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原　　著

Effects of Monochloramine and Free Chlorine Disinfection on Alkaline Hot Spring Water as Shown by Inactivation Experiments with *Mycobacterium phlei*, *Bacillus subtilis*, and *Escherichia coli*

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高アルカリ温泉水における *Mycobacterium phlei*，*Bacillus subtilis*，*Escherichia coli* のモノクロラミンと遊離塩素の消毒効果

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要　　旨

高アルカリ温泉水に対するモノクロラミン消毒下の浴槽水では，様々な雑菌が繁殖してしまうことが知られている．特に，実地試験で増殖が見られる非結核性抗酸菌の *Mycobacterium phlei* は，モノクロラミン消毒への抵抗性が，特に強いことが懸念されている．これまでに筆者らは，モノクロラミン消毒下の *M. phlei* を対象として，その実地および試験管内試験の両面から，消毒効果を報告してきた．本研究では，アルカリ性の温泉（pH＝9.6）を使ったモノクロラミン消毒試験を行い，*M. phlei*，*Bacillus subtilis*，*Escherichia coli* の不活化を比較した．実験の結果，*E.coli* は，モノクロラミンおよび遊離塩素のいずれの消毒でも速やかに不活化されたが，*B. subtilis* はいずれでも消毒には相応の時間を必要とした．*B. subtilis* の 1-Log 不活化に必要な CT 値は，モノクロラミン消毒でおよそ 250 mg/L・min であったが，遊離塩素消

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毒でおよそ 2,000 mg/L・min であり，モノクロラミン消毒に対する抵抗性は，*M. phlei* と同程度と評価された．本研究により，アルカリ性の温泉では，遊離塩素消毒の効果が極めて弱く，モノクロラミン消毒の方が有効であることが示された．

キーワード：モノクロラミン，高アルカリ温泉水，*Mycobacterium phlei*，*Bacillus subtilis*，*Escerichia coli*，レジオネラ属菌，循環ろ過

Abstract

Monochloramine disinfection of alkaline hot spring water appears to allow the growth of various bacteria, including *Mycobacterium phlei*, which is a non-tuberculous mycobacterium. Reportedly, *M. phlei* is particularly resistant to monochloramine disinfection. In this study, the efficacy of monochloramine disinfection was examined by performing inactivation experiments using an alkaline hot spring containing *Bacillus subtilis* and *Escherichia coli*. Our results showed that *E. coli* was rapidly inactivated by both monochloramine and free chlorine. However, increased time duration and a higher concentration of monochloramine and free chlorine were required to inactivate *B. subtilis*. The CT value (concentration × time value) required for 1-Log inactivation of *B. subtilis* was approximately 2,000 mg/L·min for free chlorine and 250 mg/L·min for monochloramine. The results showed that *M. phlei* and *B. subtilis* were equally resistant to these disinfectants. In addition, free chlorine disinfection was very weakly effective in alkaline hot spring water, indicating that monochloramine disinfection was more effective than free chlorine disinfection.

Key words : Monochloramine disinfection, alkaline hot spring, *Mycobacterium phlei*, *Bacillus subtilis*, *Escherichia coli*, *Legionella* spp., circulation filtration system

1. Introduction

Free chlorine disinfection with sodium hypochlorite is widely used in hot spring facilities because of its low cost and ease of use. However, it has become clear that free chlorine disinfection is not always effective for all hot spring waters, such as highly alkaline hot springs. Monochloramine disinfection was developed as an alternative method to sodium hypochlorite disinfection, and its usage was added to the Japanese Ministry of Health, Labor and Welfare's notification (Minister of Health, Labor and Welfare, Deputy Vice-Minister for Public Health and Food Safety, 2019 ; Minister of Health, Labor and Welfare, Pharmaceutical Safety and Environmental Health Bureau, Environmental Health Division, 2019). The disinfection efficacy of monochloramine has been tested against *Legionella* spp. and has been used to control their growth (Jakubek *et al*., 2013).

Hot spring development through deep drilling in nonvolcanic areas is increasing nationwide (Mori and I. Matsumoto, 2021), which often yields highly alkaline hot spring waters. Therefore, the demand for effective disinfection methods for alkaline hot spring water is expected to increase.

Previously, we conducted field tests (Mori *et al*., 2019 ; Mori *et al*., 2020) and *in vitro* tests (Mori *et al*., 2022) of monochloramine disinfection in alkaline hot spring water and continued to verify the disinfection efficacy and practicality of monochloramine in alkaline hot springs.

In field tests, the growth of heterotrophic bacteria was observed in bathtub water under monochloramine disinfection (Mori *et al*., 2019). *Mycobacterium phlei* was reported to be the dom-

inant species detected in other hot spring facilities under monochloramine disinfection (Watanabe *et al*., 2018).

Mycobacterium phlei is a species of non-tuberculous mycobacteria (NTM). In recent years, the increase in non-tuberculous mycobacteriosis (NTM disease) caused by NTM has become a major concern (Wada, 2017). Although *M. phlei* is considered to have low pathogenicity against healthy individuals, opportunistic infection in immunocompromised patients in Japan has been reported (Tanaka *et al*., 2019).

In this study, we focused on the difficulty of *M. phlei* disinfection under monochloramine rather than its virulence. Although *M. phlei* does not form endospores, it is highly resistant to disinfection because of the high lipid content in its cell wall (Oriani *et al*, 2018). Results of previous field studies on monochloramine disinfection have shown that *M. phlei* is difficult to control.

In general, the growth of bacteria promotes the growth of amoebae (Thomas *et al*., 2010), which consequently promotes the growth of *Legionella* spp. From the viewpoint of preventing Legionnaires' disease, the control of bacteria, including *M. phlei*, should be considered an important issue in bathtub water hygiene.

The disinfection resistance of *M. phlei* should be evaluated by comparison with other typical bacteria. In this study, *in vitro* tests were conducted using a gram-negative bacterium, *Escherichia coli*, and a gram-positive bacterium, *Bacillus subtilis*, as comparison targets. *E. coli* was selected as an indicator of cleanliness because it is one of the criteria for hygiene control of bathtub water quality. Meanwhile, *B. subtilis* was selected as one of the representative endospore-forming bacteria to compare with the disinfection resistance of *M. phlei* and endospores.

The results of this study provide useful insights into monochloramine disinfection.

2. Methods

This study followed the methods described in Mori *et al*. (2022). As the details can be found in that published paper, a brief overview is provided here.

An alkaline hot spring with a pH of 9.64 and electrical conductivity of 39.4 mS/m was used for the *in vitro* tests. The alkaline hot spring was identical to the hot spring water used in the field test (Mori *et al*., 2019) and previous *in vitro* tests (Mori *et al*., 2022). The test solution was autoclaved at 121℃ for 15 min prior to testing. The results of the post-sterilization chemical analysis reported by Mori *et al*. (2022) showed no change in the chemistry related to disinfection inhibition, including pH and ammonium ion concentration. The disinfectant solution for monochloramine was a mixture of sodium hypochlorite (K-MIX ; KI Chemical Industry Co., Ltd., Shizuoka, Japan) and ammonium sulfate (Legicide ; KI Chemical Industry Co., Ltd., Shizuoka, Japan), as appropriate.

In vitro tests were conducted in a laboratory controlled at 25℃. The bacterial strains used were stored at −80℃. Both *E. coli* (ATCC25922) and *B. subtilis* (NBRC3134) were pre-cultured on standard agar medium (Eiken Chemical Co., Ltd., Tokyo, Japan), and the bacterial solution was prepared by scraping off the growing bacteria with a disposable inoculation loop. *B. subtilis* was observed with a microscope after the strain was cultured under the same conditions as in the experiment. Gram staining was performed using Gram stain kits (FUJIFILM Wako Pure

Chemical Corporation, Osaka, Japan) to differentiate between endospores and vegetative cells, and the resultant slides were observed under an optical microscope (BX50, Olympus Corporation, Tokyo, Japan) with a DP20 camera.

The bacterial solution, adjusted for turbidity, was added to 150 mL of hot spring water prepared in sterile cups to obtain a concentration of approximately 10^5–10^6 CFU/mL. Monochloramine and sodium hypochlorite were added to the sterile cups to achieve low (approximately 5 mg/L as monochloramine or free chlorine), medium (approximately 10 mg/L as monochloramine or free chlorine), and high (approximately 20 mg/L as monochloramine or free chlorine) concentrations. The samples were collected from the cups 15, 30, 60, 90, and 120 min after disinfection. Each collected sample was divided into two parts : one was used to measure the disinfectant concentration, and the other was used for incubation. The disinfection concentration was measured, and the sample solution (culture test solution) was then neutralized with sodium thiosulfate, diluted, mixed with standard agar medium (Eiken Chemical Co., Ltd.), and incubated at 37℃ for 24 h.

The monochloramine concentration was measured using the indophenol method with a pocket colorimeter (HACH DR300, CLRMTR, monochloramine model), and the free chlorine concentration was measured using the DPD method with a pocket colorimeter (HACH Pocket Colorimeter II chlorine model, Hach Company, Loveland, CO, USA). The disinfectant concentration was used to calculate the CT (concentration × time) value, i.e., the product of the disinfectant concentration and contact time.

3. Results and Discussion

3.1 Stability of disinfection concentration

The changes in monochloramine and free chlorine concentrations in the test solution are shown in Fig. 1.

Both monochloramine and free chlorine remained at a concentration of at least 80% from the time of bacterial addition to the end of the experiment. Some hot spring waters contain diverse chemical components that interfere with disinfection. However, we confirmed that the alkaline hot spring water did not contain any chemically interfering components.

Therefore, there were no concerns regarding the stability of the disinfectant concentration.

3.2 Inactivation of *B. subtilis* and *M. phlei* in relation to CT values

Correlation plots between the inactivation ratios of *B. subtilis* and *M. phlei* and CT values from the disinfection test are shown in Fig. 2. Experimental data for *M. phlei* were obtained from a study by Mori *et al.* (2022).

The degree of inactivation of *M. phlei* and *B. subtilis* was almost the same. For 1-Log inactivation, monochloramine required a CT value of approximately 250 mg/L·min, whereas free chlorine required a CT value of approximately 2,000 mg/L·min.

In this experiment, approximately 1-Log of bacteria was inactivated by free chlorine disinfection, and the CT value required for inactivation above 2-Log was unknown. In contrast, 3-Log inactivation was achieved by monochloramine disinfection, with a CT value of approximately

750 mg/L·min. The disinfection efficacy of monochloramine against *M. phlei* and *B. subtilis* was estimated to be about eight times higher than that of free chlorine.

Thus, the efficacy of monochloramine in highly alkaline hot spring water was confirmed.

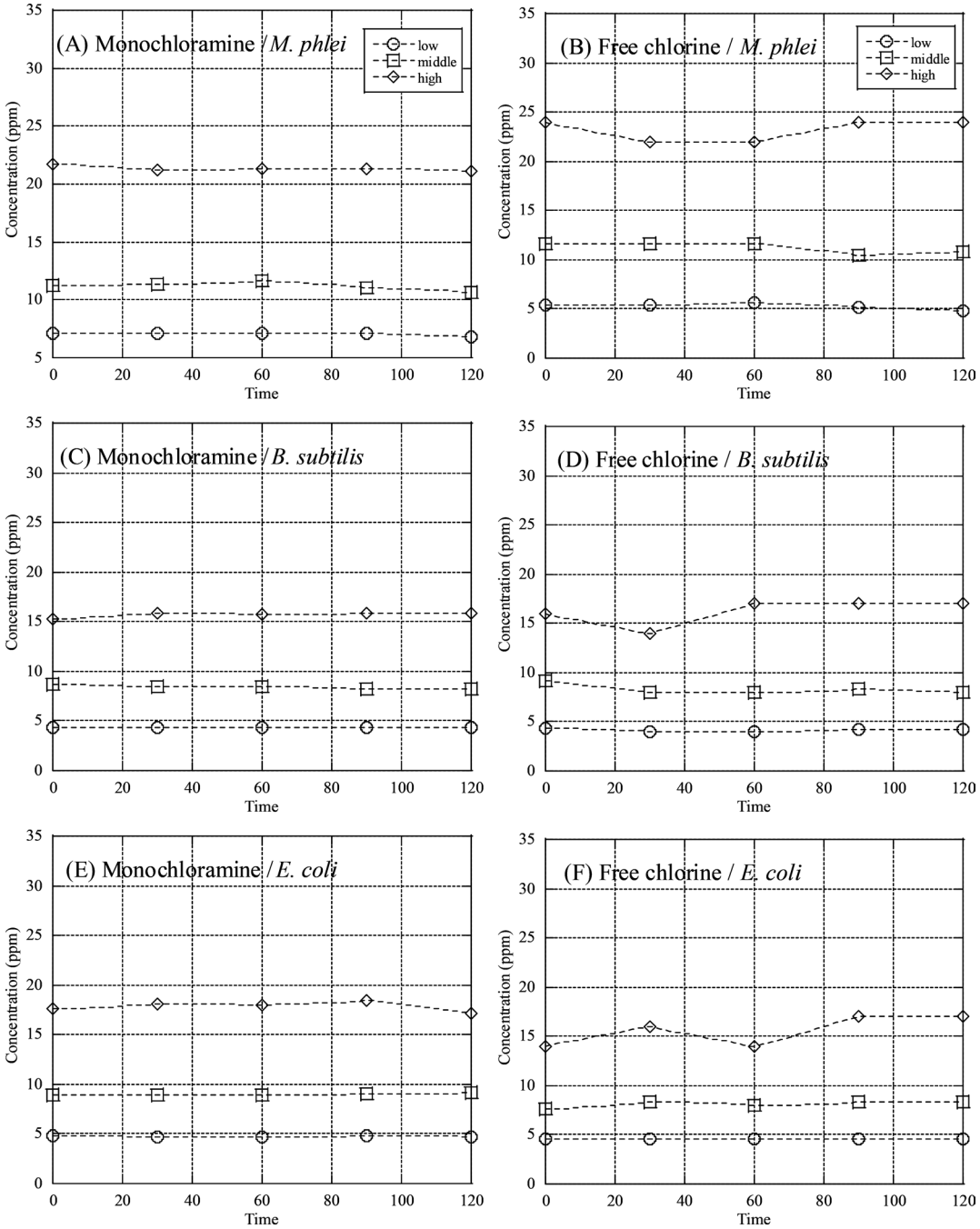

Fig. 1　Changes in monochloramine and free chlorine concentrations in the test solution.

Previous studies have reported that monochloramine disinfection can be applied to bathtub water with a wide pH range, from slightly acidic to alkaline (Yanagimoto *et al*., 2021 ; Izumiyama *et al*., 2022). The results of that study further support our current findings.

A photograph of the bacterial solution cultured under the same conditions as those used in the experiment is shown in Fig. 3.

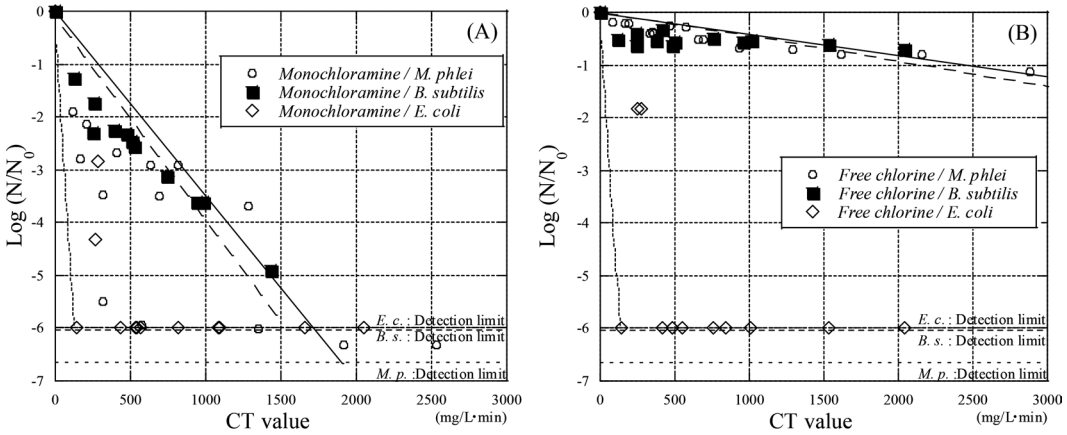

Fig. 2　Inactivation curves by (A) monochloramine and (B) free chlorine disinfection of *M. phlei*, *B. subtilis*, and *E. coli*. The vertical axis "Log (N/N_0)" indicates the logarithm of N_0 (the number of colonies at the beginning of the experiment) and N (the number of colonies at each CT value). The horizontal axis "CT value" indicates the CT (concentration × time) value, i.e., the product of the disinfectant concentration and contact time. Experimental data for *M. phlei* were taken from Mori *et al*. (2022). The solid line indicates the inactivation of *M. phlei*, the dashed line indicates the inactivation of *B. subtilis*, and the dotted line indicates the inactivation of *E. coli*. Under the conditions employed here, colony numbers of *E. coli* in many samples were below the detection limit. For such cases, the line connecting the intersection of the origin and the plot with the lowest CT value is displayed as the inactivation curve for reference.

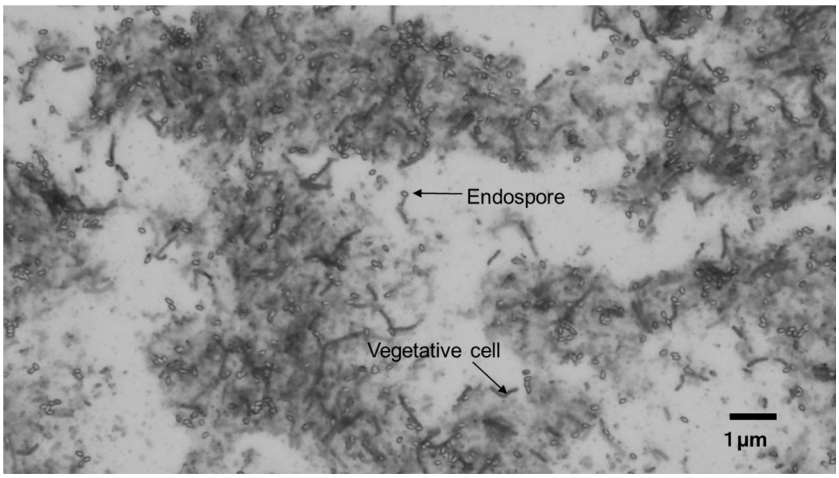

Fig. 3　Image of the bacterial solution cultured under the same conditions as the solution.

This image shows that endospores were formed and were mixed with vegetative cells. Based on this observation, the mixture of endospores and vegetative cells was considered to interpret the experimental results of this study.

The detailed correlation between *B. subtilis* and free chlorine disinfection is shown in Fig. 4.

The results shown in Fig. 4 indicate that inactivation proceeded immediately up to a CT value of approximately 400 mg/L·min ; however, it did not progress beyond approximately 500 mg/L·min.

The change in slope of the inactivation curve could have resulted from the addition of the bacterial solution containing the endospore and vegetative cell mixture. The vegetative cells with low resistance to free chlorine were inactivated first, and the endospores with high resistance to free chlorine became dominant. Furthermore, endospore inactivation was extremely low under free chlorine disinfection in alkaline hot spring waters.

This two-step inactivation of *B. subtilis* was also observed in *B. subtilis* under disinfection with monochloramine (Fig. 2 (A)). Under the conditions employed here, inactivation proceeds immediately up to a CT value of approximately 250 mg/L·min, but after that, the slope of the inactivation curve appears to change slightly to a smaller slope. The reason for this may be the same as that of *B. subtilis* under free chlorine disinfection.

These results indicate that the resistance of *M. phlei* to disinfection was comparable to that of *B. subtilis* endospores. The disinfection resistance of *M. phlei* is presumably due to its lipid-rich cell wall. In highly alkaline hot spring water disinfected with free chlorine, it is difficult to control *M. phlei* without cleaning the bathtub and piping or washing them with a highly concentrated disinfectant.

3.3　Inactivation of *E. coli* in relation to CT values

The correlation between the inactivation ratio of *E. coli* and the CT values from the disinfection test is shown in Fig. 2.

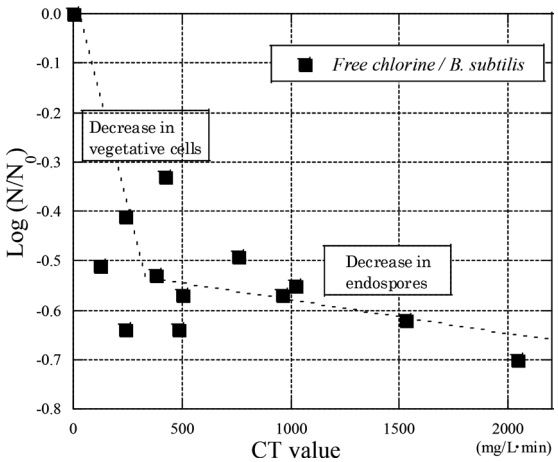

Fig. 4　Inactivation curves by free chlorine disinfection of *B. subtilis*. This figure is an enlarged portion of Fig. 2 (B).

E. coli colonies could not be counted 15 min after first sampling, indicating that colony numbers in many samples were below the detection limit.

A minimum disinfectant concentration of 5 mg/L and a 15 min disinfection time resulted in the inactivation of 5 to 6-Log or more at a CT value of 75 mg/L·min. The gram-negative bacterium *E. coli* was inactivated to a reasonable degree, even with free chlorine disinfection, in alkaline hot spring waters.

3.4 Control of *M. phlei* and *B. subtilis* in a highly alkaline hot spring

The results of this study indicate that approximately 1-Log of *M. phlei* and *B. subtilis* can be inactivated at a CT value of approximately 2,000 mg/L·min under free chlorine disinfection of alkaline hot spring water conditions.

Assuming that 0.5 mg/L free chlorine disinfectant was used, the calculations revealed that 1-Log disinfection could be achieved after 4,000 min (67 h or approximately 3 days). The time required for disinfection is too long, and the bacterial count may increase rather than decrease during this time.

Therefore, controlling *M. phlei* and *B. subtilis* growth is difficult using free chlorine in alkaline hot spring waters. This could lead to advancing biofilm formation in pipes and filters disinfected with free chlorine.

However, we assumed that a monochloramine concentration of 3 mg/L in alkaline hot spring waters would suffice. In this case, the time required for 3-Log disinfection was estimated to be 250 min (4.2 h or approximately 0.2 days), which is considered practical.

Considering that there have been cases where *M. phlei* was detected in large numbers when monochloramine was used continuously, an effective treatment for *M. phlei* may be an important research topic for future widespread use of monochloramine.

4. Conclusion

In this study, alkaline hot spring water was disinfected using free chlorine and monochloramine at pH 9.6 for the removal of *B. subtilis*, *E. coli*, and *M. phlei*.

Based on the experimental results, we compared the inactivation of *B. subtilis* and *E. coli*. with that of *M. phlei*. One-Log inactivation of these bacteria except *E. coli*. required a CT value of approximately 250 mg/L·min for monochloramine versus 2,000 mg/L·min for free chlorine.

The results showed that the resistance of *M. phlei* and *B. subtilis* against disinfection was approximately the same.

These results indicate that monochloramine is more effective than free chlorine for inactivating these bacteria in highly alkaline hot spring waters.

Better statistical strength and more reliable findings can be obtained by increasing the number of experiments. Accumulation of more experimental data will be the subject of future work on this aspect.

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