absorbance was measured in a Spectrophotometer Junior (Coleman Instruments, Maywood, IL) at 515 nm. A standard curve was constructed using low range chlorine standards (Hach Co., Loveland CO). Ozone was additionally
measured by the indigo trisulfonate method (Hach Co., Loveland, CO).

**Disinfection of M. avium RAF with Chlorine**

Two hundred ml of CDFPB was inoculated with \(6 \times 10^5\) colony-forming units (CFU) from the RAF. Sodium hypochlorite (Aldrich Chemical Co. Milwaukee WI.) was added to a final concentration of 1.0 ppm. The disinfection assay was carried out at 23°C, under reduced light levels and with gentle stirring. When sample was removed for microbiological analysis, 0.1 % \(\text{sodium thiosulfate}\) equal to 10% of sample volume was added to suspensions to neutralize free chlorine. Chlorine-demand-free solutions and glassware were prepared using methods described in Standard Methods for the Examination of Water and Wastewater (1).

Samples for measurement of chlorine, and for CFU were collected before, and 0.5, 10, 20, 40 and 60 min after addition of chlorine to the cell suspensions.

**Disinfection of E. coli with Chlorine**

Experiments with E. coli were performed similarly to those for M. avium. Two hundred ml of CDFPB was inoculated with \(6 \times 10^5\) colony-forming units. Chlorine was added to a final concentration of 0.1 ppm (10 fold lower). Sample times were changed to before addition of chlorine, and 10-sec, 30-sec, 60-sec, and 300 sec after addition of chlorine.
Disinfection of M. avium RAF with Monochloramine

Two hundred ml of CDFPB was inoculated with ≈ 6 x 10⁵ CFU from the RAF. Sodium hypochlorite (Aldrich Chemical Co. Milwaukee WI.) was added to ammonium chloride (Sigma Chemical, St. Louis MO.) at a 3:1 ratio to generate monochloramine. The resulting solution was then tested for presence of dichloramine and chlorine using methods described in Standard Methods for the Examination of Water and Wastewater (1). Chloramine was added to a final concentration of 10 or 1 ppm. The disinfection assay was carried out at 23°C, under reduced light levels and with gentle stirring. When sample was removed for microbiological analysis, 0.1 % w/v sodium thiosulfate equal to 10% of sample volume was added to suspensions to neutralize monochloramine. Chlorine-demand-free solutions and glassware were prepared using methods described in Standard Methods for the Examination of Water and Wastewater (1).

Samples for measurement of monochloramine, and for CFU were collected before, and 0.5, 10, 20, 40 and 60 min after addition of monochloramine to the cell suspension.

Disinfection of E. coli with Monochloramine

Experiments with E. coli were performed as described for M. avium Two hundred ml of CDFPB was inoculated with ≈ 6 x 10⁵ colony-forming units. Monochloramine was added to a final concentration of 1 ppm. Sample times remained the same as the M. avium RAF experiments.

Disinfection of M. avium RAF with Chlorine Dioxide

Two hundred ml of CDFPB was inoculated with ≈ 6 x 10⁵ CFU from the RAF. Chlorine dioxide was generated by the acidification of a sodium hypochlorite solution as described
in Standard Methods for the Examination of Water and Wastewater (1). Chlorine dioxide was added to a final concentration of 1 or 0.1 ppm. The disinfection assay was carried out at 23°C, under reduced light levels and with gentle stirring. When sample was removed for microbiological analysis, 0.1 % \( \text{w/v} \), sodium thiosulfate equal to 10% of sample volume was added to suspensions to neutralize chlorine dioxide. Chlorine-demand-free solutions and glassware were prepared using methods described in Standard Methods for the Examination of Water and Wastewater (1). Samples for measurement of chlorine dioxide, and for CFU were collected before, and 0.5, 5, 10, 20 and 40 min after addition of chlorine dioxide to the cell suspension.

**Disinfection of E. coli with Chlorine Dioxide**

Experiments with *E. coli* were performed similarly to those for *M. avium*. Two hundred ml of CDFPB was inoculated with \( \approx 6 \times 10^5 \) colony-forming units. Chlorine dioxide was added to a final concentration of 0.1 ppm. Sample times were changed to before addition of chlorine, and 10, 30, 60, and 300 sec after the addition of chlorine dioxide.

**Disinfection of M. avium RAF with Ozone**

Ozone was generated by an M-1500 corona discharge ozonator (Clearwater Tech. Inc., San Luis Obispo, CA.) fed with commercial grade oxygen. Ozone-demand-free glassware was prepared by soaking glassware in a solution of >2ppm ozone for 1 hour. Glassware was subsequently air dried and sterilized. Ozone-demand-free phosphate buffer (ODFPB) was made from phosphate buffer as described in Standard Methods for the Examination of Water and Wastewater (1). However, the buffer was rendered ozone-demand-free by bubbling ozone through it, keeping a ozone residual >2ppm for 1 hour. The ODFPB was then boiled for 30 min to expel the ozone. That buffer was then sterilized and kept in sealed, ozone-demand free bottles until use. ODFPB
was used within 2 weeks after creation. A stock solution of ozone was generated by continuously
bubbling ozone through deionized water for 15 min. The solution was then diluted for use in
disinfection experiments.

Two hundred ml of ODFPB was inoculated with \( \approx 10^6 \) CFU from the RAF. Ozone was
added from the stock solution to a final concentration of 0.1 ppm. The disinfection assay was
carried out at 23°C, under reduced light levels. When sample was removed for microbiological
analysis, 0.1 % w/v sodium thiosulfate equal to 10% of sample volume was added to suspensions to
neutralize ozone. Samples for microbiological analysis were removed before and 10, 30, 60, and 600
sec after addition of the ozone. Samples for measuring ozone levels were taken at 80, 300, and 600
sec after addition of ozone.

**Disinfection of E. coli with Ozone**

Experiments with E. coli were performed as those for M. avium. One hundred ml of
ODFPB was inoculated with \( \approx 6 \times 10^5 \) colony-forming units. Ozone was added to a final
concentration of 0.1 ppm, and microbiological sample times were changed to before addition of
ozone, and 10, 30, 60, and 300 sec after the addition of ozone. Ozone sampling times were changed
to 60 and 300 sec after the addition of ozone to the cell suspension.

**Measurement of Ozone**

Measurement of ozone was determined by the N,N-diethyl-p-phenylenediamine (DPD)
colorimetric method and the indigo trisulfonate colorimetric method. (Hach Co. Loveland CO).
Initially, the indigo trisulfonate method was used. However, when the DPD method was found to
be as accurate, and more easily quantifiable, all further tests utilized it.
**Enumeration of M. avium**

Surviving *M. avium* CFU were enumerated by spreading 0.1 ml of a diluted in or undiluted suspension on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville MD) containing 10% (v/v) OAA enrichment and 0.5% (v/v) glycerol (M7H10E). Dilutions were made in sterile, 0.1 M phosphate buffer pH 7.0. Middlebrook 7H10 agar medium containing 0.5% (v/v) Tween 80 (Sigma Chemical, St. Louis MO) and 0.5% (v/v) glycerol (M7H10Tw) was also employed in some experiments as an additional plating media to examine the effect of chlorine-induced stress. Plates were incubated at 37°C for 7 days and colonies counted. Each dilution and time point was plated in triplicate.

**Enumeration of E. coli.**

Surviving *E. coli* CFUs were enumerated by spreading 0.1 ml of a diluted (sterile, 0.1 M phosphate buffer pH 7.0) or undiluted suspension on Plate Count Agar (Difco Laboratories, Detroit MI) and counted after 24 hours incubation at 37°C.

**Growth of M. avium in Biofilms.**

Biofilms of *M. avium* strain 1060 were grown on 23 mm x 47 mm pieces of sterile polyvinyl chloride (PVC aka. PVC wafers) suspended in 450 ml Middlebrook 7H9 broth media (BBL Microbiology Systems, Cockeysville, MD) containing 10% (v/v) OAA enrichment and 0.5% (v/v) glycerol in a 500 ml screw-capped Erlenmeyer flask. Biofilm cultures were incubated at 37°C, shaken 55 reciprocations per min for 10 days.
Biofilm Harvesting.

PVC wafers were removed from the broth aseptically and were soaked for 1 min in CDFPB to wash off growth media and remove loosely bound cells. Cells in the biofilm were scraped off one side of the PVC wafer with a sterile fluoroarbon policeman (Fisher Scientific, Hampton NH) and suspended in 20ml CDFPB. That suspension served as a pre-chlorine control. Following exposure of the PVC wafer to chlorine, the cells on the opposite side were harvested as described here.

Biofilm Disinfection and Enumeration.

The once scraped PVC wafer was suspended in CDFPB and exposed to between 3 and 8.75 ppm chlorine residual for 60 min under reduced light levels. Cells were then harvested as described above. A dilution series of the treated and untreated cell suspensions (from PVC) were prepared and plated on M7H10E. Plates were incubated for 10 days at 37°C and colonies counted.

Statistical methods.

For the RAF disinfection studies, for chlorine, monochloramine, and chlorine dioxide, the data represents the average of a minimum of three replicates. For ozone RAF disinfection studies, data represents a minimum of 2 replicates. Statistical regressions based on the logarithm of the percent survival with time (in minutes) were calculated for each strain. Those regressions were subsequently used to calculate CT values for each strain. Ninety-five percent confidence intervals were computed using Microsoft™(Redmond, WA) Excel. SigmaPlot® (SPSS Inc. Chicago, IL) was used to graph the data.
Results

Chlorine, Chloramine, Chlorine Dioxide, and Ozone Susceptibility of E. coli

E. coli susceptibility to chlorine, chloramine, chlorine dioxide, and ozone was tested and CT_{99.9\%} values are given in Table 1. Susceptibility of E. coli was highly dependent on the disinfectant used, with CT_{99.9\%} values spanning 4.5 logs for the various disinfectants. The disinfectants showed the following disinfection efficiency order, from strongest to weakest: ozone, chlorine dioxide, chlorine, monochloramine.

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>CT_{99.9%} ± C.I. 95%</th>
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<tr>
<td>Chlorine</td>
<td>0.088 ± 0.003</td>
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<tr>
<td>Monochloramine</td>
<td>72.7 ± 28.1</td>
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<tr>
<td>Chlorine Dioxide</td>
<td>0.015 ± 0.003</td>
</tr>
<tr>
<td>Ozone</td>
<td>0.00192 ± 0.002</td>
</tr>
</tbody>
</table>

Table 1: Experimental CT_{99.9\%} values for E. coli. Experimental conditions: pH 7.0, 23°C.

Chlorine Susceptibility of M. avium

Reduced-aggregate fractions of M. avium cultures were significantly more resistant to free chlorine than were single cell suspensions of E. coli. CT_{99.9\%} values for E. coli and the five M. avium strains are tabulated in Table 2. The M. avium strains exhibited CT_{99.9\%} values 571- to 2318- times greater than that for E. coli C (Table 2). M. avium strains exhibited the following chlorine-resistance order, from most resistant to most susceptible (average percent survival at 60 min): M. avium strain 1060 (12 %), M. avium strain 5002 (35 % @ 40 min), M. avium strain 1508 (6.15 %), M. avium strain A5 (4.9 %), M. avium strain 5502 (1.2 % @ 40 min). The range of chlorine susceptibility represented
by the \textit{M. avium} strains is shown in Figures 2 and 3. Note log scale on Figure 2 and linear scale on Figure 3. The clinical isolates of \textit{M. avium} (i.e., 1060, 5002) were more resistant to chlorine than their PFGE-matched, water isolates (i.e., 1508, 5502, respectively). \textit{M. avium} strain 1060 had a CT$_{99.9\%}$ value that was 24\% higher than that for \textit{M. avium} strain 1508 and \textit{M. avium} strain 5002 has a CT$_{99.9\%}$ that was 250\% higher than that for \textit{M. avium} strain 5502.
Table 2: $CT_{99.9\%}$ Values for RAF of M. avium strains and E. coli exposed to Chlorine, Monochloramine, Chlorine Dioxide, and Ozone. Experimental conditions: pH 7.0, 23°C

<table>
<thead>
<tr>
<th>Organism</th>
<th>Chlorine $CT_{99.9%}$ ± C.I. 95%</th>
<th>Monochloramine $CT_{99.9%}$ ± C.I. 95%</th>
<th>Chlorine Dioxide $CT_{99.9%}$ ± C.I. 95%</th>
<th>Ozone $CT_{99.9%}$ ± C.I. 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. avium strain A5</td>
<td>106 ± 8.6</td>
<td>96.5 ± 8.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. avium strain 1060</td>
<td>204 ± 36</td>
<td>458 ± 152</td>
<td>7.9 ± 2.8</td>
<td>0.173 ± 0.144</td>
</tr>
<tr>
<td>M. avium strain 1508</td>
<td>164 ± 28</td>
<td>548 ± 62.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. avium strain 5502</td>
<td>50.3 ± 9.9</td>
<td>91.0 ± 34.1</td>
<td>2.0 ± 0.13</td>
<td>0.102 ± 0.005</td>
</tr>
<tr>
<td>M. avium strain 5002</td>
<td>126 ± 26.6</td>
<td>1710 ± 814</td>
<td>10.6 ± 1.7</td>
<td>0.117 ± 0.009</td>
</tr>
<tr>
<td>E. coli C</td>
<td>0.088 ± 0.003</td>
<td>72.7 ± 28.1</td>
<td>0.015 ± 0.003</td>
<td>0.00192 ± 0.0002</td>
</tr>
</tbody>
</table>
Figure 2: M. avium disinfection kinetics at 1.0 ± 0.2 ppm chlorine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Figure 3: *M. avium* disinfection kinetics at 1.0 ± 0.2 ppm chlorine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
**Monochloramine Susceptibility of M. avium**

The *M. avium* strains exhibited a wide range of susceptibility to monochloramine. Figures 4, 5, 6, and 7 show disinfection kinetics for *M. avium* exposed to monochloramine. *M. avium* strains exhibited the following monochloramine resistance order, from most resistant to most susceptible: 5002, 1508, 1060, A5, and 5502. The most resistant *M. avium* strains: 1060, 1508, 5002 exhibited CT<sub>99.9%</sub> values 6.3, 7.5, and 23.5 times higher, respectively, than *E. coli* C (Table 2). However, two *M. avium* strains, A5 and 5502, exhibited chloramine CT<sub>99.9%</sub> values similar to that of *E. coli* C.

Cells and RAFs were initially exposed to 10 ppm monochloramine for up to 60 min. This exposure was adequate for *M. avium* strains 1060, 1508, and 5002 (Figures 4 and 6). However, when *M. avium* strains A5 and 5502 were initially tested, their CT<sub>99.9%</sub> values fell below the detection limit of the experiments. These strains were then exposed to 1 ppm monochloramine for 60 min (Figures 5 and 7)
Figure 4: M. avium strains 1508, 1060, and 5002 disinfection kinetics at 10 ± 1 ppm monochloramine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Figure 5: M. avium strains 5502 and A5 disinfection kinetics at 1 ± 0.1 ppm monochloramine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Figure 6: M. avium strains 1508, 1060, and 5002 disinfection kinetics at 10 ± 1 ppm monochloramine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Figure 7: M. avium strains 5502 and A5 disinfection kinetics at 1±0.1 ppm monochloramine. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Chlorine Dioxide Susceptibility of M. avium

M. avium exhibited significant resistance to disinfection by chlorine dioxide when compared to E. coli (Table 2). Figures 8 and 9 show disinfection kinetics for M. avium when exposed to chlorine dioxide on logarithmic and linear scales respectively. M. avium strains 1060, 5502, and 5002 possessed CT$_{99.9\%}$ values 527-, 133-, and 706- times higher than E. coli C, respectively (Figures 4, 5). M. avium strain 5002, a clinical isolate, was 5.3-times more resistant than its PFGE-matched environmental isolate, strain 5502. The increased resistance of the clinical strain over its PFGE-fragment identical environment pair parallels the relationship between CT$_{99.9\%}$ for chlorine and monochloramine resistance. In contrast, the clinical strain M. avium strain 5002 had an increased relative CT$_{99.9\%}$ value 5.3 times greater over its environmental pair when compared to chorine and monochloramine.

M. avium CT$_{99.9\%}$ values are much lower for chlorine dioxide compared to chorine. M. avium CT$_{99.9\%}$ values are on the order of 1.1 to 1.4 logs less for chlorine dioxide than chlorine (Figure 4). This agrees with the observation that chlorine dioxide is a slightly better disinfectant than chlorine at equal concentrations (7).
Figure 8: M. avium strains 1060, 5502, and 5002 disinfection kinetics at 0.1 ± 0.02 ppm chlorine dioxide. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Figure 9: M. avium strains 1060, 5502, and 5002 disinfection kinetics at 0.1 ± 0.02 ppm chlorine dioxide. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Ozone Susceptibility of M. avium

M. avium strains demonstrated a CT$_{99.9\%}$ values 53- to 90- times that of E. coli when exposed to 0.1 ppm ozone (Table 2). M. avium strains 1060, 5502, and 5002 exhibited nearly the same CT$_{99.9\%}$ values for 0.1 ppm ozone. Unlike chlorine, chlorine dioxide, and monochloramine, the clinical isolate M. avium strain 5002 showed no measurable increased resistance over its environmental PFGE paired isolate M. avium strain 5502 when exposed to ozone. The three strains tested showed overlap of at least one other isolate’s CT$_{99.9\%}$ within their 95% confidence intervals (Table 2).

Disinfection kinetics are shown for M. avium strain 5002 in Figure 10. The plot of percent survival on logarithmic axis vs. time does not give the expected straight line. Rather, susceptibility is biphasic. Beyond 30 sec, the cell inactivation increases greatly over the rate of cell inactivation in the first 30 sec.
Figure 10: M. avium strain 5002 disinfection kinetics at 0.1 ± 0.02 ppm ozone. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C
Chlorine Susceptibility of M. avium in Biofilms

*M. avium* strain 1060 biofilms were exposed to chlorine residuals between 3 and 8.75 ppm. *M. avium* strain 1060, when grown on PVC wafers, exhibited a large variation in CT values and cfu density (Table 3). When *M. avium* strain 1060 grown in biofilm was compared to *M. avium* strain 1060 grown in liquid culture, it exhibited between a 4% and 230% increase in CT value.

As a verification of efficiency of recovery, a PVC wafer was aseptically slid over the surface of an M7H10EG plate to enumerate remaining cells. Approximately $10^3$ cfu were observed on the plate, corresponding to 0.3% of the total cfu recovery. Thus, the cells remaining on the wafer represent a statistically insignificant portion of the biofilm cells.

<table>
<thead>
<tr>
<th>Biofilm #</th>
<th>CT 99.9%</th>
<th>Biofilm Density (cfu/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>421</td>
<td>1.5 x 10⁶</td>
</tr>
<tr>
<td>2</td>
<td>675</td>
<td>3.8 x 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>212</td>
<td>2.6 x 10⁴</td>
</tr>
<tr>
<td>Average</td>
<td>436</td>
<td>5.0 x 10⁴</td>
</tr>
</tbody>
</table>
Effect of Cell Stress on M. avium

M. avium was plated on two different media M7H10Tw and M7H10E. M7H10Tw contained Tween-80, a detergent making the medium more stressful to the cells than M7H10E. The effect of cell stress on recovery of M. avium was to be expressed by lower recovery of CFU on the Tween 80-containing media. However, recovery of CFU on M7H10Tw was infrequent and significantly lower for some strains than others. Therefore, no conclusions could be confidently drawn.
Discussion

Maintenance of Disinfectant Concentration

Chlorine, monochloramine, chlorine dioxide, and ozone, when in dilute solutions, tend to decrease in concentration over time. Cell number was kept low to minimize loss of disinfectant to bacterial binding as well as to use numbers similar to those in drinking water. While every effort was made to maintain a constant residual, a decrease in disinfection concentration did occur in every experiment. When the concentration of disinfectant dropped below 20% of its original concentration during microbiological sample times, the experiment was not used for disinfection data.

Reproducibility of M. avium Disinfection Curves

Reproducibility of M. avium disinfection curves varied with the disinfectant and strain used (Figure 12, Table 2). Reproducibility of survival counts could be affected by several factors, including the high resistance of M. avium aggregation and other enumeration problems, and the efficiency of the disinfectant. The high resistance of M. avium to disinfectants when compared to E. coli, suggests that for slight differences in cell properties to influence survival of the cells. For example, if cell wall composition is the main determinant of M. avium disinfectant resistance, then slight differences in cell wall composition might result in large differences in disinfection susceptibility. Aggregation of cells may prevent contact of disinfectant with cells internal to the aggregate. Large degrees of aggregation have been documented in mycobacteria (39). This phenomenon may allow cells within the aggregate to survive despite cytotoxic levels of disinfectant in solution outside of the aggregate. Aggregation of cells can also prevent accurate enumeration of
cells. For example an aggregate of 25 cells may form a single CFU, or upon plating may be broken up producing up to 25 CFU. Previous studies (39) have shown a large enrichment of CFU (up to 15 times) when petri plates used for enumeration were vigorously spread as opposed to gently spread. Efficiency of the disinfectant may also play a role in effecting the reproducibility of disinfection counts. If a disinfectant is more efficient in inactivating cells, the shorter contact time needed may compress the percent cell survival counts.

*M. avium* as a species, contains a large degree of physiological diversity. Differences have been found in antimicrobial sensitivity in *M. avium* isolates from the same patient (40). Differences in disinfectant susceptibility between strains of *M. avium* as seen here can be expected as a result of this diversity.

**Correlation of Results to Published Values**

E. *coli* C was used to determine if the CT values for chlorine, monochloramine, chlorine dioxide, and ozone determined in these experiments agreed with other published values. In Table 4, CT$_{99.9\%}$ values complied from published data, and experimental CT$_{99.9\%}$ values are shown. For all disinfectants, the experimental value is either within the published range of values or is within 40% of the published value. For CT$_{99.9\%}$ values, which can range from a difference of 15 percent to several logs for the same organism for different studies (2), agreement to within 40% of the published value can be considered a close match. The small discrepancies between the published and experimental values may be accounted for by different disinfection conditions, or inherent susceptibility differences for different strains of *E. coli*.
Table 4: Comparison of experimental and published CT values for E. coli. Experimental conditions: pH 7.0, 23°C. Differences in conditions for published data are noted. Data taken from references 37, 16, and 14.

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Published CT (_{99.9%})</th>
<th>Experimental CT (_{99.9%})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine</td>
<td>(\approx 0.1 \text{ @ pH 6 @ 5°C})</td>
<td>0.088 ± 0.003</td>
</tr>
<tr>
<td>Monochloramine</td>
<td>64 - 180 @ pH 8-9 @ 20°C</td>
<td>72.7 ± 28.1</td>
</tr>
<tr>
<td>Chlorine Dioxide</td>
<td>(\approx 0.025)</td>
<td>0.015 ± 0.003</td>
</tr>
<tr>
<td>Ozone</td>
<td>0.002</td>
<td>0.00192 ± 0.002</td>
</tr>
</tbody>
</table>

**Chlorine susceptibility of M. avium**

M. avium is much more resistant to chlorine than is E. coli (Table 2, Figure 12). Factors that may form the physiologic basis for the high resistance of M. avium to chlorine include impermeability to free chlorine and the existence of resistance (or repair) mechanisms. M. avium is highly impermeable due to the thick cell wall with a high percentage of lipids (3). The impermeability may prevent access of polar disinfectant to vital cell components. By nature of their slow growth relative to other bacteria, a resistance (or repair) mechanism if present may have sufficient time to operate and thus allow mycobacteria to survive. This repair mechanism may act synergistically with cell wall impermeability to allow the cell sufficient time to utilize any repair mechanisms. Possible resistance mechanisms include: (1) impermeability to disinfectants, (2) expression of a dehalogenation activity, and (3) possession of proteins whose activity or function is resistant to oxidation and halogenation. Impermeability to disinfectants would allow critical cellular targets to avoid damage by disinfectants. Chlorine can act as a disinfectant in two ways, oxidation and chlorination (37). If a vital cell component is chlorinated, expression of a dehalogenation activity may allow that component to be
repaired. Also, possession of critical cell components that are resistant to oxidation and halogenation would allow these components to remain active in the presence of chlorine.

**Monochloramine Susceptibility of M. avium**

Monochloramine is recognized as a less powerful disinfectant than chlorine, chlorine dioxide and ozone. Surprisingly, monochloramine was relatively more effective in inactivating *M. avium* than the other disinfectants, relative to *E. coli* (Table 2, Figure 12). The other disinfectants show a CT$_{99.9\%}$ value at least 53 times that of *E. coli* whereas monochloramine values range only from 23.5 times to nearly equal to that of *E. coli*.

Monochloramine’s relative efficacy in disinfecting *M. avium* could be due to its ability to penetrate cell layers more effectively to reach vital cell components. LeChevallier et al. (25) have shown that monochloramine is nearly as effective as chlorine in disinfecting biofilms, suggesting that monochloramine was better at penetrating hydrophobic biofilm material to reach vital cell components. This same mechanism may occur in *M. avium*. Resistance of *M. avium* to other disinfectants may largely be due to reduced diffusion of the polar disinfectants through the *M. avium* cell wall.

The PFGE-paired isolates, 1060 and 1508, exhibited similar relative monochloramine CT$_{99.9\%}$ values when compared to chlorine and CT$_{99.9\%}$ values. The value for the clinical strain (1060) was 24% and 20% higher than the environmental isolate (1508) for chlorine and monochloramine respectively. In contrast, the other paired isolates, the clinical isolate (5002) had an 18-fold greater CT$_{99.9\%}$ value than that for the environmental isolate 5502. This could indicate that the clinical isolate 5002 was less permeable to monochloramine than was strain 5502.
Chlorine Dioxide Susceptibility of M. avium

M. avium \( CT_{99.9\%} \) values were much lower for chlorine dioxide compared to chlorine. M. avium \( CT_{99.9\%} \) values are on the order of 1.1 to 1.4 logs lower for chlorine dioxide than for chlorine (Figure 4). This agrees with the observation that chlorine dioxide is a slightly better disinfectant than chlorine at equal concentrations (7).

Ozone Susceptibility of M. avium

M. avium strain 5002 disinfection kinetics for ozone differed from disinfection kinetics observed with other disinfectants. Figure 10 illustrates disinfection kinetics for M. avium 5002 exposed to ozone. For ozone, a log - linear based graph did not give the expected straight line. Disinfection efficiency increased over time, resulting in faster inactivation as time progressed. This may indicate that there are multiple critical sites on the cell surface, or that multiple hits on a critical site are necessary for inactivation. This may also indicate that ozone increases the permeability of the cell wall thus opening critical sites for oxidative attack.

Differences in Susceptibility between Clinical and Environmental Isolates

Significant differences, as judged by 95% C.I. of the \( CT_{99.9\%} \) values (Table 2, Figure 12 ), were evident between both matched pairs (1060 clinical, 1508 environmental and 5002 clinical, 5502 environmental) for chlorine. Statistically significant differences were also observed for 5002 and 5502 for chloramine and chlorine dioxide. Significant differences were not observed for 5002 and 5502 when treated with ozone.
Differences between the clinical and environmental isolates may have a clinical or ecological basis. Resistance to free chlorine may be a virulence factor for *M. avium* because an intra cellular pathogen would need to be relatively resistant to killing by hypohalides produced by phagocytic cells during the respiratory burst. This may explain the increased resistance exhibited by the clinical isolates of *M. avium* which share the same DNA fingerprint pattern with the water isolates (Figure 12). Clinical isolates may also have been selected for chlorine resistance by passage through the distribution system. Only cells that persisted through the system, in the presence of chlorine, would survive to produce infection in patients.
Comparison of *M. avium* resistance to other Water Borne Pathogens

*M. avium* is significantly more resistant to chlorine, chlorine dioxide, and ozone than is *E. coli* as shown above. Table 5 shows CT\(_{99\%}\) (note 99% cell inactivation, or 2 logs) values of other resistant water based microorganisms. *M. avium* chlorine resistance approximates that of water based protozoa *Giardia muris* cysts and *Entamoeba hystolytica* cysts. *M. avium* monochloramine resistance approximates that of *E. coli* as shown above, and possesses a CT\(_{99\%}\) on the low range of Polio 1 virus and below that of *G. muris*. For chlorine dioxide, *M. avium* CT\(_{99\%}\) values were just below those of *G. muris* and were in the range of Polio 1 virus. For ozone, *M. avium* was significantly below *G. muris* and on the low range of Polio 1 virus. Protozoan cysts are known to be highly resistant to disinfectants (2). *M. avium* as its CT\(_{99\%}\) values approach those of *G. muris* cysts, can also be thought of as highly resistant.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Chlorine CT(_{99%})</th>
<th>Monochloramine</th>
<th>Chlorine Dioxide</th>
<th>Ozone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio 1</td>
<td>1.1 - 2.5</td>
<td>770 - 3740</td>
<td>0.2 - 6.7</td>
<td>0.1 - 0.2</td>
</tr>
<tr>
<td><em>Giardia muris</em> cysts</td>
<td>30 - 630</td>
<td>1400</td>
<td>7.2 - 18.5</td>
<td>1.8 - 2.0</td>
</tr>
<tr>
<td><em>Entamoeba hystolytica</em> cysts</td>
<td>50</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>33.3 - 136</td>
<td>60.6 - 1142</td>
<td>1.3 - 7.06</td>
<td>0.068 - 0.115</td>
</tr>
</tbody>
</table>
Chlorine Susceptibility of M. avium in Biofilms

Large variations in experimental biofilm density and in biofilm susceptibility prevent any firm conclusions to be drawn from the data (Table 3). Large increases in the resistance of cells in a biofilm when compared to cells in suspension, as seen in two of the three biofilm experiments, could be due to decreased permeability of the biofilm to chlorine, or physiological changes of the cell when in a biofilm. Biofilms could act as a barrier to diffusion of disinfectant to cells on the interior surface of the biofilm. Also when within a biofilm cells may experience reduced oxygen tension and reduced nutrient levels, factors that may produce a resistant stressed cell.

Chlorine Dioxide Concentration Based Threshold Effect

When first attempting chlorine dioxide disinfection experiments, a concentration of 1 ppm chlorine dioxide was used. That concentration proved to be too high for disinfection kinetics to be observed. However, approximate CT$_{99.9\%}$ values could be determined. After disinfection experiments at 0.1 ppm chlorine dioxide indicated a CT$_{99.9\%}$ value significantly greater than the approximate values for 1 ppm chlorine dioxide, a critical concentration threshold level for chlorine dioxide was hypothesized. That is a concentration level of chlorine dioxide above which cell inactivation greatly increases. Initial experiments using a range of concentrations from 0.1 ppm to 0.5 ppm chlorine dioxide show a biphasic curve, illustrated in figure 11, with the inflection point (indicating the critical concentration) between 0.3 and 0.4 ppm chlorine dioxide.

Cells possessing multiple sensitive sites for oxidative attack could explain a threshold effect by chlorine dioxide. Only a few sites might be critical to cell survival. When the concentration of disinfectant reaches a certain level, critical sites would be attacked faster than they can be repaired, and thus bulk
Figure 11: Surviving *M. avium* strain 5002 CFU after exposure to chlorine dioxide for 10 min at indicated concentration. All concentrations began with equal numbers of CFU from a RAF at time zero. Note the biphasic curve with inflection point between 0.3 and 0.4 ppm. Experimental conditions: pH 7.0 and 23°C.
cell survival decreases rapidly. Thus cell damage and repair maybe in equilibrium until the threshold concentration, when damage events begin to outpace repair events, leading to cell death.

This effect may also indicate that CT$_{99.9\%}$ values may be significantly different for experiments at different concentration of chlorine dioxide. This may invalidate the use of CT values for the comparison of disinfection kinetics.
Figure 12: Comparison of $CT_{99.9\%}$ values M. avium strains for chlorine, monochloramine, chlorine dioxide, and ozone. Error bars illustrate the 95% confidence interval. Experimental conditions: pH 7.0, 23°C.
Application of M. avium CT\textsubscript{99.9\%} Value to an Idealized Municipal Water System

M. avium's high resistance when exposed to chlorine may allow M. avium to pass through a water treatment plant and into the distribution system. Using an idealized water treatment system we can observe how this might occur. In this idealized water system we must make several assumptions: (1) Chlorine levels remain constant in primary treatment and distribution system residual, (2) disinfection of takes place at 23°C and pH 7, (3) there is no re-growth of M. avium in the plant or in the distribution system, (4) there is no aggregation of M. avium at any time, (5) that the initial concentration of M. avium in the raw water is $10^3$ CFU/ml and, (6) that the CT\textsubscript{99.9\%} value for M. avium the system is chosen as the highest experimental value: 204 reported here for M. avium strain 1060. The idealized water systems are designed to replicate the disinfectant concentration and contact times of average water systems, with one treatment system representing the upper disinfection range, and one in the lower disinfection range. Two systems will be examined. System 1, using 1.6 ppm chlorine for 21 min as the primary treatment and 0.1 ppm chlorine residual in a distribution system where the contact time is 20 hours. Thus, system 1 could be considered to be using low levels of disinfectant and short contact times, when compared to the majority of municipal water systems. System 2 utilizes a primary treatment of 2 ppm with a contact time of 128 minutes, and a distribution system residual of 0.3 ppm for 24 hours. System 2 could be considered to use higher levels of disinfectant and longer contact times than used in most water treatment systems.

To calculate survival, the concentration (in ppm) times the time (in minutes) for one log of cell inactivation of M. avium strain 1060 needs to be calculated. Since the CT value to 3 logs cell inactivation for M. avium strain 1060 is 204, the CT value to 1 log cell inactivation is $204/3 = 68$. 

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To calculate the log percent of cell inactivation for a specific treatment, the concentration times the contact time was calculated. This value was then divided by the CT value to 1 log inactivation for *M. avium* to determine the logs of cell death. This was then subtracted from the original value.

Results for Systems 1 and 2 models are in table 6.

For System 1, the primary treatment only inactivates 0.49 logs of *M. avium* allowing 323 CFU to pass through as viable cells. For System 2, the primary treatment eliminates 3.76 logs of *M. avium* cells, so less than 1 CFU remains, eliminating all CFU prior to entry into the distribution system. In System 1 the distribution system residual inactivates another 1.8 logs, reducing the number of viable CFU to 5. According to the model for System 1, it is possible for *M. avium* CFU to reach the end of the distribution system in spite of the disinfectant levels used for treatment.

Assumptions made for the idealized systems may significantly alter the ability of *M. avium* to persist to the distribution system end. In the first assumption, we assume that distribution system residuals remain constant, this is not the case. Chlorine levels drop in response to many variables including carbon content, temperature, and pH (37). Lower chlorine concentrations in the distribution system would allow more *M. avium* CFU to reach the end of the distribution system. Also assumed is that there is no re-growth of *M. avium* in the distribution system. *M. avium* has been found to grow in natural waters (18), and it may be possible for it to grow in municipal water. Re-growth of *M. avium* in the distribution system would again allow *M. avium* increased chance to reach the distribution system end by providing more CFU. Assumption 4, that there is no aggregation of *M. avium* at any time is almost certainly false. Mycobacteria form aggregates as they grow (32). Aggregation might prevent diffusion of disinfectants to vital cell components in cells on the interior of the aggregate enhancing survival through the distribution system. Thus, the idealized systems depicted here might under-estimate the numbers of CFU persisting to the distribution system end under conditions provided by the other assumptions.
Table 6: Comparison of two hypothetical idealized water treatment systems.

<table>
<thead>
<tr>
<th></th>
<th>System 1 (lower range)</th>
<th>System 2 (higher range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary Treatment: 1.6 ppm for 21 min</td>
<td>Primary Treatment: 2 ppm for 128 min</td>
</tr>
<tr>
<td></td>
<td>Distribution System: 0.1 ppm for 1200 min</td>
<td>Distribution System: 0.3 ppm for 1440 min</td>
</tr>
<tr>
<td>Natural Water</td>
<td>CFU 1000          Log CFU 3</td>
<td>CFU 1000          Log CFU 3</td>
</tr>
<tr>
<td>Primary Treatment</td>
<td>0.49 logs cell inactivation</td>
<td>3.76 logs cell inactivation</td>
</tr>
<tr>
<td>Post Treatment (Plant effluent)</td>
<td>323          2.51</td>
<td>&lt;1              &lt;0</td>
</tr>
<tr>
<td>Distribution System Residual</td>
<td>1.8 logs cell inactivation</td>
<td>6.35 logs cell inactivation</td>
</tr>
<tr>
<td>Distribution System End</td>
<td>5              0.71</td>
<td>&lt;1              &lt;0</td>
</tr>
</tbody>
</table>
Summary

Reduced-aggregate fractions of *Mycobacterium avium* and *Escherichia coli* were exposed to chlorine, monochloramine, chlorine dioxide, and ozone and the kinetics of disinfection (i.e. loss of CFU over time) measured. Chlorine disinfection kinetics of *M. avium* cells in biofilms were also measured.

**Chlorine Disinfection**

*M. avium* exhibited a high resistance to chlorine compared to *E. coli*. *M. avium* CT \(_{99.9\%}\) (disinfectant concentration \(\times\) time to 3 logs cell inactivation) values were between 571- and 2318 - times those of *E. coli* (Table 2, Figure 12). Clinical isolates of *M. avium* showed 0.25 and 2.5 times increased resistance to chlorine when compared to their pulsed-field-gel-electrophoresis- (PFGE) matched environmental isolates (Table 2, Figure 12).

**Monochloramine Disinfection**

*M. avium* strains exhibited a mixed response to exposure to monochloramine. The CT \(_{99.9\%}\) values of three strains (2 clinical, 1 environmental) were between 6.3- and 23.5- times that of *E. coli* (Table 2). Two strains (1 clinical, 1 environmental) exhibited CT \(_{99.9\%}\) values approximately the same as *E. coli*, a difference from all the other disinfectants which were much less effective on *M. avium* than on *E. coli*.

**Chlorine Dioxide Disinfection**

*M. avium* strains exhibited a high resistance to chlorine dioxide when compared to *E. coli*. *M. avium* CT \(_{99.9\%}\) values of between 133- and 706- times higher that that of *E. coli*. In the paired isolates tested, the clinical isolate was 5.3 times more resistant than the matched environmental isolate.
Ozone Disinfection

*M. avium* exhibited a high resistance to ozone when compared to *E. coli*. *M. avium* strains exhibited a CT\textsubscript{99.9\%} value of between 52 and 90 times higher that that of *E. coli*. In the paired isolates tested, the clinical isolate was nearly identical as judged by CT\textsubscript{99.9\%} values. The disinfection rate of *M. avium* strain 5002 increased by non-logarithmic factor, indicating that inactivation efficiency was increasing over time.

Biofilm Disinfection with Chlorine

*M. avium* strain 1060 showed between a 4 and 230\% increase in CT\textsubscript{99.9\%} value when in biofilms as opposed to planktonic (free living) culture. The large increase in resistance of cells in a biofilm when compared to cells in suspension, as seen in two of the three biofilm experiments, could be due to decreased permeability of the biofilm to chlorine, or physiological changes of the cell when in a biofilm. Due to the large variance in biofilm density and and CT\textsubscript{99.9\%} values, any conclusions based on these experiments should be considered tentative at best.

Comparison of *M. avium* resistance to other Water Borne Pathogens

*M. avium* resistance to chlorine and chlorine dioxide approaches that of the protozoan cysts of *Giardia muris* and *Entamoeba hystolytica*. *M. avium* is much less resistant, relatively, to monochloramine possessing values similar to *E. coli*. Ozone resistance of *M. avium* two orders of magnitude greater than *E. coli* and one order of magnitude of less than *G. muris* cysts.
Chlorine Dioxide Concentration Based Threshold Effect

A critical concentration threshold level for chlorine dioxide was found. That is, a concentration level of chlorine dioxide above which cell inactivation greatly increases. Initial experiments using a range of concentrations from 0.1 ppm to 0.5 ppm chlorine dioxide show a biphasic curve, illustrated in figure 11, with the inflection point (indicating the critical concentration) between 0.3 and 0.4 ppm chlorine dioxide.

Future Research Possibilities

1. Identification of factors responsible for higher chlorine, monochloramine and chlorine dioxide resistance of clinical M. avium isolates.
2. Determine differences in chlorine susceptibility of M. avium cells grown in laboratory media and in drinking water.
3. Determine whether M. avium cells exposed to chlorine have been stressed.
4. Identify the mechanism(s) of the bactericidal effect of chlorine on M. avium.
5. Identify determinants of M. avium sensitivity to monochloramine.
6. Determine inactivation kinetics of M. avium biofilms for monochloramine, chlorine dioxide, and ozone.
References:


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