Inactivation of Environmental *Mycobacteria* by Free Chlorine and UV

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Abstract
Environmental mycobacteria (EM) are common saprophytes in all ecosystems, including water, soil, food, and aerosols. Several species of EM, such as *M. kansasii, M. avium, M. intracellulare, M. marinum,* and *M. xenopi,* are opportunistic pathogens causing pulmonary, cutaneous disease, and lymphadenitis for humans or animals. We examined the resistance of mycobacteria against the chlorine and UV and determined the Ct value and UV dose to inactivate EM. Chlorine disinfection experiments were done on *M. fortuitum* in oxidant demand-free buffered water at the worst condition (pH 8.5, 4℃) and normal condition (pH 7.0, 20℃). As a result, the CT value for 3 log inactivation of *M. fortuitum* was 600 times above greater than that of *E. coli*. Also, UV experiments were performed for various species of *M. avium, M. fortuitum, M. intracellulare and M. lentiflavium*. A UV collimated-beam device was used to suspension of four species in phosphate buffered saline with doses of 5, 10, 20, 50, 100 mJ/cm^2. UV sensitivity of mycobacteria was species specific. More than 3 log of *M. avium, M. intracellulare and M. lentiflavum* could be inactivated at 20 mJ/cm^2. But *M. fortuitum* was so most resistant that 3 log inactivation required a dose of 100 mJ/cm^2. *Mycobacteria* were shown to be two to ten times more resistant to UV than *E. coli* for 3 log inactivation.

Keywords
Collimated-Beam Device; Free chlorine; Mycobacteria; UV

INTRODUCTION

Environmental Mycobacteria (EM) have been recovered from a wide variety of environmental sources, including water, soil, dust, and aerosols. Mycobacteria is found in the natural water environment as well as in artificial water systems such as plumping systems, cooling towers, swimming pools and drinking water. Most of them are saprophytic, although some are potential pathogens and may be involved in pulmonary or cutaneous diseases or in lymphadenitis. (Le Dantec et al., 2002). Mycobacterium avium complex (MAC) consisted of *M. avium* and *M. intracellulare* is currently listed on U.S. Environmental Protection Agency's Candidate Contaminant List and was included because it is potentially a waterborne pathogen and has been detected in drinking water sources. The high concentration of mycolic acid and the hydrophobic surface characteristics of

mycobacteria are primarily responsible for the high resistance of mycobacteria to chemical disinfection (WHO, 2004).

Most species of mycobacteria can survive in water with 1mg/L free chlorine (WHO, 2004). Du Moulin et al. (1998) found *M. marinum* to be resistant to 10mg/L free chlorine. Haas et al. (1983) found that there was no reduction in acid-fast bacterial numbers following the chlorination in the water treatment plant examined (WHO, 2004). Taylor et al. (2000) reported that CT99.9% value for the *M. avium* strains were 700- to 3000-times greater than that for *E. coli*. Data published by Le Dantec et al. (2002) also demonstrated CT99.9% value for the *M. aurum* and *M. gordonae* are 100 and 330 times more resistant to chlorine than *E. coli*. This indicates that the more effective disinfectants more than the chlorine need to control mycobacteria in water.

There is growing interest among public water systems on ultraviolet (UV) light, based on its ability to inactivate certain microorganisms without harmful disinfection-by-products (DBPs). Some pathogens, such as *Cryptosporidium*, are resistant to commonly used disinfectants, whereas UV light has proven effective against these microorganisms. (USEPA, 2003)

We examined the resistance of mycobacteria against the free chlorine and UV and determined the CT value and UV dose to inactivate EM.

MATERIALS AND METHODS

Microorganism

Pure cultures of *M. avium* (ATCC 35717), *M. fortuitum* (ATCC 23010), *M. intracellulare* (ATCC 700662) and *M. lentiflavium* (isolated at tap water) were incubated in Middlebrook 7H9 broth (Difco Laboratories, USA) containing 10%(vol/vol) oleic acid albumin enrichment (ADC enrichment; BBL, USA) at 37℃, over 7 days. *E. coli* (B-EC9001, equivalent ATCC 11775, BTF) was incubated in tryptic soy broth (Difco) at 37℃ overnight. The microorganisms were harvested by centrifugation at 6,000 rpm for 10min, washed twice with a sterilized phosphate buffered saline and subsequently suspended in the phosphate buffered saline at an initial concentration of approximately 106 CFU/mL.

Chlorine disinfection

The three reactor vessels were needed for chlorine disinfection experiment of *M. fortuitum*

and *E. coli*. First reactor (control) contained organisms plus the oxidant demand-free buffered water, but no chlorine. This was used to observe if there was a microbial decay in the absence of chlorine and to determine the initial microbial density. Second reactor (chlorine decay) contained organisms plus the oxidant demand-free buffered water and chlorine. This reactor was used to determine the residual chlorine concentration. Third reactor contained the same materials as second reactor and was used to determine the inactivation of organisms in the presence of chlorine. A freshly prepared free chlorine stock solution was added to the bacterial suspension at a final concentration of 0.5 mg/L, 1 mg/L and 2 mg/L. After 0 to 120 minutes of reaction time with chlorine at 4℃ and 20℃ with gentle shaking, samples of each 10mL were taken and chlorine residuals were quenched with sterile 10% sodium thiosulfate. The disinfection experiments were done in free chlorine stock solution at a final concentration of 0.5 mg/L, 1 mg/L and 2 mg/L at the worst condition (pH 8.5, 4℃) and 1 mg/L at normal condition (pH 7.0, 20℃).

UV source

A bench-scale collimated beam apparatus (Calgon Carbon Corp.) was used to irradiate the samples. This apparatus contained interchangeable low pressure (10W) and medium pressure (1kW) UV lamps. For both types of lamps, the irradiance was measured with a radiometer (International light IL1400A, detector SED240, International light Inc.) equipped with an SED 240 UV detector. Low pressure UV irradiance was measured at 254nm whereas medium pressure UV irradiance was measured at 200~300nm.

The 60 mm petri-dish with the microorganism suspension was placed on a stir plate under the collimated beam apparatus, where the suspension was thoroughly mixed for 2 minutes with the aid of a magnetic stir bar. All samples were exposed at room temperature. Immediately following UV exposure, the entire sample volume was collected. The exposure time was determined by the control of the manually operated shutter. The suspensions of mycobacteria in phosphate buffered saline were irradiated with UV doses of 5, 10, 20, 50, 100 mJ/cm^2 from low pressure and medium pressure UV lamps. The suspension of *E. coli* was irradiated with UV doses of 2, 6, 8, 10 mJ/cm^2 from low pressure UV lamp.

Inoculation and count of microorganism

Chlorine experiment. First reactor and third reactor samples were serially diluted and plated on M7H10 agar (*M. fortuitum*) and tryptic soy agar (*E. coli*) to count the organism levels

following exposure. Free chlorine (second reactor) was measured by the N,N-diethyl-p-phenylenediamine colorimetric method at end point. The number of colonies was counted after 7 days (*M. fortuitum*) or 1day (*E. coli*) at 37℃.

UV experiment. The irradiated samples were serially diluted and plated on M7H10 agar (mycobacteria) and tryptic soy agar (*E. coli*) to count the organism levels following exposure. Non-irradiated samples were plated to determine the initial organism levels. The number of colonies after the incubation was counted and the log inactivation was determined as follows:

$$\text{Log } (N_0/N) = \text{Log inactivation} \quad (1)$$

Where N = the number of colonies at the time t (after UV irradiation)
N_0 = the number of colonies before UV irradiation

Chlorine disinfection kinetics

The chlorine residual data were analyzed for the value of constant k^* according to the first-order decay rate equation:

$$C=C_0 \exp(-k^* t) \quad (2)$$

Where C = observed chlorine residual (mg/L)
C_0 = initial chlorine residual (mg/L)
t = time from start of experiment to time of sample (min)
k^* = first-order decay rate constant (min^{-1})

The values of constant k^* were determined by the method of nonlinear regression. Using the value of constant k^*, the inactivation data were fitted using five inactivation models (Chick, Chick-Watson, Hom, Power law, Hom-Power law). By comparing the goodness of fit, best-fit value of the parameters resulted in minimum residual sum of square (RSS), the model that represented the best inactivation kinetics were determined.
Parameters calculated by the best model were inputted to the equation (3) and Ct value was evaluated.

$$Ct = \int_{t=0}^{t=t} C_0 \, e^{(-k^* t)} dt \quad (3)$$

RESULTS AND DISCUSSION

Inactivation by free chlorine

The inactivation characteristics of *M. fortuitum* and *E. coli* at various contact times of chlorine are presented in Fig.1, 2, 3 and Fig.4. More than 3 log of *E. coli* was inactivated in 30 seconds and 7-log of *E. coli* was inactivated in 1 minute. But to inactivate more than 3 log of *M. fortuitum*, over 160 minutes of contact time needed at pH 8.5 and 4℃ and the curve of log inactivation for *M. fortuitum* exhibited a "tailing" unlike *E. coli*.

For *M. fortuitum* at pH 8.5, the HOM model described best its inactivation by chlorine disinfection. The Chick-Watson (CW) model described best its inactivation by chlorine disinfection at pH 7.0. The Chick model described best its inactivation by chlorine disinfection for *E. coli* (Table 1).

The predicted Ct values for the inactivation of *M. fortuitum* and *E. coli* using best-fit model were presented in Table 2. Ct value for 2~4 log inactivation of *M. fortuitum* at pH 8.5 and 4℃ was from 36 mg·min/L to 278 mg·min/L at 2 mg/L of residual chlorine, and the inactivation at 2 mg/L was rapider than that at 1mg/L. But Ct value at pH 7.0 and 20℃ was 15~100 mg·min/L for 2~4 log inactivation and inactivation rate was higher than at the condition of pH 8.5 and 4℃.

When Giardia Ct value of treatment technique of Korea and surface water treatment rule (SWTR) of U.S. compared with calculated *M. fortuitum* Ct value, Ct value of *Giardia* was 2 to 5 times higher than that of *M. fortuitum*.

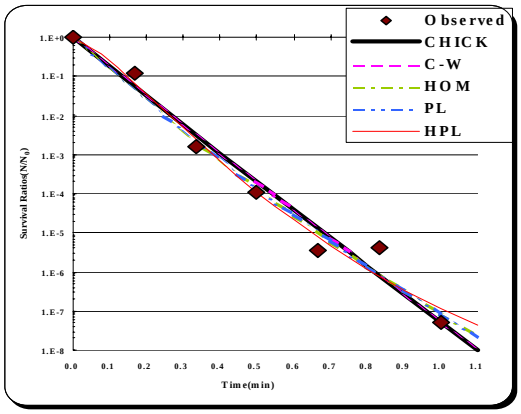

Fig. 1. Inactivation of *E. coli* at pH 8.5 and 4℃ (free chlorine residuals 0.5 mg/L)

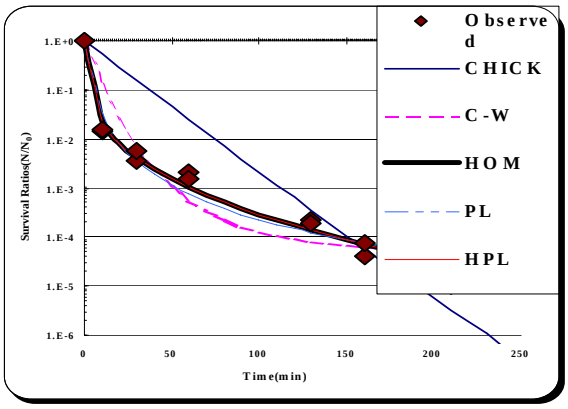

Fig. 2. Inactivation of *M. fortuitum* at pH 8.5 and 4℃ (free chlorine residuals 2 mg/L)

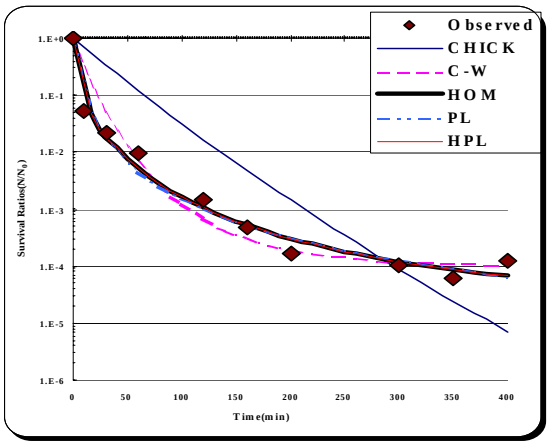

Fig. 3. Inactivation of *M. fortuitum* at pH 8.5 and 4℃ (free chlorine residuals 1 mg/L)

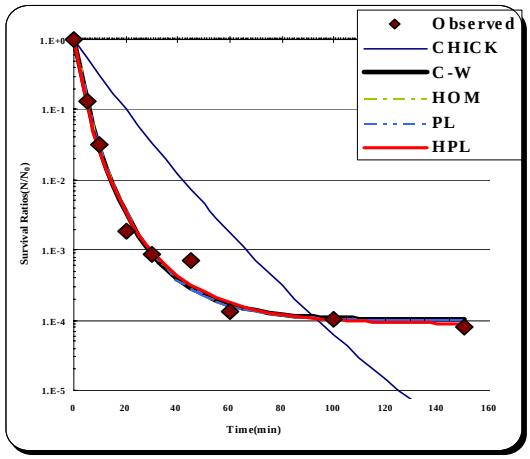

Fig. 4. Inactivation of *M. fortuitum* at pH 7.0 and 20℃ (free chlorine residuals 1 mg/L)

Table 1. Best-fitting inactivation kinectic models for *M. fortuitum* and *E. coli*

Bacteria	Condition	Kinetic model	Parameter(constant)			RSS	R^2(%)
			k	n	m		
M. fortuitum	pH 8.5, 4℃, Cl_2 2 mg/L	HOM	4.699	1.00E-07	0.3336	2.954	97.54
	pH 8.5, 4℃, Cl_2 1 mg/L	HOM	0.9607	2.880	0.4596	1.620	98.28
	pH 7.0, 20℃, Cl_2 1 mg/L	Chick-Watson	0.2940	10.57		1.342	98.53
E. coli	pH 8.5, 4℃, Cl_2 1 mg/L	Chick	31.29			5.738	97.37

Table 2. Comparison of predicted Ct value for *M. fortuitum* and *E. coli* and SWTR Ct value for *Giadia* cysts by free chlorine

Inactivation	*Giardia* cysts			*E. coli*		*M. fortuitum*	
	pH8.5,5℃, (Cl_2 2mg/L)	pH8.5,5℃, (Cl_2 1mg/L)	pH7.0,20℃, (Cl_2 2mg/L)	pH8.5,4℃, (Cl_2 1mg/L)	pH8.5,4℃, (Cl_2 2mg/L)	pH8.5,4℃, (Cl_2 1mg/L)	pH7.0,20℃, (Cl_2 1mg/L)
2 log	196	173	37	0.15	36	52	15
3 log	294	260	56	0.22	120	139	28
4 log	-	-	-	0.29	278	333	100

Inactivation by UV

The inactivation characteristics of mycobacteria and *E. coli* by low pressure or medium pressure UV lamp exposure are presented in Fig. 5, 6 and 7.

Low pressure dose of 8 mJ/cm^2 needed for 3.5 log inactivation of *E. coli*. But UV dose of 100mJ/cm2 for 3.5 log inactivation of *M. fortuitum* and 50 mJ/cm2 for 3.5 log inactivation of *M. avium* needed.

The irradiation with 20 mJ/cm^2 from low and medium pressure could inactivate 3~4 log of *M. intracellulare* or 3~4 log of *M. avium* or more than 4 log of *M. lentiflavum* or 2.5 log of *M. fortuitum*.

M. intracellulare inactivated more than 2 log at UV dose of 5 mJ/cm^2 under low pressure and medium pressure lamp. The others except *M. intracellulare* showed lower inactivation under medium pressure lamp than low pressure lamp at 5~10 mJ/cm^2 but a similar or higher inactivation under medium pressure lamp than low pressure lamp at more than 20 mJ/cm^2.

M fortuitum showed the most resistance among species examined for low pressure and medium pressure lamp. Though there was some difference according to species, the inactivation rate of mycobacteria was no statistically significant difference ($P>0.05$) following low pressure or medium pressure UV radiation exposure.

Relationship between UV dose and inactivation rate for *E. coli* showed good fitting to a linear regression (y=kx, y: log inactivation rate, k: linear regression constant, x: UV dose; y=0.4596x, r2=0.988). K-value was 0.44 and 0.506 in study of Kumiko Oguma et al. (2004) and Hijnen et al. (2006), respectively. Previous studies and our study showed a similar k-value for *E. coli*.

Log- inactivation rate for mycobacteria was observed tailing at UV dose of more than 20 mJ/cm^2. This case could be described by log-log relationship.

As the predicted UV dose for 3 log inactivation of mycobacteria and *E. coli* using regression equation, UV dose of 12~66 mJ/cm^2 for mycobacteria and that of 6.5 mJ/cm^2 for *E. coli* was needed. *Mycobacteria* showed to be two to ten times more resistant to UV than *E. coli* (Table 2).

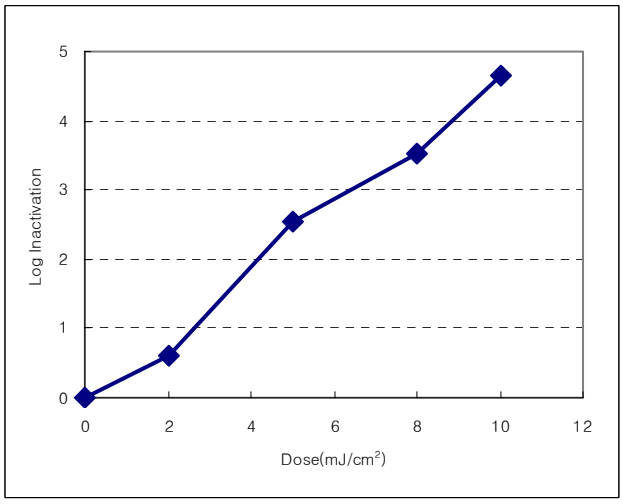

Fig. 5. Inactivation of *E. coli* by Low-Pressure UV lamp

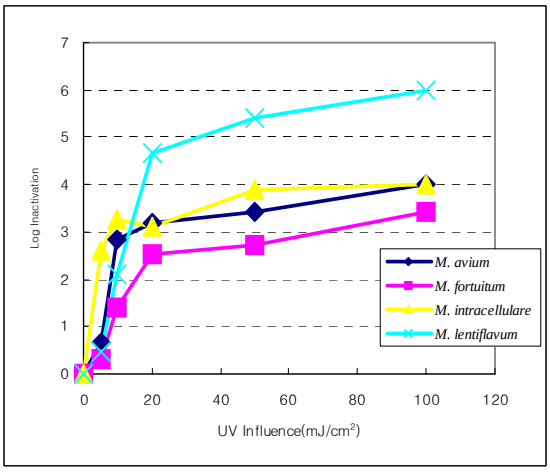

Fig. 6. Inactivation of *mycobacteria* by Low-Pressure UV lamp

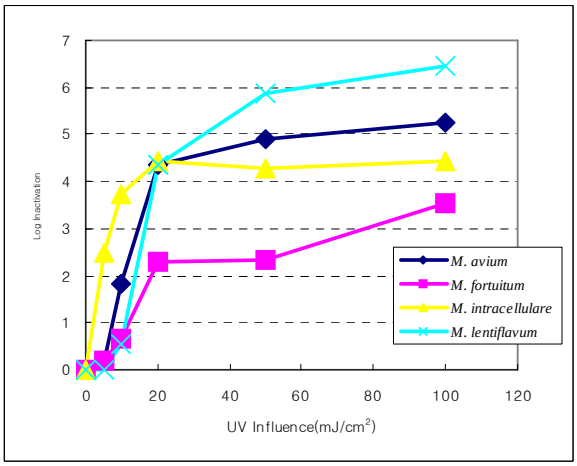

Fig. 7. Inactivation of *mycobacteria* by Medium-Pressure UV lamp

Table 2. Predicted UV dose for 3 log inactivation of mycobacteria and *E. coli*

UV lamp	*Mycobacteria*				*E. coli*
	M. fortuitum	*M. avium*	*M. intracellulare*	*M. lentiflavum*	
Low-pressure	66	26	16	12	6.5
Medium-pressure	84	16	10	14	-

Ct value for 3 log inactivation of *M. fortuitum* was 28 mg·min/L at pH 7.0, 20℃ and 1 mg/L of residual chlorine. Le Dantec et al. (2002) showed that *M. fortuitum* has Ct value of 135 mg·min/L for 3 log inactivation at pH 7.0 and 23℃. Jacangelo et al. (2002)

reported Ct value of 1330 mg·min/L for 2 log inactivation of *M. fortuitum* at pH 8.0, 5℃ and 1 mg/L of residual chlorine and Ct value of 861 mg·min/L at pH 7.0, 25℃ and 1mg/L of residual chlorine. These studies appeared higher Ct value than our study.

In this study, predicted Ct value of *M. fortuitum* was higher than *E. coli* and lower than Giardia. Several studies were reported *M. avium* was more resistant to chlorine than *M. fortuitum* and *Giardia* (Taylor et al. 2000; WHO, 2004).

At UV experiment, more than 3 log of *M. avium*, *M. intracellulare* and *M. lentiflavum* could be inactivated at 20 mJ/cm^2. But *M. fortuitum* had the most resistance among examined species and 3 log could be inactivated at 100 mJ/cm^2.

1 log of *M. fortuitum* was inactivated and 2 log of *M. avium* was inactivated at 5~10 mJ/cm^2. This result was similar to results by other researchers. David et al. (1971, 1973) reported that UV dose of 3.2~8.9 mJ/cm^2 inactivated 1 log of *M. fortuitum* and McCarthy et al. (1974) reported that UV dose of 7 mJ/cm^2 inactivated for 2 log of *M. avium* (WHO, 2004).

Wilson et al. (1993) reported 3 log inactivation of *E. coli* at 7.3 mJ/cm^2. In our study, like the result of Wilson et al., 2.5 log of *E. coli* was inactivated at 5 mJ/cm2 and 3.5 log of *E. coli* was inactivated at 8 mJ/cm^2.

These results showed that as the resistance of mycobacteria to chlorine and UV was different according to species, it was difficult to standardize Ct value and UV dose to eliminate waterborne *mycobateria*, and therefore it was important to decide the removal target species.

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