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Title: Fate of coliphage in a wastewater treatment process

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Running title: Bacteriophage in the wastewater treatment process

ABSTRACT

Fate of coliphage in a wastewater treatment process in the central part of Japan were investigated from March to December 2001. Relatively abundant coliphage, 1,000-10,000 PFU/ml determined by three different *E. coli* strains, was detected in the influent to the process. But, no remarkable seasonal change of phage concentration in the influent was observed during the ten-month test period. Almost ten times higher coliphage concentration was determined by the F^+ *E. coli* strain than by the other two F^- strains. The RNA phage was more stable than the DNA phage against the aerobic treatment using activated sludge. Most of the phages in the influent and primary settling tank were detected as suspended forms. Anaerobic-aerobic treatment enhanced adsorption of the phage into the solid particle. Almost no phage was detected in the effluent from the process. Aerobic treatment using activated sludge and/or addition of flocculants such as PAC were effective for the removal of coliphage, an index of enteric viral pollution.

Key words: *E. coli*, domestic wastewater, wastewater treatment process, coliphage, activated sludge

Domestic wastewater has been documented as a vector of viral diseases, but many of the primarily involved agents are difficult to detect. For practical purposes such as monitoring of the wastewater treatment process and formulation of quality standards for water effluent, a model indicator would be desirable. Bacteriophage has the potential for use as a process indicator of viruses (1, 2, 3). The physical structures of certain types of bacteriophage resemble that of enteroviruses and such types of bacteriophage can be cultivated by a simple, rapid, accurate and inexpensive method. Considerable attention has been given to bacteriophages capable of infecting *Escherichia coli* because of its specific faecal nature. Detection of somatic coliphage, which binds to receptors located on the cell wall (somatic receptors), varies with the host strain. *E. coli* C is the most productive host for somatic coliphage (4). Alternatively, F-specific bacteriophages have been proposed as suitable model indicators of the concentration of human enteric viruses in a water treatment process because of their size and their relatively high resistance to inactivation (5).

Activated-sludge treatment is known to reduce the concentration of enteric pathogens. It was suggested that virus loss during this treatment is due to attachment of viruses to wastewater particulates, which subsequently settle and become a component of sludge (6). Other evidence indicates that mixed liquor suspended solids (MLSS) of activated sludge inactivate and remove virucidal agents (7). Most studies of bacteriophages in a wastewater treatment process are based on the characterization of pure phages seeded to the process or model environments. There are marked differences between the removal and/or inactivation of seeded and naturally occurring bacteriophages. In this study, seasonal change of the coliphage concentration was determined by three different *E. coli* host strains in the influent of a certain domestic wastewater treatment process and fate of

phages in the process were monitored for ten months.

Samples for phage enumeration were obtained from an urban wastewater treatment process in the central part of Japan, approximately monthly from March to December 2001. A schematic diagram, indicating ports for sampling, is shown in Fig. 1. The number of inhabitants around this process was about 200,000. Most of the contamination was of human origin. No animal farms and industries were located around the plant. The daily volume of the influent was 28,000 m^3. There was no rainwater inflow to the process. Hydraulic retention times in each step were: 55 min in the primary settling tank, 60 min in the anaerobic tank, 6 h in the aerobic tank, and 4 h in the secondary settling tank. Polyaluminum chloride (PAC), at a final concentration of 2.5 mg-Al/ *l*, was added to the secondary settling tank as a flocculant. Sodium hypochlorite (NaClO) was added to the chlorination tank to a final concentration of 0.02 mg-free-Cl/*l*. The average parameter values for influent quality for the period of one year were BOD_5, 260 mg/*l*; COD, 120 mg/*l*; and total suspending solids (TSS), 190 mg/*l*; and the average parameter values for effluent quality were: BOD_5, 3 mg/*l*; COD, 10 mg/*l*; and TSS, 5 mg/*l*. Samples were pooled in sterile 100 m*l* tubes at 4 ℃ and transported to the laboratory within 24 h. An aliquot 50 m*l* of each sample was centrifuged (1,000 g, 10 min), and then the obtained supernatant was carefully transferred to a new sterilized tube, centrifuged (13,000 g, 5 min) and then subjected to enumeration of suspended phages. The solid content in a sample was defined as the wet pellet concentration mg (wet weight)/g after the 1,000-g centrifugation in each sample. The pellet obtained after 1,000-g centrifugation was resuspended in an elution buffer (pH = 10) containing 0.5 wt% NaCl and 0.05 M boric acid, vortexed for 1 min and allowed to stand for 90 min at room temperature to detach the phages from the solid matter. Then, the sample was centrifuged

(13,000 g, 5 min) and the obtained supernatant was subjected to enumeration of phages attached to solid substances.

The coliphage concentration was analyzed by a double-layer plaque assay after 16 h incubation at 37 ℃ with three *E. coli* strains, *E. coli* K-12 (W3110) F^-, *E. coli* K-12 HfrH (IAM 12017) F^+, and *E. coli* C. Luria-Bertani (LB) broth was used for culturing and plaque assay. *E. coli* W3110 is a female (F^-) strain of K12 derivative. *E. coli* C is also a female strain. On the other hand, *E. coli* HfrH is a male (F^+) strain of K-12 derivative. When required, RNase (from bovine pancreas) was added to the plaque assay mixture to a final concentration of 100 μg/m*l* and incubated at 37 ℃. RNase was dissolved in water at a concentration of 10 mg/m*l* and boiled for 5 min before use. The values for each experiment are based on the average of triplicate enumeration.

Coliphage infectious to the three *E. coli* strains was constitutively detected in the supernatant of the influent from March to December 2001. It was reported that *E. coli* C was generally found to yield the highest plaque counts among *E. coli* species (8). This can be explained by the absence of a DNA restriction system in this strain and by the presence of a broad range of receptors. However, the most abundant phage detected in the influent in this study was that infectious to *E. coli* HfrH (IAM 12017) F^+ strain, with a value of $1x10^3 \sim x10^4$ PFU/m*l*, which was almost ten times larger than those of *E. coli* K-12 (W3110) F^- and *E. coli* C phages (Fig. 2). When the *E. coli* HfrH F^+ strain was used for the examination, the resulting plaque counts reflected both the number of somatic phages and the number of F-specific ones. Theoretically, a count of F-specific phages could be obtained by subtracting the results for an F^- strain from those for an isogenic F^+ strain. When the RNase was premixed with the plaque assay mixture, *E. coli* HfrH (IAM 12017) F^+ specific phage concentration was reduced from 2/3 to 1/2 compared to the

value obtained without RNase treatment, indicating that the proportion of the RNA phage was 1/3-1/2.

The phage concentration in each step of a wastewater treatment process was determined to monitor the fate of phage in the wastewater treatment process. The arithmetical mean of the phage concentration during the test period of the process is shown in Fig. 3. General trends of the fate of phage in the process in each month were almost identical. Since the phage concentration in the effluents from the secondary settling tank and the chlorination vessel became below the identification limit of plaque assay in some cases, arithmetical mean instead of geometrical mean was used for the analysis. The phage concentrations infectious to *E. coli* K-12 (W3110) F^-, *E. coli* HfrH (IAM 12017) F^+ and *E. coli* C remain almost constant in the influent and primary settling tank. A slight decrease was observed in the anaerobic tank. On the other hand, a significant decrease in the phage concentration was observed in the aerobic tank and in the secondary settling tank. Only a few phages (PFU/m*l*) were detected in the supernatant of the secondary settling tank. RNase treatment of the sample from the aerobic tank reduced the phage concentration infectious to *E. coli* HfrH (IAM 12017) F^+ to 1/50, indicating that phages infectious to *E. coli* HfrH (IAM 12017) F^+ in the aerobic tank are predominantly RNA phages. This shows that the RNA phage was more stable than the DNA phage against aerobic treatment using activated sludge. Phage concentration in the returned sludge was higher than that of aerobic tank. No phage was detected in the effluent from the process except phages infectious to the *E. coli* HfrH (IAM 12017) F^+ strain without RNase treatment.

The average fraction of suspended phage in the supernatant and attached phage to solid substances through test period in the process is shown in Fig. 4. The average solid

content in each step during the test period is shown in Fig. 1B. Even though the detached efficiency of the phage from the solid particle by mixing with the elution buffer was not 100 %, large number of the phage was detached from the centrifuged pellet by this procedure. Most of the phages in the influent and primary settling tank were detected as suspended forms. Even though the total phage concentration in the anaerobic tank was reduced compared with that of primary settling tank, attached phage concentration in the this tank was increased. Significant reduction of suspended phages in the aerobic tank was observed. Two independent mechanisms are thought to be involved in the virus removal by the activated sludge. In the first stage, virus is removed from the liquid phase by adsorption onto the flock, whereas in the second stage, virus is removed by predation by other microbes, i.e., protozoa or metazoa (9, 10). The increase of the attached phage concentration in the anaerobic tank was thought to be caused by the phage transition to the solid particle from the liquid phase and the inflow of the returned sludge from the secondary settling tank. The drastic reduction of the suspended phage in the aerobic tank would be due to the phage adsorption onto the flock followed by predation by other microbes. Adsorbed phage onto the flock settled down by the addition of PAC in the secondary settling tank and remained stably in the sludge. Almost no phage was detected in the supernatant of the secondary settling tank and effluent from the process.

As far as we know, the seasonal change and fate of coliphage in a domestic wastewater treatment process have not been well studied. The wastewater treatment process investigated in this study was one of the typical facilities in Japan with the daily capacity of 28,000 m^3 for 200,000 inhabitants. No remarkable seasonal change of phage concentration in the influent to the process was observed during the ten-month test period. Even though the phage concentration in the influent to the process was estimated

1,000-10,000 PFU ml^{-1} order, almost no phage was detected in the effluent from the process. Aerobic treatment using activated sludge and/or addition of flocculants such as PAC were effective for the removal of coliphage, an index of enteric viral pollution.

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FIGURE LEGENDS

Fig. 1 (A) Schematic diagram indicating ports for sampling. Numbers in the diagram indicate the position of the sampling ports: 1-influent, 2- supernatant from the primary settling tank, 3-anaerobic tank, 4-aerobic tank, 5-returned sludge, 6-supernatant from the final settling tank, and 7-effluent. (B) Averages of solid content.

Fig. 2 Average phage concentration in influents from March to December 2001.

Fig. 3 Average phage concentration in each step of the wastewater treatment process. Numbers in the x-axis indicate the positions of the sampling ports in conformity with those shown in Fig.1 (A).

Fig. 4 The fraction of suspended phage in the supernatant and attached phage to solid substances in each step of the wastewater treatment process. Open bar indicates suspended phage concentration and solid bar indicates attached phage concentration. Numbers in the x-axis indicate the positions of the sampling ports in conformity with those shown in Fig.1 (A).