

## Usefulness of monitoring tropical streams for male-specific RNA coliphages

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### ABSTRACT

The objective of this study was to evaluate the usefulness of monitoring streams in Hawaii for FRNA coliphages as a reliable indicator of sewage contamination. This study was undertaken as a result of our previous findings that monitoring streams in Hawaii for traditional faecal indicator bacteria (faecal coliform, *Escherichia coli*, enterococci) was not useful in determining when streams are contaminated with sewage, because environmental (soil) sources rather than sewage accounted for the high concentrations of faecal bacteria in streams. Two perennial streams, sewage and soil samples were monitored for traditional faecal indicator bacteria (faecal coliform, *E. coli*, enterococci) and FRNA coliphages. The results showed that sewage treatment processes and disinfection drastically reduced the concentrations of traditional faecal indicator bacteria but FRNA coliphages were still present in significant concentrations in the treated sewage effluents. These results indicate that monitoring sewage effluents and environmental waters for only traditional faecal indicator bacteria may not be adequately protective of human health effects. Ambient concentrations of traditional faecal indicator bacteria in soil and streams of Hawaii were consistently high but consistently low for FRNA coliphages, indicating that monitoring streams of Hawaii for FRNA coliphages can be used to determine when streams are contaminated with sewage.

**Key words** | coliphage, faecal indicator bacteria, sewage contamination

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### INTRODUCTION

Concentrations of US Environmental Protection Agency (USEPA)-approved faecal indicator bacteria (*Escherichia coli*, enterococci) in environmental waters are used to determine the extent of sewage contamination and to establish recreational water quality standards. These water quality standards are used to determine the risk or probability that someone using that body of water for primary contact recreational use (e.g. swimming) will become ill from sewage-borne pathogens. The current USEPA-recommended marine recreational water quality standards were developed from the results of previously completed epidemiological and water quality studies at three beach sites (New York City, Boston Harbor and Lake Pontchartrain) in the United States. These sites were selected because concentrations of faecal bacteria due to nearby sewage discharges were high but acceptable. Simi-

lar studies were conducted for fresh recreational waters at lakes in Pennsylvania and Oklahoma. Based on the results of these studies (Cabelli *et al.* 1982; USEPA 1986), USEPA recommended a new recreational water quality standard for marine waters not to exceed a geometric mean concentration of 35 enterococci 100 ml<sup>-1</sup> based on five weekly samples taken over a month. For fresh waters, USEPA-recommended standards are based on similar geometric mean concentrations of 33 enterococci 100 ml<sup>-1</sup> or 126 *E. coli* 100 ml<sup>-1</sup>.

The results of these USEPA studies showed that concentrations of enterococci in marine and fresh waters correlated with incidences of swimming-associated gastroenteritis, whereas concentrations of *E. coli* correlated with swimming-associated gastroenteritis only in fresh waters. A significant conclusion of the USEPA studies was

that concentrations of faecal coliforms in marine and fresh waters did not correlate with swimming-associated gastroenteritis. USEPA concluded that the most likely sewage-borne pathogens causing gastroenteritis were Norwalk type viruses (Cabelli *et al.* 1982). It was concluded that *E. coli* was too unstable in marine waters to serve as a reliable surrogate for the presence of sewage-borne pathogens. It should be noted that USEPA conducted a similar epidemiological study at a lake in Connecticut. This lake was not contaminated with sewage, and faecal indicator bacteria in the lake were from non-point sources such as wild animals rather than from a point source (sewage). Under these conditions, the concentrations of enterococci and *E. coli* did not correlate with swimming-associated gastroenteritis (Calderon *et al.* 1991).

An analysis of the USEPA studies shows that the source of faecal indicator bacteria must be known to determine the relative risk to swimmers. If recreational waters are contaminated with sewage, the risk to swimmers is high and the concentrations of enterococci and/or *E. coli* in the water are likely to be correlated to swimming-associated gastroenteritis. Under these conditions, the incidences of gastroenteritis as predicted by USEPA recreational water quality standards are reasonable. However, if recreational waters are contaminated by non-point source pollution, the risk to swimmers will be lower and the concentrations of faecal indicator bacteria will probably not be correlated with swimming-associated gastroenteritis. Under these conditions, the incidences of swimming-associated gastroenteritis as predicted by USEPA recreational water quality standards should not be applicable. In this regard, many beaches in the United States are contaminated with non-point source rather than point source (sewage) pollution (Novotny 1988; USEPA 1998). However, since monitoring data for faecal indicators cannot differentiate between non-point source and point source pollution, most states take the conservative approach and assume that the source of contamination is sewage.

In the application and interpretation of recreational water quality standards, USEPA makes two assumptions, both of which are not applicable to the state of Hawaii. The first assumption is that the source of faecal indicator bacteria is primarily animal faeces and there are no signifi-

cant environmental (non-faecal) sources of these bacteria. However, in Hawaii these faecal indicator bacteria were consistently present in soil samples taken throughout the state (Hardina & Fujioka 1991; Fujioka & Byappanahalli 2001). For many of these sites, faecal contamination is not evident. The second assumption is that these faecal indicator bacteria will not multiply under environmental conditions. However, in Hawaii, compelling evidence has been obtained to show that *E. coli* and enterococci are able to multiply in soil. Moreover, soil conditions in Hawaii provide for the essential growth conditions (temperature, moisture, nutrients) to support the growth of these faecal bacteria. Based on these results, Byappanahalli and Fujioka (1998) reported that these faecal bacteria have become established as minor populations of the soil microflora. However, the populations of faecal indicator bacteria in soil are significant because rainfall washes these soil-associated faecal bacteria into streams, where the concentrations of all faecal bacteria consistently exceed recreational water quality standards.

In summary, all streams and storm drains on Hawaii and Guam contain concentrations of faecal indicator bacteria that exceed recreational water quality standards (Fujioka *et al.* 1988, 1999). Moreover, streams or storm drains which discharge into coastal waters often result in elevated levels of faecal bacteria in waters at swimming beaches. Although we (Hardina & Fujioka 1991; Fujioka & Byappanahalli 2001) provided evidence that the source of these faecal indicator bacteria is primarily soil (low risk) and not sewage (high risk), the method used to monitor these faecal bacteria cannot determine whether the source is soil or sewage. Under these conditions, the probability that the source of faecal bacteria is sewage must always be considered.

It is clear that there is a need to develop methods that can determine the source of faecal bacteria because the source of faecal bacteria determines the probable presence of pathogens. If the source of faecal bacteria is sewage, all human pathogens may be present and the health risk to humans would be very high. If the source of faecal bacteria is animals only, pathogenic human enteric viruses could not be present although many bacterial and protozoan pathogens may be present. Thus, the health risk to humans would be moderate. If the source of faecal bacteria is

multiplication in the environment (e.g. soil), human viruses and protozoan parasites could not be present and the health risk to humans would be low.

The inability of existing methods to determine the source of faecal bacteria has been recognized as a limitation by USEPA. As a result, USEPA is now supporting a programme called bacterial source tracking to develop methods to identify the source of the faecal bacteria (USEPA 2001). Most of the current approaches use molecular methods to further characterize the isolated faecal bacteria to determine their source (Scott *et al.* 2002). The approach taken in Hawaii is to monitor environmental waters for alternative faecal indicator bacteria such as *Clostridium perfringens* (*C. perfringens*), which are more specifically related to sewage than traditional faecal indicator bacteria. Using this approach, evidence for the presence of sewage in streams in Hawaii can be determined based on concentrations of *C. perfringens* but not by determining the concentrations of faecal indicator bacteria (Fujioka & Shizumura 1985; Fujioka *et al.* 1997). Elevated levels of *C. perfringens* and faecal indicator bacteria indicate that the source of faecal indicator bacteria is sewage. Low concentrations of *C. perfringens* and elevated concentrations of faecal indicator bacteria indicate that the source of faecal bacteria is the environment (soil).

A review of the literature shows that many environmental microbiologists believe that the best alternative faecal indicator for sewage and for possible presence of human enteric viruses is the group of male-specific RNA viruses (FRNA coliphages), which infect pilated strains of enteric bacteria such as *E. coli* and *Salmonella* bacteria. Numerous studies (Havelaar *et al.* 1990; IAWPRC 1991; Sobsey *et al.* 1995; Schaper *et al.* 2002) have reported that monitoring for FRNA viruses in environmental waters and shellfish provides reliable data for the presence or absence of human enteric viruses. Some of the compelling reasons for the usefulness of FRNA coliphages are as follows: (1) FRNA coliphages have the same shape, size and type of nucleic acid as human enteric viruses; (2) FRNA coliphages are more resistant to disinfection and to environmental factors than faecal bacteria, and this stability is similar to human enteric viruses; (3) FRNA coliphages are consistently present in relatively high concentrations in human sewage; and (4) the methods to

enumerate FRNA coliphages are feasible, sensitive and selective. The goal of this study was to evaluate the feasibility of monitoring streams in Hawaii for FRNA coliphages as reliable indicators of faecal contamination as compared with the traditional faecal indicator bacteria.

## METHODOLOGY

The study was conducted on the island of Oahu, Hawaii. Sewage samples were obtained from two wastewater treatment plants. The first plant (Sand Island) only treats sewage to the primary level, whereas the second plant (Wahiawa) processes sewage by primary treatment, secondary treatment (activated sludge) and disinfection by chlorination. Stream samples were obtained from two typical perennial streams (Manoa, Nuuanu), which flow through urbanized areas of Honolulu and do not receive sewage effluent discharges. Both streams are designated for recreational use. Soil samples were obtained at least 12 cm below the surface from the following sites: (1) near the banks of streams, (2) from a grassy area on the campus of the University of Hawaii, (3) from a farm owned by the University of Hawaii, and (4) from the backyards of private homes. Human faeces were collected from 20 persons (9 males, 11 females) whose age ranged from 4 months to 71 years. The ethnicity of the human subjects was as follows: nine Asians, seven Caucasians, three Asian/Caucasians and one Samoan. Faecal samples from several animals were obtained from Animal Service of the University of Hawaii. The pigeon faeces were collected from a nearby park, whereas faeces from cow, sheep, pig and quail were collected from the farm owned by the University of Hawaii. All samples were collected in sterile plastic bottles. Ten per cent sodium thiosulfate was added to samples containing chlorinated sewage samples. All samples were stored in a covered iced chest during transport and assayed within 5 hours of collection.

The elution method described by Camper *et al.* (1985) was used to elute bacteria and phages from soil samples. The membrane filtration methods described in *Standard Methods* (1992) were used to assay for faecal coliform, *E. coli* and enterococci. The method described by Bisson

**Table 1** | Geometric mean concentrations of faecal indicator bacteria and FRNA phages in sewage

Sewage sample	Bacteria (CFU 100 ml <sup>-1</sup> )				FRNA phage (PFU 100 ml <sup>-1</sup> )
	Faecal coliform	<i>E. coli</i>	Enterococci	<i>C. perfringens</i>	
Raw ( <i>n</i> = 4)	3.20 × 10 <sup>7</sup>	2.72 × 10 <sup>7</sup>	2.77 × 10 <sup>5</sup>	7.14 × 10 <sup>4</sup>	4.32 × 10 <sup>6</sup>
Primary treated ( <i>n</i> = 10)	3.19 × 10 <sup>7</sup>	1.52 × 10 <sup>7</sup>	2.96 × 10 <sup>5</sup>	7.96 × 10 <sup>4</sup>	1.57 × 10 <sup>6</sup>
Secondary treated ( <i>n</i> = 4)	1.39 × 10 <sup>5</sup>	1.39 × 10 <sup>5</sup>	1.66 × 10 <sup>4</sup>	5.75 × 10 <sup>2</sup>	8.51 × 10 <sup>3</sup>
Chlorinated ( <i>n</i> = 4)	8	7	11	1.78 × 10 <sup>2</sup>	2.03 × 10 <sup>3</sup>

*n* = number of samples assayed

and Cabelli (1979) was used to assay for *C. perfringens*. Male-specific RNA viruses were assayed on piliated strains of *E. coli* HS(pFamp)R and *Salmonella typhimurium* (*S. typhimurium*) WG-49 using the double agar method described by Havelaar *et al.* (1990) and by Debartolomeis and Cabelli (1991).

## RESULTS

### Concentrations of faecal indicator bacteria and FRNA coliphages in sewage

Since faecal bacteria are naturally present in high concentrations in sewage, they are called faecal indicator bacteria, especially when they are recovered from environmental waters. The relative concentrations of faecal indicator bacteria (faecal coliform, *E. coli*, enterococci, *C. perfringens*) and FRNA coliphages were determined in raw, primary treated, secondary treated and chlorinated sewage effluent samples from the Sand Island Sewage Treatment Plant and from the Wahiawa Sewage Treatment Plant. The results (Table 1) show that the relative geometric mean concentrations of all faecal microorganisms were similar in raw and primary treated sewage. In these samples the concentrations of faecal coliform and *E. coli* were highest (~10<sup>7</sup> 100 ml<sup>-1</sup>), followed in decreasing order by concentrations of FRNA coliphages (~10<sup>6</sup> 100 ml<sup>-1</sup>), enterococci (~10<sup>5</sup> 100 ml<sup>-1</sup>) and *C. perfrin-*

*gens* (~10<sup>4</sup> 100 ml<sup>-1</sup>). Secondary treatment (activated sludge process) reduced the concentrations of all faecal microorganisms by approximately 99%. The chlorination step resulted in the greatest reduction of faecal coliform and *E. coli* (~99.99%), followed by enterococci (~99.9%). In contrast, chlorination did not substantially reduce the concentrations of *C. perfringens* and FRNA coliphages. As a result, the secondary treated and chlorinated sewage effluent contained geometric mean concentrations of <10 CFU 100 ml<sup>-1</sup> of faecal coliform and *E. coli*, ~11 CFU 100 ml<sup>-1</sup> of enterococci, 178 CFU 100 ml<sup>-1</sup> of *C. perfringens* and 2,000 PFU 100 ml<sup>-1</sup> of FRNA coliphages.

### Concentrations of indicator bacteria and FRNA coliphages in two model streams

The relative concentrations of all faecal microorganisms were determined by collecting water samples from Manoa and Nuuanu streams as they flowed through the urbanized area of Honolulu. Both were considered model streams because they are perennial streams flowing out of a pristine watershed area directly through an urbanized area and do not receive sewage effluent or other point source discharges. The results (Table 2) show that geometric mean concentrations of approximately 10<sup>5</sup> CFU 100 ml<sup>-1</sup> of faecal coliform, *E. coli* and enterococci were recovered from Manoa Stream, whereas slightly lower (10<sup>2</sup> CFU 100 ml<sup>-1</sup>) concentrations of these same faecal bacteria

**Table 2** | Geometric mean concentrations of faecal indicator bacteria and FRNA phages in streams

Stream site	Bacteria (CFU 100 ml <sup>-1</sup> )				FRNA phage (PFU 100 ml <sup>-1</sup> )
	Faecal coliform	<i>E. coli</i>	Enterococci	<i>C. perfringens</i>	
Manoa	2.55 × 10 <sup>3</sup> (13/13)*	2.49 × 10 <sup>3</sup> (13/13)*	1.69 × 10 <sup>3</sup> (13/13)*	12–14 (10/13)*	2–6 (7/13)*
Nuuanu	5.06 × 10 <sup>2</sup> (13/13)*	4.41 × 10 <sup>2</sup> (13/13)*	3.38 × 10 <sup>2</sup> (13/13)*	7–9 (9/13)*	8–34 (6/13)*

\*(No. of positive samples/No. of samples assayed)

were recovered from Nuuanu Stream. The three faecal indicator bacteria were recovered in high concentrations in 13 of 13 samples from both streams. In these same 13 stream water samples, *C. perfringens* was detected in 10 of 13 samples from Manoa Stream and in 9 of 13 samples from Nuuanu Stream. The geometric mean concentrations of *C. perfringens* for both streams ranged from 7 to 14 CFU 100 ml<sup>-1</sup>. In comparison, FRNA coliphages were detected in only 7 of 13 samples from Manoa Stream and 6 of 13 samples from Nuuanu Stream. The geometric mean

concentrations of FRNA coliphages in both streams ranged from 2 to 34 PFU 100 ml<sup>-1</sup>.

### Concentrations of faecal indicator bacteria and FRNA coliphages from soil samples

The relative concentrations of all faecal microorganisms in soil were determined by analysing soil samples from six distinct sites (stream banks, farm, backyards, campus). The results (Table 3) show that faecal bacteria (faecal

**Table 3** | Concentrations of faecal indicator bacteria and FRNA phages in soil

Soil site	Bacteria (CFU g <sup>-1</sup> )					FRNA phage (PFU g <sup>-1</sup> )
	Faecal coliform	<i>E. coli</i>	Enterococci	<i>C. perfringens</i>		
Manoa stream bank No. 1	2.10 × 10 <sup>3</sup>	3.20 × 10 <sup>3</sup>	1.60 × 10 <sup>1</sup>	6.24 × 10 <sup>2</sup>	< 3	
Backyard of a house no. 1	3.20 × 10 <sup>2</sup>	1.28 × 10 <sup>2</sup>	1.12 × 10 <sup>2</sup>	3.52 × 10 <sup>2</sup>	< 3	
Manoa stream bank No. 2	3.20 × 10 <sup>1</sup>	1.60 × 10 <sup>1</sup>	3.20 × 10 <sup>0</sup>	8.00 × 10 <sup>2</sup>	< 3	
Backyard of a house no. 2	3.20 × 10 <sup>1</sup>	3.20 × 10 <sup>1</sup>	3.04 × 10 <sup>1</sup>	1.12 × 10 <sup>2</sup>	< 3	
Farm	1.60 × 10 <sup>3</sup>	6.40 × 10 <sup>3</sup>	5.60 × 10 <sup>4</sup>	5.60 × 10 <sup>3</sup>	< 3	
Grassy area on University campus	ND	ND	ND	ND	< 3	

ND=Not done

**Table 4** | Concentrations of faecal indicator bacteria and FRNA phages recovered from animal faeces

Animal faeces	Bacteria (CFU g <sup>-1</sup> )				FRNA phage (PFU g <sup>-1</sup> )
	Faecal coliform	<i>E. coli</i>	Enterococci	<i>C. perfringens</i>	
Guinea pig ( <i>n</i> = 2)	8.00 × 10 <sup>5</sup>	1.17 × 10 <sup>5</sup>	1.57 × 10 <sup>5</sup>	< 67	< 3
Rat ( <i>n</i> = 2)	1.08 × 10 <sup>7</sup>	1.03 × 10 <sup>7</sup>	6.33 × 10 <sup>6</sup>	< 67	< 3
Chicken ( <i>n</i> = 3)	1.62 × 10 <sup>8</sup>	1.62 × 10 <sup>8</sup>	3.81 × 10 <sup>7</sup>	67	4.1 × 10 <sup>1</sup>
Cat ( <i>n</i> = 2)	1.32 × 10 <sup>6</sup>	1.46 × 10 <sup>6</sup>	3.57 × 10 <sup>5</sup>	5.50 × 10 <sup>4</sup>	< 3
Rabbit ( <i>n</i> = 2)	1.55 × 10 <sup>7</sup>	1.57 × 10 <sup>7</sup>	3.23 × 10 <sup>6</sup>	< 67	< 3
Mice ( <i>n</i> = 2)	2.03 × 10 <sup>6</sup>	3.83 × 10 <sup>6</sup>	1.99 × 10 <sup>6</sup>	< 67	< 3
Monkey ( <i>n</i> = 2)	1.45 × 10 <sup>7</sup>	2.35 × 10 <sup>7</sup>	1.35 × 10 <sup>5</sup>	< 67	< 3
Pig ( <i>n</i> = 1)	5.73 × 10 <sup>7</sup>	5.80 × 10 <sup>7</sup>	9.33 × 10 <sup>6</sup>	1.73 × 10 <sup>5</sup>	< 3
Quail ( <i>n</i> = 1)	5.40 × 10 <sup>7</sup>	3.40 × 10 <sup>7</sup>	5.80 × 10 <sup>6</sup>	< 67	2.36 × 10 <sup>2</sup>
Sheep ( <i>n</i> = 1)	3.47 × 10 <sup>5</sup>	2.80 × 10 <sup>5</sup>	1.67 × 10 <sup>6</sup>	4.07 × 10 <sup>4</sup>	< 3
Cow ( <i>n</i> = 1)	1.87 × 10 <sup>6</sup>	1.87 × 10 <sup>6</sup>	4.27 × 10 <sup>4</sup>	2.67 × 10 <sup>2</sup>	< 3
Dog ( <i>n</i> = 1)	6.47 × 10 <sup>4</sup>	7.13 × 10 <sup>4</sup>	5.33 × 10 <sup>4</sup>	1.47 × 10 <sup>4</sup>	< 3
Pigeon ( <i>n</i> = 1)	1.87 × 10 <sup>7</sup>	1.71 × 10 <sup>7</sup>	5.00 × 10 <sup>6</sup>	< 67	< 3

*n*=number of samples assayed

coliform, *E. coli*, enterococci, *C. perfringens*) were consistently detected in soil samples, whereas FRNA coliphages were below detectable levels (<3 per 10 g of soil) in all soil samples.

### Concentrations of faecal indicator bacteria and FRNA coliphages in animal faeces

The relative concentrations of all faecal microorganisms in animal faeces were determined by analysing faeces from 13 different warm-blooded animals. The results (Table 4) show that faecal indicator bacteria (faecal coliform, *E. coli*, enterococci) were consistently recovered in elevated concentrations from faeces of all 13 animals. In contrast, *C. perfringens* was detected in only 46% (6 of 13)

of the animal faeces, whereas FRNA coliphages were detected in faeces of only two animals (chicken, quail).

### Concentrations of faecal indicator bacteria and FRNA coliphages in human faeces

The relative concentrations of all faecal microorganisms in human faeces were determined by analysing faeces from 20 human subjects of various gender, age and ethnic make-up. The results (Table 5) show that faecal coliform and *E. coli* were consistently recovered in elevated geometric mean concentrations (10<sup>7</sup> CFU g<sup>-1</sup>) from faeces of all 20 human subjects, whereas enterococci were recovered in 19 of 20 subjects at a lower geometric mean concentration (10<sup>4</sup> CFU g<sup>-1</sup>). In contrast, *C. perfringens*

**Table 5** | Geometric mean concentrations of faecal indicator bacteria and FRNA phages recovered from 20 human faeces

<b>Bacteria (CFU g<sup>-1</sup>)</b>				
<b>Faecal coliform</b>	<b><i>E. coli</i></b>	<b>Enterococci</b>	<b><i>C. perfringens</i></b>	<b>FRNA phage (PFU g<sup>-1</sup>)</b>
2.93 × 10 <sup>7</sup> (20/20)*	2.8 × 10 <sup>7</sup> (20/20)*	8.57 × 10 <sup>4</sup> (19/20)*	24.5–247 (9/20)*	< 3 (0/20)*

\*(No. of positive samples/No. of samples assayed)

Faecal samples from: 9 males and 11 females; age 4 mo.–71 years; 1 Samoan, 9 Asian, 7 Caucasian, 3 Asian/Caucasian

was detected in only 9 of 20 faecal samples at an even lower geometric mean concentration (10<sup>2</sup> g<sup>-1</sup>) and FRNA coliphages were not detected in any of the 20 human faecal samples.

### Temperature dependence of formation of pili by *E. coli* HS(pFamp)R

It has been previously reported that formation of pili by *E. coli* HS(pFamp)R and *S. typhimurium* WG-49 occurs only at a temperature >25°C and that this is the reason for the production of FRNA coliphages in animals but not under environmental conditions. To assess the effect of temperature on pili formation, pilated *E. coli* HS(pFamp)R as well as two environmental *E. coli* isolates (J-2, L-1) were grown at 25°C, 30°C, 34°C and 37°C. These cultures were then spot-tested with 54 presumptive FRNA coliphages, which were isolated either on *E. coli* HS(pFamp)R or *S. typhimurium* WG-49. The results (Table 6) show that all 54 FRNA phages produced plaques in *E. coli*

HS(pFamp)R grown at temperatures ranging from 30 to 37°C, but 0 of 54 phages produced plaques when *E. coli* HS(pFamp)R was grown at 25°C. These results confirmed previous reports that *E. coli* HS(pFamp)R will produce pili at 30°C but not at 25°C. The inability of the 54 phages to produce plaques in *E. coli* isolate L-1 grown at all temperatures (25 to 37°C) supports previous reports that most environmental isolates of *E. coli* do not have the genes or plasmid to produce pili. However, environmental *E. coli* isolate J-2 was unusual in that 2 of 54 phages produced plaques when this strain was grown at 37°C. These results suggest that environmental isolate J-2 was unusual and may be able to produce limited pili only at 37°C.

### Host range of environmental isolates of FRNA coliphages

In this study, FRNA coliphages were detected based on their ability to infect and cause plaque formation on a

**Table 6** | Effect of growth temperature on three *E. coli* strains to support plaque production following inoculation by 54 phages recovered by pilated *E. coli* and *S. typhimurium*

<b><i>E. coli</i> host</b>	<b>Fraction of 54 FRNA phages producing plaques in <i>E. coli</i> hosts grown at:</b>			
	<b>25°C</b>	<b>30°C</b>	<b>34°C</b>	<b>37°C</b>
HS(pFamp)R	0/54	54/54	54/54	54/54
J-2 isolate	0/54	0/54	0/54	2/54
L-2 isolate	0/54	0/54	0/54	0/54

**Table 7** | Host range and plaque production by 55 phages recovered from the environment by piliated *E. coli* and *S. typhimurium*

Host bacteria	Growth conditions	Plaque produced by fraction of phages recovered on piliated:	
		<i>E. coli</i> HS(pFamp)R	<i>S. typhimurium</i> WG-49
<i>E. coli</i> HS(pFamp)R (piliated strain)	37°C	20/20	35/35
<i>E. coli</i> HS(pFamp)R (piliated strain)	37°C + RNase	3/20	4/35
<i>E. coli</i> (non-piliated strain)	37°C	1/20	0/35
<i>S. typhimurium</i> (WG-49) (piliated strain)	37°C	19/20	35/35
<i>S. typhimurium</i> (WG-49) (piliated strain)	37°C + RNase	2/20	4/35
<i>S. typhimurium</i> (non-piliated strain)	37°C	0/20	0/35
Fraction of 45 <i>E. coli</i> environmental isolates susceptible to all FRNA phages	37°C	1/45	2/45

specially developed piliated strain of *E. coli* HS(pFamp)R or piliated strain of *S. typhimurium* WG-49. Both piliated bacteria were developed to specifically support the growth of FRNA coliphages. However, since some FDNA phages can also infect these piliated bacteria and some somatic phages can occasionally infect these piliated bacteria, the isolated phages were further characterized by two methods. The first method was the addition of RNase in the agar medium, which prevents plaque formation by FRNA but not FDNA phages. The second method was to determine the ability of the isolated phages to infect piliated and nonpiliated bacterial hosts. Using these two methods, the percentage of the 20 phages recovered on *E. coli* HS(pFamp)R and the 35 phages recovered on *S. typhimurium* WG-49 that were FRNA and FDNA coliphages, could be determined. The results (Table 7) show that all (20 of 20) of the phages recovered on *E. coli* HS(pFamp)R formed plaques on *E. coli* HS(pFamp)R and 19 of 20 phages formed plaques on *S. typhimurium* WG-49. In the presence of RNase, 3 of 20 (15%) phages formed plaques on *E. coli* HS(pFamp)R whereas 2 of 20 (10%) phages formed plaques on *S. typhimurium* WG-49, indicating that 10 to 15% of the phages recovered on *E. coli* HS(pFamp)R were

actually FDNA phages. None of the 20 phages produced plaques on nonpiliated *S. typhimurium*, whereas 1 of 20 phages produced plaques on nonpiliated *E. coli*. This same phage produced plaques in one of the 45 environmental isolates of *E. coli*. Taken together, these results indicate that of the three phages determined to be DNA phage, one was atypical and could even be a DNA somatic phage.

The 35 phages isolated on *S. typhimurium* WG-49 were similarly characterized. The results (Table 7) show that all 35 phages produced plaques on *E. coli* HS(pFamp)R and on *S. typhimurium* WG-49, indicating that these phages required pili to produce plaques. In the presence of RNase, 4 of 35 phages formed plaques on *E. coli* HS(pFamp)R and on *S. typhimurium* WG-49, indicating that 11% of the phages recovered on *S. typhimurium* WG-49 are FDNA phages. None of the 35 phages recovered on *S. typhimurium* WG-49 formed plaque on nonpiliated *E. coli* or nonpiliated *S. typhimurium*, indicating that none of the 35 phages was capable of infecting nonpiliated enteric bacteria. When the 45 environmental isolates of *E. coli* were used as potential hosts, two phages formed plaques and both on the same *E. coli* isolate (J-2). Based on previous data (Table 6), J-2 isolate appears to be

unusual and may be capable of forming some kind of pili at 37°C or may have unusual somatic receptors.

## DISCUSSION

Traditional faecal indicator bacteria (faecal coliform, *E. coli*, enterococci) as well as alternative faecal microorganisms (*C. perfringens*, FRNA coliphages) are found in high concentrations in raw and primary treated sewage. Today, most sewage must undergo secondary treatment and chlorination before it can be discharged into environmental waters. Most wastewater treatment plants receive National Pollution Discharge Elimination System (NPDES) permits, which require that the final sewage effluent be monitored for traditional faecal indicators as evidence that the sewage has been adequately treated and disinfected. The results (Table 1) of this study confirm that secondary treatment and chlorination of sewage effluent can reduce the densities of traditional faecal indicator bacteria to very low levels ( $\sim 10$  CFU 100 ml<sup>-1</sup>), which provides assurance that this sewage effluent has been adequately treated for disposal into environmental waters. However, the same sewage effluent contained moderate concentrations ( $10^2$  CFU 100 ml<sup>-1</sup> and  $10^3$  PFU 100 ml<sup>-1</sup>, respectively) of alternative faecal indicators such as *C. perfringens* and FRNA coliphages (Table 1). These results question the current procedure of relying on monitoring sewage effluents for faecal indicator bacteria because the expected resistance of sewage-borne pathogens (e.g. human enteric viruses) to disinfection and to environmental factors is similar to FRNA coliphages and is greater than faecal indicator bacteria (IAWPRC 1991).

Environmental waters designated for recreational use are also monitored for traditional faecal indicators to determine whether the water meets recreational water quality standards. When concentrations of faecal indicator bacteria exceed recreational water quality standards, USEPA recommends that the body of water be posted as unsafe for swimming and be placed off limits. Under most conditions, it is assumed that environmental waters are contaminated with sewage and therefore the risk to swimmers is very high. However, on Oahu, Hawaii, all streams

have been previously reported (Fujioka *et al.* 1988; Hardina & Fujioka 1991) to contain elevated concentrations of all traditional faecal indicator bacteria, which routinely exceed recreational water quality standards. The results of the current study (Table 2) confirm the high concentrations of traditional faecal indicators in the two streams in Oahu, under ambient conditions. In these same stream samples *C. perfringens* and FRNA coliphages were detected in only 46 to 77% of the water samples and their geometric mean concentrations were low, ranging from 2 to 34 PFU 100 ml<sup>-1</sup> (Table 2). These results confirm the previous conclusion (Hardina & Fujioka 1991) that, because of high concentrations of faecal indicator bacteria under ambient conditions, sewage contamination of streams cannot be determined by monitoring for faecal indicator bacteria only. These results also confirm the previous conclusion (Fujioka *et al.* 1997) that, because of low ambient concentrations of *C. perfringens* in streams, monitoring streams for elevated concentrations of *C. perfringens* is a reliable means to determine when streams are contaminated with sewage.

In Hawaii, soil has been reported (Byappanahalli & Fujioka 1998) as the natural habitat of faecal indicator bacteria. Rain, the source of all streams, transports the soil-associated faecal bacteria to streams. The soil environment in Hawaii provides the conditions (temperature, moisture, nutrients) that enable these faecal bacteria to multiply and become part of the soil microflora. These results indicate that the concentrations of faecal indicator bacteria in streams represent soil contamination rather than sewage contamination. The results of the current study (Table 3) confirm the presence of high concentrations of faecal indicator bacteria in soil environments of Hawaii. In contrast, soil concentrations of *C. perfringens* were relatively low and FRNA coliphages could not be detected in soil. In summary, the same ratio of high concentrations of faecal indicator bacteria and relatively low concentrations of *C. perfringens* and FRNA coliphages in soil was observed in streams. These results support the conclusion that soil is the source of faecal indicator bacteria in streams.

The non-point source of faecal indicator bacteria pollution in streams is usually attributed to animal faeces. The results of the current study (Table 4) show that animal

faeces consistently contain high concentrations of faecal indicator bacteria. In contrast, fewer animals were sources of *C. perfringens* and only a few animal faeces (chicken, quail) were sources of FRNA coliphages. Similar results were obtained for human faeces. The absence of FRNA coliphages in most human faeces as well as many animal faeces has been reported by others (Havelaar *et al.* 1990; IAWPRC 1991), and is indicative of the current mystery regarding the source of the high concentrations of FRNA coliphages in all sewages. Thus, elevated concentrations of FRNA coliphages in environmental waters are indicative of sewage contamination but may not be indicative of direct contamination by faeces. Evidence was obtained to show that multiplication of FRNA coliphages under environmental conditions was not likely because host bacteria such as *E. coli* HS(pFamp)R did not form pili at environmental temperatures (<25°C) but did so at elevated temperatures (>30°C). Moreover, most environmental isolates of *E. coli* did not produce pili even at elevated temperatures and are generally unsuitable hosts for the production of FRNA coliphages (Tables 6 and 7).

## CONCLUSIONS

The results of this study confirm many of the conclusions reported previously. The first conclusion is that the current procedure of monitoring treated sewage and environmental waters for traditional faecal indicator bacteria only (faecal coliform, *E. coli*, enterococci) as a means of determining the hygienic quality of treated sewage and recreational waters is unreliable. This concern is based on the observation that sewage, which had been disinfected to reduce the concentrations of traditional faecal indicator bacteria to very low levels, still contained substantial concentrations of alternative faecal indicators such as *C. perfringens* and FRNA coliphages. Since FRNA coliphages are similar to pathogenic human enteric viruses in terms of their resistance to disinfection and to environmental factors, monitoring sewage and environmental waters for FRNA coliphages is a better method for predicting the presence and absence of pathogenic enteric viruses than monitoring for traditional faecal indicator bacteria.

The second conclusion is that monitoring streams in Hawaii for faecal indicator bacteria provides unreliable data to determine when the streams are contaminated with sewage. This is due to the high ambient concentrations of traditional faecal indicator bacteria in the streams of Hawaii, which routinely exceed recreational water quality standards. The primary source of these faecal indicator bacteria is soil, a non-point source of pollution, rather than sewage. As a result, the high concentrations of these soil-associated faecal indicator bacteria in streams have less health significance than faecal bacteria whose source is sewage. The third conclusion is that monitoring streams in Hawaii for alternative faecal indicators (*C. perfringens*, FRNA coliphages) provides reliable data to determine when the streams are contaminated with sewage. This is due to the low ambient concentrations of these alternative faecal indicators in streams as well as in soil. As a result, an increase in concentrations of these alternative faecal indicators provides reliable data that the streams are contaminated with sewage.

This is the first study to evaluate the usefulness of monitoring sewage, human and animal faeces as well as soil and environmental waters in Hawaii for FRNA coliphages. The results obtained from this study indicate that FRNA coliphages are reliable markers of sewage or point source contamination in tropical streams. However, more sources of environmental waters must be monitored for FRNA coliphages to determine the expected ambient levels in environmental waters throughout the state of Hawaii. The consistent and high concentrations of FRNA coliphages in sewage but generally low incidences and concentrations of this group of coliphages from human faeces as well as animal faeces indicate that FRNA coliphages are good markers of sewage but may not be good markers for direct contamination by human or animal faeces. Moreover, since there is little evidence that FRNA coliphages can multiply under environmental conditions, the source of the high concentrations of FRNA coliphages in sewage has not been fully determined. In summary, there is a need to determine how FRNA coliphages, which are present in low concentrations in most human faeces, can reach such high densities in raw sewage. There is also a need to determine the ambient concentrations of FRNA coliphages in environmental waters and to establish a

health-related concentration in streams so that standards can be developed to determine when that body of recreational water is significantly contaminated with sewage.

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