Fluctuation of densities of bacteriophages and *Escherichia coli* present in natural biofilms and water of a main channel and a small tributary

Hiroshi Hirotani, Ma Yu and Takeshi Yamada

ABSTRACT

Fluctuation of bacteriophage and *Escherichia coli* densities in naturally developed riverbed biofilms were investigated for a 1-year period. *E. coli* ranged from 1,500 to 15,500 most probable number (MPN)/100 mL and from 580 to 18,500 MPN/cm² in the main channel in the river water and biofilms, respectively. However, the fluctuations were much greater in the tributary, ranging from 0.8 to 100 MPN/100 mL and from 0.3 to 185 MPN/cm² in water and biofilms, respectively. The fluctuations of coliphages were also greater in the tributary than in the main channel. FRNA phage serotyping results indicated no significant differences in the source type of the fecal contamination in the main channel and tributary sampling stations. Significant correlations between phage groups in biofilms and water were found at both main channel and tributary. It was assumed that natural biofilms developed in the streambed captured and retained somatic phages in the biofilms for a certain period of time in the main channel site. At the location receiving constant and heavy contamination, the usage of phage indicators may provide additional information on the presence of viruses. In the small tributary it may be possible to estimate the virus concentration by monitoring the *E. coli* indicator.

Key words | fecal indicators, microbial source tracking, natural biofilms, somatic phage, tributary

INTRODUCTION

To evaluate the sanitary condition of the river, it has been a common practice to measure indicator organisms to detect fecal contamination. In Japan, total coliforms serve as a water quality standard indicating fecal pollution for river, lake and ocean water. Since total coliform counts inevitably contain false positive results, more than 70% of the locations still do not meet the coliform standard (http://www.env.go.jp/water/suiiki/h15/index.html). Today, it is widely known that total coliforms include bacteria not related to fecal contamination, and therefore the *Escherichia coli* indicator has been suggested as a better alternative for detecting fecal contamination in water (Toranzos et al. 2002). However, this indicator bacterium has been found to exist in the pristine environment in the tropics (Carrillo et al. 1985; Fujioka & Shizumura 1985) and it is suspected that biofilms harbor *E. coli* to result in false positive results (Balzer et al. 2010; Hirotani & Yoshino 2010, 2012).

Another indicator system that has been suggested as an alternative to the coliform bacteria are the bacteriophages, which may indicate a better relation with the presence of viral pathogens (Dutka 1973; Wu et al. 2011). There is still some need for information on the propagation and survival of bacteriophages in the environment, including the relation of those with natural biofilms. Doolittle et al. (1995) observed that phage T4 are able to infect the host bacterium cells within the extracellular matrix of the experimentally formed biofilms, thus abundance of bacteriophages may be augmented in the presence of biofilms. Flood & Ashbolt (2000) suggested that virus-sized particles can be entrapped and concentrated in the wetland biofilms.

Natural biofilms are complex communities developed in the solid–water interface in the natural river environment. Formation of river biofilms and their activities have been studied in many aspects including nutrient condition (Blenkinstopp & Lock 1990; Mohamed et al. 1998; Battin et al. 2003) and flow velocity (Hall-Stoodley et al. 2004; Hirotani et al. 2008). Since characteristics of natural biofilms are altered by the surrounding conditions during development, the effect of biofilms on indicator organisms may be differed by the environmental conditions of the sampling stations. In
this study, indicator bacteria and bacteriophages in the natural river biofilms and in the river water are measured monthly over a 1-year period. The investigation was done in the main channel and the tributary to compare the behavior of indicators in those environments taking into account the affection of the biofilms.

MATERIALS AND METHODS

Study site and sampling

The study was done in the Yamatogawa watershed in Osaka, Japan. Water samples and stones were collected monthly from June 26, 2009 to May 26, 2010 from two sampling stations. Station 1 (Sta. 1) was located in the main channel (discharge = ca. 10 m²/s) of the Yamato River, Japan, which receives treated sewage water from a population of ca. 1.2 million distributed in a catchment 800 km² in size. Sta. 2 was located in a small tributary (discharge = ca. 0.5 m²/s), the Hara River, assumed to have a daytime population in the order of a few hundred within its watershed of 10 km² (Figure 1). Sampling was done about the same hour during the day. Water samples were taken directly to the sterile polypropylene bottles. Small stones (usually three), which were emergent on the streambed under the river water, were grab-sampled from the river bed and stored in Ziploc bags (SC Johnson & Son, Racine, WI, USA). The samples were brought to the laboratory within 3 h on ice. Water temperature was measured on site using calibrated alcohol thermometers.

Biofilm developed on the surface of the sampled stones was scrubbed off with nylon toothbrushes into sterile water (200 mL) in the laboratory. The biofilm samples thus prepared and the water samples were subjected to microbial analyses. The surface areas of the stones were measured by the following empirical formula (Hirotani et al. 2008):

\[
\text{Surface area (cm}^2\) \; = \; 3.51 \times \text{weight (g)}^{2/3}
\]

The total surface area of the stones in each batch sample ranged from 231.1 to 341.5 cm².

Sample analyses

Total coliforms and E. coli were enumerated by the most probable number (MPN) method using Colilert 18 and Quanti-Tray/2000 (Idexx, Westbrook, ME, USA). Although total coliforms and E. coli in water are usually expressed as numbers per 100 mL, they were expressed per one milliliter in this study to compare their densities in biofilm surface (per one cm²). Bacteriophages were measured by the single-layer plaque method (Grabow & Coubrough 1986), using E. coli ATCC 15597 and ATCC 13706 as hosts. Each sample (50 mL) was distributed in five Petri dishes. Salmonella Typhimurium WG49 were also used as a host to monitor the fluctuation of F+ RNA coliphages, but we failed to assay them in some instances during the monthly survey due to the experimental conditions. Therefore the fluctuation of F+ RNA coliphages is not included in this study.
Microbial source tracking

Source tracking of fecal pollution was done by genotyping of F+ RNA coliphages (Vinjé et al. 2004). Briefly, plaque obtained on layer of Salmonella Typhimurium WG49 was assayed by reverse transcription-PCR (PrimeScript One Step RT-PCR Kit Ver. 2, Takara, Kyoto, Japan) to classify the coliphages into subgroups I through IV, which are known to associate with either animal or human wastes (Osawa et al. 1981).

RESULTS AND DISCUSSION

Overview of fluctuation of indicators

The range of the fluctuation of E. coli densities in water at Sta. 1 was within one order of magnitude (Figure 2). The fluctuation of the densities of somatic phages in the main channel, which are detected with E. coli ATCC 13706, ranged even smaller, within one order of magnitude. However, the densities of the coliphages detected with E. coli ATCC 15597, the total of somatic and FRNA phages, fell in the summer months, and the difference between the maximum and the minimum reached almost two logs. At Sta. 2, the range of fluctuation of E. coli was greater than two orders of magnitude, in which no seasonality was assumed. Two phage groups fluctuated rather similarly, and the decrease in bacteriophage densities corresponded with the incidence of E. coli decrease. Since in some instances more coliphages were detected with E. coli ATCC 13706 than with ATCC 15597, not all the somatic phages that infected E. coli ATCC 13706 seem to infect ATCC 15597.

Monthly fluctuations of the densities of E. coli and somatic phage were rather small in the river water in the main channel, whereas they were rather large and were in correspondence in the small tributary. Since the catchment size is much smaller in the tributary, a change in the fecal load may affect the indicator densities in water immediately once it had occurred. On the contrary, the main channel with a huge catchment area and a greater river flow is hardly affected by each contamination event in the upper reaches within the catchment.

The fluctuations of E. coli densities in biofilms were more obvious at Sta. 2 than at Sta. 1 (Figure 3). Coliphages detected in the biofilm from the main channel site using both hosts fluctuated almost identically. E. coli and coliphages in the tributary biofilms also indicated somewhat similar fluctuation. The range of fluctuation was greater in the biofilm than in the water for all indicators.

Source of fecal contamination

FRNA phages belonging to three serological groups were detected in the sample from the main channel, whereas in sample from the tributary all the determinable phage belonged to the single serogroup (Table 1). According to Fisher’s exact probability test, however, the difference in the assumed origin of the fecal contamination in two sample stations was not significant (P = 0.22). The source of contamination in the main channel and tributary was considered to be chiefly human, and the difference was not distinguishable. Hence, the difference between the features observed at Sta. 1 and 2 was considered to be independent of the types of the contamination source.

Relation of phages and E. coli

The correlations between E. coli and phage groups were not significant at Sta. 1 either in river water or biofilms,
whereas they were all significant at Sta. 2 (Table 2). In a small tributary with rather small stream flow, a fluctuation in fecal contamination load may have affected both bacterial and phage indicator level. However, in a rather large river receiving a constant contamination throughout the reach, \textit{E. coli} densities indicated a fairly steady amount within one order of magnitude. Some authors report the positive correlation of coliphages and coliforms in natural water (Kernard \& Valentine 1973; Araujo et al. 1991), but some vice versa (Hilton \& Stotzky 1973; Toranzos 1991). In this study the relation was significant only at one station with a smaller catchment with a relatively small discharge. Since the fecal contamination level was similar and there was no significant difference in the contamination source, the reason causing this discrepancy could be the condition of the river environment including the biofilms. To our knowledge, this is the first study reporting on the relation of phages and a fecal indicator bacterium in natural biofilms. In the small tributary the estimation of the virus concentration may be achieved only by monitoring \textit{E. coli}.

### Relation of indicator microorganisms in water and biofilms

The correlation of \textit{E. coli} in water and biofilms was not significant either in the main channel or the tributary (Table 3). This may be due to the growth of \textit{E. coli} in the biofilm matrix during the elongated retention time in the environment. The correlations of phage groups were significant except for the somatic phages in the tributary. This suggests the entrapment and the prompt release of phage particles from and to the water and biofilm matrix. Replication of coliphages in rivers seems unlikely (Lucena et al. 2003). The effect of predation was not obvious. However, the somatic phages, whose correlation was not significant in the main channel,

<table>
<thead>
<tr>
<th>Serological group</th>
<th>Sta. 1 (Main channel)</th>
<th>Sta. 2 (Tributary)</th>
<th>Assumed origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0</td>
<td>Animal</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>5</td>
<td>Human</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
<td>Human</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>0</td>
<td>Animal</td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

out of ca. 10 pfu/mL.

<table>
<thead>
<tr>
<th>Phage host</th>
<th>Sta. 1 (Main channel)</th>
<th>Sta. 2 (Tributary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>\textit{E. coli} 15597</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>\textit{E. coli} 13706</td>
<td>0.44</td>
</tr>
<tr>
<td>Biofilm</td>
<td>\textit{E. coli} 15597</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>\textit{E. coli} 13706</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*\(P < 0.05\).

### Table 3 | Correlation coefficient (r) of \textit{E. coli} and coliphages between water and biofilms

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Sta. 1 (Main channel)</th>
<th>Sta. 2 (Tributary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli}</td>
<td>(-0.07)</td>
<td>0.44</td>
</tr>
<tr>
<td>Phage (host \textit{E. coli} 15597)</td>
<td>0.66*</td>
<td>0.59*</td>
</tr>
<tr>
<td>Phage (host \textit{E. coli} 13706)</td>
<td>(-0.07)</td>
<td>0.64*</td>
</tr>
</tbody>
</table>

*\(P < 0.05\).
may have survived for a certain period of time. The reason for the lack of correlation of somatic phages in the main channel is not clear, but the size of the viral particles and the difference in the structure composition of the biofilms from the tributary could be the cause. From the above results, it can be assumed that some virus groups are captured and retained in the biofilms for some period, and therefore it can be said that biofilms may protect the virus from the inactivation, for example, caused by sunlight.

**CONCLUSION**

Fluctuation of phage densities and *E. coli* in naturally developed riverbed biofilms were investigated in the main channel and tributary of a river over a 1-year period. The fluctuations of *E. coli* and somatic phage were moderate in the river water in the main channel, whereas they were rather large and were in correspondence in the small tributary. The difference in the indicator densities observed in the tributary and in the main channel was independent of the contamination source types. Biofilms may capture and retain some groups of viruses, which may assist survival of viruses in the river environment. Generally, the phage indicators are expected to provide information on the presence of viruses, whereas in the small tributary the monitoring of viral indicators in addition to *E. coli* indicator may not be necessary to assure the absence of the viral risks.

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**REFERENCES**


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