EFFECT OF SUNLIGHT ON COLIPHAGES IN AN OXIDATION POND

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ABSTRACT

Escherichia coli B were used as host cells for isolation and multiplication of coliphages. The coliphages were isolated from a small canal which receives the effluent from an oxidation pond in Bangkok, Thailand, and were used as a virological indicator. Coliphages were assayed by the plaque forming technique (PFU method). The coliphage which was used in this study had no resistance to acid (pH 3) and weak resistance to alkali (pH 10). The rate of coliphages in the oxidation pond was investigated by field measurement and laboratory experiments.

Batch experiments showed the adsorption of coliphage to microbial particulates (mainly algae) occurred under aerobic conditions. The desorption of coliphage from the particulates was observed under anaerobic conditions. The adsorption-desorption process was reversible and was controlled by dissolved oxygen concentration. The same mechanism of adsorption-desorption was observed in the oxidation pond as well. During day-time, the concentration of coliphage decreased under high concentration of dissolved oxygen caused by photosynthesis. On the other hand, the concentration of coliphage increased after sunset because the dissolved oxygen concentration in the pond decreased to zero due to respiration of algae.

The degree of removal of coliphage in the oxidation pond (design retention time = 20 days) was 90% (1 log). Field measurement with submerged bottles in the oxidation pond and sunlight-exposure experiment using beakers showed that coliphage could be inactivated by sunlight only near the water surface (less than 10 cm depth, the highest estimate) in the oxidation pond.

KEYWORDS

Coliphage, oxidation pond, sunlight, inactivation, adsorption-desorption, dissolved oxygen.

INTRODUCTION

Increase in water reuse has necessitated an understanding of the inactivation process or survival mechanism of enteric viruses in natural waters and in water/wastewater treatment plants for preventing waterborne diseases caused by pathogenic viruses. The inactivation of enteric viruses is affected by many factors (temperature, pH, turbidity, sunlight intensity and cation concentration). There is a lack of information on the effect of various factors on the inactivation process. Especially, the virological investigation in
natural waters is difficult due to low concentration of virus. The investigation of virus survival in oxidation ponds or lagoons will be useful for understanding the fate of virus both in natural water and in water/wastewater treatment processes because oxidation ponds or lagoons have high concentration of virus and are exposed to natural environmental conditions. Mahdy (1979) and Yano et al. (1985) pointed out that human viruses has been readily and repeatedly detected in sewage effluents which, after various degrees of treatment, enter waterways and become a part of rivers and streams that are the sources of drinking water. Smith et al. (1978) and Dahling and Safferman (1979) showed the long survival of enteric viruses under natural environmental conditions.

Coliphages and other bacteriophages have been discussed as indicators for survival of pathogenic enteric viruses. Some recent studies showed the possibility of using coliphage as a viral model in water pollution (Kott et al., 1978, IAWPRC Study Group, 1983 and Grabow et al., 1984), due to the following reasons: coliphages outnumber enteric viruses in normal water environments, they are more resistant to unfavorable environmental conditions, detectable by simple and economical techniques which yield results within one day, and are no risk to human health. Different viruses have naturally different characteristics, so one virus, such as coliphage, cannot be representative of all pathogenic enteric viruses. Some studies indicated that under all circumstances the coliphages could not suit all criteria (Bell, 1976, and Neimi, 1976). The controversial results are no doubt caused by different methods, media and phage strains. So further investigations are required for developing a precise standard method and assessing the advantages and/or disadvantages of coliphage as a viral indicator. Information on the fate of coliphage in natural waters would be useful not only for routine virological surveillance but also for further insight into virus transport and survival in aquatic environments.

This study was composed of three parts: 1) Field measurement of coliphage in an oxidation pond, 2) Laboratory experiments on the adsorption-desorption mechanism of coliphage to suspended particulates caused by dissolved oxygen concentration change, 3) Inactivation tests of coliphage by sunlight.

MATERIAL AND METHODS

Oxidation Pond

The oxidation ponds for wastewater treatment of Asian Institute of Technology (AIT) were investigated for field measurement. AIT is situated in Bangkok (lat. 14°N), Thailand. The oxidation ponds treat the wastewater from the research and education activity of the Institute and from the dormitory (about 900 residents). The oxidation pond system is composed of two ponds (design retention time = 8 days and 20 days) in series (see Fig. 1).
Coliphage and Determination

Media. Phage agar for the isolation of coliphages and coliphage assays consisted of 0.8% Nutrient Broth (Difco, or 0.1% Bacto peptone, 0.3% beef extract), 0.5% NaCl, 0.02% MgSO₄·7H₂O, 0.005% MnSO₄·4H₂O, 0.15% dextrose and 1.1% Bacto agar (Difco, adjusted to a final pH = 7.2). All plates were dried at 45°C before use. The formula of the soft agar was the same as above, except that 0.6% instead of 1.1% Bacto-agar was used. Phage broth contained the same composition as phage agar but without the Bacto-agar. This medium was used for culturing the host strains (E. coli B) and also for diluting phage samples.

Bacterial culture. Escherichia coli B was employed in a pure culture as the host bacterium for quantitative determination and propagation of coliphage. E. coli B was obtained from the Division of Agricultural and Food Engineering, Asian Institute of Technology, Thailand. The culture of E. coli B was inoculated into 10 mL of phage broth and incubated at 37°C for 3.5-4.0 h before use for phage isolation and plaque forming method.

Isolation of coliphage. Natural water was collected from a canal Klong Nueng in Bangkok. This water sample (10 mL) was centrifuged at 12 000 rpm for 10 min at 4°C. Supernatant was then filtered through a 0.45 µm membrane filter. Coliphage in the filtrate was isolated by the Agar-Layer method. Equal volume (0.1 mL) of filtrate was inoculated on the surface of phage agar, and then 4 mL of melted soft agar including 0.3 mL of young bacterial culture supplemented with 0.2 mL of 0.1 mol/L CaCl₂ was added. After thorough mixing the plates were incubated at 37°C overnight. The coliphage was purified by repeated single-plaque isolation as follows: Single plaques with surrounding medium were transferred by sterilized loop to 2 mL of phage broth and incubated at 37°C for 4 h. The broth was centrifuged at 12 000 rpm for 10 min at 4°C. The supernatant was then serially diluted ten-fold and plated as before but using the appropriate E. coli B host cells. The second and the third single-plaque isolation followed the same procedure.

Propagation of coliphage. After the third single-plaque isolation, the equal size of single plaques were transferred into phage broth (ratio = one plaque to 2 mL) and this phage broth was mixed with bacterial culture (volume ratio = 20:1). The mixture was then incubated at 37°C for 2-3 h until becoming clear. The mixture was centrifuged at 12 000 rpm for 10 min at 4°C and filtered with a sterilized 0.45 µm membrane filter. Filtrate was kept at 4°C as stock phage. Propagation of coliphage by this method yielded 10⁸-10⁹ plaques/mL.

Coliphage determination. Coliphage concentration measurement was carried out by the agar-layer method (plaque forming method). The sample (0.1 mL) was mixed with 0.3 mL of young bacterial culture, 0.2 mL of CaCl₂ solution (CaCl₂·2H₂O, 15 g in 1000 mL of distilled water) and 4.0 mL of melted soft agar were mixed and poured over the surface of an ordinary phage agar plate and allowed to set to form a thin layer. After incubation at 37°C overnight, each plaque appeared as a clear area in the plaque lawn of E. coli B. Suitable dilution was made to obtain 30 to 300 plaques on a plate. PFU (plaque forming unit) per volume was used as the unit for coliphage concentration.

Sensitivity of coliphage to pH. The inactivation of coliphage at different pH is shown in Fig.2 (Ohgaki et al 1985). The coliphage used in this research was stable in the pH range of 6.0-9.0. At this pH range, the inactivation of coliphage was between 5% to 30%. It could be seen that this coliphage is more sensitive in the acid side than in the alkali side. The inactivation of coliphage was larger than 99.9% at pH = 4.4.

Adsorption-Desorption Experiments with Dissolved Oxygen Concentration Change

The microbial particulates (mainly algae) in the first pond were harvested. Solution containing 65 mg/L of the microbial particulates was aerated in 300 mL bottles by air pump for 3 hours after addition of the appropriate amount of coliphage stock solution. After aeration, the bottles were allowed to stand at room temperature (about 25°C) for 21 hours, and were aerated again. All bottles were covered with aluminum foil.
Dissolved oxygen concentration was measured by a DO-meter or Azide-modification method. Coliphage concentration in liquid phase and the amount of coliphage adsorbed to particulates were determined by the following method. The microbial particulates were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was used for coliphage concentration measurement. The residue was suspended again with 3% beef extract (the ratio of beef extract to sample = 1:10–1:20) at pH 9.0 and was then shaken for 10 min (75 rpm) at room temperature (about 25°C). After shaking, it was filtered through a 0.45μm membrane filter. The filtrate (eluate) was used for coliphage concentration measurement. This elution method for solid-combined coliphage was based on Seeley's method (1979).

Inactivation Experiments by Sunlight

Sunlight exposure laboratory experiment. Coliphages in pH buffer solution were exposed to sunlight. The conditions and results of four experimental runs (Run A, B, C and D) are summarized in Table 1. The sunlight intensity, or sunlight radiation received, was measured by a bimetallic actinograph.

Submerged bottles experiments in pond. Glass bottles (300mL) which contained pH buffer solution inoculated with coliphages were submerged in the second pond at depth just below the surface, 10 cm depth below the surface and 100 cm depth below the surface, from 9:30 am to 4:00 pm (6 hours 30 minutes). The bottles used were the same as Run C. All bottles were supported by bamboo rods as shown in Fig.3. Half the bottles were covered with aluminum foil and were protected from exposure to sunlight.
RESULTS AND DISCUSSION

Coliphage Concentration Change in Ponds

The variation of coliphage concentration, DO, water temperature and pH during daytime in the first pond and in the second pond are shown in Fig. 4(a) and Fig. 4(b) respectively. DO increased to 20 mg/L at 3:00 pm from 0 mg/L at 6:00 am and pH reached around 9.0 at 3:00 pm caused by photosynthetic activity of algae (the suspended solid concentration was 77.5 mg/L in the first pond and 66.3 mg/L in the second pond). "Total coliphage concentration" in Fig.4 represents the value of sample without any treatment. "Filtrate coliphage concentration" in Fig.4 represents the value of the sample filtered by a glass fiber filter (GF/C). Both values showed a minimum value at 3:00 pm. However, after sunset, the concentration recovered to the level of 6:00 am. The variation pattern of coliphage concentration in the second pond was similar to that in the first pond. The decrease of coliphage concentration from dawn to 3:00 pm would not have been either due to inactivation by sunlight, pH increase or temperature increase. If the inactivation had caused the decrease of coliphage, the coliphage concentration would not have recovered after sunset. This phenomenon suggests that a kind of reversible process on coliphage change exists in ponds.

The ratio of coliphage concentration in the first pond to the second pond was about ten (1 log). The coliphage concentration decreased approximately to only 90% in 20 days (design retention time value of the second pond). Omura et al. (1984) investigated the coliphage removal in the same ponds and reported similar results. This result shows that the coliphage removal was not high in the second pond in spite of strong sunlight intensity and high water temperature.

![Graphs showing changes in DO, temperature, pH, and coliphage concentration over time](image-url)

Fig.4 Variation of coliphage concentration (9 May 1985)

(a) 1st pond, ss=77.5, COD_{cr}(Total)=110.7, COD_{cr}(Filtrate)=20.9mg/L,

(b) 2nd pond, ss=66.3, COD_{cr}(Total)=78.2, COD_{cr}(Filtrate)=22.9mg/L,
The results of adsorption-desorption experiments with microbial particulates of oxidation pond are presented in Fig. 5. Dissolved oxygen concentration (DO) change in bottles is shown in Fig. 5(a). After aeration was stopped, the DO decreased and reached zero after 3-4 hours due to the respiration of algae and other microbial organisms. During aeration, the coliphage concentration in supernatant decreased and the coliphage concentration in eluate increased caused by adsorption of coliphage to algae and other microbial particulates (Fig. 5(b)). On the other hand, when DO decreased, the coliphage concentration
in supernatant increased and the coliphage concentration in eluate decreased due to desorption of coliphage from algae and others. During anaerobic condition, the coliphage concentration in supernatant regained the initial value. The second aeration showed the same result as the first one (see Fig.6).

The increased amount of coliphage in the eluate (maybe adsorbed amount) was only about 1% of the decreased amount in the supernatant (Fig.5(b)). The imbalance in the change of coliphage count between the supernatant and the eluate (Fig.5(b)) was due to the low recovery-efficiency of elution method. However, both coliphage concentrations in the supernatant and in the eluate changed simultaneously.

These results show that a reversible adsorption-desorption process was the principal cause of coliphage concentration change in this system, and the process was controlled by DO. A similar phenomenon in activated sludge process was reported by Shimohara, Sugishima and Kaneko (1984). They showed that nitrogen gas aeration instead of aeration with air inhibited the virus (poliovirus 1) removal and increased slightly the virus concentration later in the activated sludge system.

Inactivation by Sunlight

Survival analysis. The "one hit one target" model (Kondo, 1972) was used for the analysis of coliphage inactivation by sunlight. The survival is expressed by the model as follows;

\[ S = e^{- \frac{23 \cdot 10^6}{R - R_{10}}} \]

where S: survival of coliphage [-]

R: sunlight radiation received [cal/cm²]

R10: inactivation dose (the sunlight dose required to reduce survival to 10%, that is, the sunlight dose required to inactivate 90% of initial value) [cal/cm²]

R10 was used as a quantitative indicator of inactivation by sunlight in this study.

<table>
<thead>
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<th>Run</th>
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<th>depth in vessel, cm</th>
<th>sunlight exposure condition</th>
<th>pH</th>
<th>temperature, °C</th>
<th>suspended cont., mg/L</th>
<th>R₁₀ ** (inactivation dose), cal/cm²</th>
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*NOTE*) (D) means "direct expose" and (G) means "through glass".

*NOTE **) R₁₀ in parentheses is the value of R₁₀ under dark condition.
Sunlight exposure experiment and submerged bottles experiment. Both results of exposure experiment and submerged bottles experiments are summarized in Table 1. The indicator for inactivation rate in dark condition (case with cover) should be expressed as a function of time instead of a function of the radiation received (R) because the inactivation rate in dark condition is not affected directly by sunlight radiation. However, it is convenient to use the "inactivation dose (R10)" for the quantitative comparison between covered conditions and uncovered conditions.

The difference between the case with aluminum foil cover (Run A) and the case without cover (Run B) is presented in Fig. 7. The R10 (over 1000 cal/cm²) of Run A implied a kind of background rate of inactivation, that is, inactivation rate due to factors other than sunlight in this experimental system. The effect of glass on inactivation rate by sunlight (Run C and B) is shown in Fig. 8. Glass decreased the intensity of sunlight and the R10 value of Run A was 230 cal/cm², almost three times that of the R10 of Run B. It was confirmed, however, that the sunlight was able to inactivate coliphage in bottles through bottle's glass wall. The results of submerged bottles experiments (Run E, F, G and H) are shown in Fig. 9. The inactivation dose (R10) of 10 cm depth bottles was the same as 100 cm depth bottles, and no difference between covered cases and uncovered cases at both measurement points was observed (Run G and H in Table 1, and Fig. 9). On the other hand, the R10 value of the uncovered bottle at surface was 700 cal/cm² and that the covered bottle was 1100 cal/cm² (Run E and F in Table 1 and Fig. 9).

These results show that coliphages were inactivated by sunlight radiation only near the water surface in the pond, and they were not inactivated at greater depth because of diminishing radiation intensity, especially ultra-violet rays, through water.
**CONCLUSION**

Escherichia coli B were used as host cells for isolation and propagation of coliphages. The coliphages were assayed by the plaque forming technique (PFU method). The coliphage which was used in this study had no resistance to acid (pH3) and weak resistance to alkali (pH10).

Field experiments with submerged bottles in the oxidation pond and sunlight-exposure experiment showed that coliphage could be inactivated by sunlight only near the water surface (less than 10 cm depth, the highest estimate) in the oxidation pond. The degree of removal of coliphage in the oxidation pond (design retention time = 20 days) was 90% (1 log).

Laboratory experiments showed that the adsorption of coliphage to microbial particulates (mainly algae) occurred in aerobic conditions. The desorption of coliphage from the particulates was observed under anaerobic conditions. The adsorption-desorption process was reversible and was controlled by dissolved oxygen concentration. The same mechanism of adsorption-desorption was observed in the oxidation ponds as well. This indicates that aerobic condition would be required for adsorption of coliphage to microbial particulates, and the biological adsorption appears to be the principal cause of the coliphage-adsorption phenomenon in natural waters containing microbial particulates. The virus combined with particulates should be considered for assessing virological pollution.

The effect of sunlight on coliphages in oxidation ponds is summarized as follows: Sunlight inactivates directly coliphages only at the surface in oxidation ponds. The photosynthesis by algae under sunlight radiation increases dissolved oxygen concentration and coliphages are adsorbed in part to microbial particulate under aerobic conditions. During the night, under the conditions without sunlight, the dissolved oxygen is diminished to zero and coliphages are desorbed from particulates under anaerobic conditions.

**ACKNOWLEDGMENT**

This work was supported in part by Japan International Cooperation Agency (JICA). Ms. Orasa Suthienkul, Lecturer, Department of Microbiology, Faculty of Public Health, Mahidol University, Thailand, is gratefully thanked for her useful advice and comments.
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