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Inactivation of two *Mycobacteria* by free chlorine: Effectiveness, influencing factors, and mechanisms

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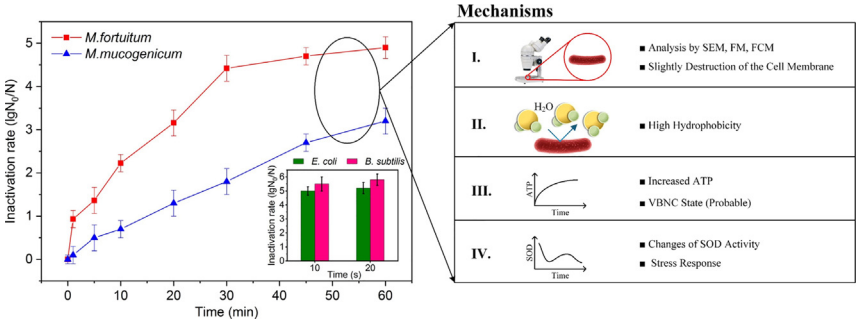
HIGHLIGHTS

- The chlorine resistance was in order: *M. mucogenicum* > *M. fortuitum*.
- Disinfection kinetics model followed Chick-Watson law.
- The microbicidal efficacy of chlorine was stronger at lower pH level.
- The presence of humic acid inhibited the inactivation efficiency.
- A comprehensive reason accounted for *Mycobacteria*'s chlorine tolerance.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorination is one of the most widely used disinfection techniques, and the problem of "chlorine-resistant bacteria" (CRB) has attracted more attention recently. In this study, the deactivation of typical CRB in water, *Mycobacterium fortuitum* (*M. fortuitum*) and *Mycobacterium mucogenicum* (*M. mucogenicum*), by free chlorine was investigated with *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) as the reference. The chlorination effectiveness of chlorine on *M. fortuitum* and *M. mucogenicum* and the effect of chlorine concentration, pH, and humic acid were studied. It was found that *M. mucogenicum* was more resistant to chlorine than *M. fortuitum*, both of which were much more resistant than *E. coli* and *B. subtilis*. The effect of disinfectant concentration on the inactivation efficiency was positive, whereas the influence of pH and humic acid was negative. The inactivation mechanisms were explored by analyzing the bacteria morphology, the destruction of cell membrane, the cell hydrophobicity, as well as total adenosine triphosphate (ATP), superoxide dismutase (SOD) activity. The slight destruction of the cell membrane was observed after deactivation with chlorine, and high hydrophobicity of the cell membrane combined with metabolic changes might lead to the chlorine tolerance of *Mycobacteria*.

1. Introduction

Drinking water quality is closely related to people's health. Since the beginning of the last century, people began to use chlorine disinfection technology to obtain sanitary drinking water. Since chlorine disinfection has many advantages, such as strong sterilization ability, low investment and operating cost, it is still the most widely used disinfection method. With the advances in detection methods, researchers have found that some bacteria can survive in drinking water plant, pipelines and other water supply system after high concentration of chlorination (Codony et al., 2005; King et al., 1988; LeChevallier et al., 1996). It was reported that *Mycobacterium avium* (*M. avium*), a kind of *Mycobacteria*, could survive after 60 min of disinfection under 4.0 mg/L of free chlorine

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and was frequently detected in the water distribution system (Miyamoto et al., 2000). These bacteria with high tolerance to chlorine are called "disinfectant-resistant bacteria" (DRB) or "chlorine-resistant bacteria" (CRB). The existence of CRB may cause the pathogenic risk to drinking water. Besides, CRB can survive in the biofilms of pipe network and lead to the decrease of biological stability (Moraga-McHaley et al., 2013).

Among the studies related to the resistance of DRB, *Mycobacteria* was reported the most. According to the pathogenicity, *Mycobacteria* can be divided into *Mycobacterium leprae* (*M. leprae*), *Mycobacterium tuberculosis* (*M. tuberculosis*) and *non-tuberculous Mycobacteria* (*NTM*). *M. leprae* mainly cause leprosy; *M. tuberculosis* can mainly cause tuberculosis. The studies of *M. leprae* and *M. tuberculosis* are mainly in the field of medicine. *NTM* can survive in soil, air and water. In water treatment, researchers focused on the study of deactivation of environmental *NTM*. And the distribution of *NTM* in water is very extensive, such as in natural water, drinking water, water distribution system biofilms, sewage, hospital water, hot water systems and so on (Behr and Falkinham, 2009; Feazel et al., 2009; Glazer et al., 2007; Moraga-McHaley et al., 2013). The species *M. avium*, *Mycobacterium chelonae* (*M. chelonae*), *Mycobacterium fortuitum* (*M. fortuitum*), *Mycobacterium gordonae* (*M. gordonae*), *Mycobacterium kansasii* (*M. kansasii*), *Mycobacterium xenopi* (*M. xenopi*) and *Mycobacterium mucogenicum* (*M. mucogenicum*) are the most frequently reported *Mycobacteria* occurring in drinking water (Covert et al., 1999; Vaerewijck et al., 2005). *NTM* cannot directly cause tuberculosis, but relate to a variety of other human infections, especially in immunocompromised people, such as AIDS. The infection rate and mortality by *NTM* are high (Wolinsky, 1979). They can also cause pulmonary and cutaneous disease, lymphadenitis, and disseminated infections by ingestion, inhalation, and inoculation from environmental sources (Le Dantec et al., 2002b).

The researchers generally believe that the *Mycobacteria* have strong tolerance to disinfectants. It was reported that the Ct value for 3.0 log inactivation of *M. fortuitum* was 600 times greater than that of *Escherichia coli* (*E. coli*) during chlorine disinfection (Lee et al., 2010). It was found that *M. chelonae* and *M. fortuitum* could survive 60% after disinfection with 0.3 mg/L of free chlorine for 60 min (Carson et al., 1978). The chlorine disinfection test showed that the $Ct_{99.9\%}$ value of *M. avium* strains ranged from 51 to 204 mg·min/L, the value of which was about 580 to 2300 times higher than that of *E. coli*, which indicated the high disinfectant resistance of *M. avium* (Taylor et al., 2000). The researchers compared the survival of *M. fortuitum* after chlorination, ozonation, and UV disinfection. The study showed that those bacteria had different tolerability for different disinfectants (Lee et al., 2015). After 2.0 mg/L of free chlorine disinfection for 30 min, the number of colonies decreased by 3.0 log, while the number of colonies reduced by 3.4 log in 20 min under 1.25 mg/L ozone. One hundred mJ/cm^2 UV irradiation could reduce the number of colonies by 3.8 log. The existing studies on inactivation of *Mycobacteria* by means of various disinfectants have mainly concentrated on the inactivation effectiveness and kinetics (Le Dantec et al., 2002a; Taylor et al., 2000), whereas the influencing factors and mechanisms of deactivation of *Mycobacteria* have rarely been reported.

This work aims at investigating the chlorination of *M. fortuitum* and *M. mucogenicum*. To the best of the authors' knowledge, there are few reports on their deactivation under free chlorine (Carson et al., 1978; Chen et al., 2012; Le Dantec et al., 2002a). In the published work, the growth capabilities of *Mycobacteria* in water environments, the efficiency of chlorination to *Mycobacteria* were studied. The objectives of the present study are to 1) investigate the disinfection efficiency and the deactivation kinetics of *M. fortuitum* and *M. mucogenicum* by free chlorine; 2) examine the influencing factors, such as chlorine concentration, pH and humic acid; and 3) explore the mechanisms of chlorine-driven inactivation of *M. fortuitum* and *M. mucogenicum* by observing the destruction of cell membrane, the morphological changes, and monitoring the cell hydrophobicity as well as the bacteria metabolic changes.

2. Materials and methods

2.1. Microorganisms

M. fortuitum and *M. mucogenicum* were chosen as the representative strains of *Mycobacteria*, because these two species were the most frequently reported *Mycobacteria* occurring in drinking water (Covert et al., 1999; Vaerewijck et al., 2005), and the disinfection characteristics of the two bacteria were unclear. *M. fortuitum* (ATCC 6841) and *M. mucogenicum* (ATCC 49650) were incubated in Middlebrook 7H9 broth (BD, USA) containing 10% (vol/vol) Middlebrook ADC enrichment (BD, USA) at 37 °C, over 7 days. *E. coli* (ATCC 25922) and *B. subtilis* (ATCC 6633) were incubated in nutrient broth (BD, USA) at 37 °C overnight. The microorganisms were harvested by centrifuging at 10,000 rpm for 2 min, then washed twice with a sterilized phosphate buffered saline (PBS; pH 7.1) and subsequently suspended in the PBS at an initial concentration of approximately 10^6 CFU/mL. Cell suspensions grown in rich laboratory media were used as sources of inocula for disinfection experiments. All assays were performed in Class II Microbiological Safety Cabinet (AC2-4S1, ESCO). Zeta potential of bacterial suspension were conducted with a Malvern Nanosizer (Zetasizer Nano ZS 90, Malvern, UK).

2.2. Chlorine disinfection procedures

Free chlorine is one of the most widely used methods for the disinfection of water. Several 300 mL beakers were used as reaction vessels for chlorine disinfection. Bacteria were inactivated with free chlorine in a phosphate buffer of a constant ionic strength under agitation. This procedure created well-controlled and reproducible experimental conditions. Samples were collected from the reactor into micro centrifugal tubes which contained excess sterile sodium thiosulfate ($Na_2S_2O_3$) to instantaneously quench the residual disinfectants. In a parallel test, 10 mL fluid was taken out to measure the residual chlorine concentration by *N*,*N* diethyl *p* phenylenediamine (DPD) method. Phosphate buffer solution was used for the regulation of pH.

2.3. Heterotrophic plate counts (HPC) detection

After disinfection, an aliquot of suspension was withdrawn and serially diluted with sterilized 0.05 M PBS. 30 μL of bacteria suspension samples were plated on 10 mL of Middlebrook 7H10 agar (for *M. fortuitum* and *M. mucogenicum*) and nutrient agar (for *E. coli* and *B. subtilis*) to count the organism levels. The number of colonies within the range of 30–300 was counted after 7 days (for *Mycobacteria*) or 1 day (for *E. coli* and *B. subtilis*) at 37 °C. Each experiment was replicated three times and the standard deviations were shown in the figures.

2.4. Disinfection kinetics model

The kinetics model was performed as described by Corinne Le Dantec (Le Dantec et al., 2002a). The Chick-Watson law was used for defining the rate of inactivation of bacteria, which was shown in Eq. (1). In this equation, N_0 is the initial concentration of microorganisms, N is the concentration remaining at time t, CFU/mL; C is the concentration of disinfectant, mg/L; and k (L·mg^{-1}·min^{-1}) is the susceptibility or lethality coefficient of the microorganism. t represents reaction time, in minutes. In order to accurately evaluate Ct values, chlorine decay was integrated as a function of time:

$$lg(N_0/N) = k\int_0^t Cdt \tag{1}$$

The Ct values are represented by the areas under the curve presented in Fig. S1 (Supporting information). And they were calculated