

## Identification of microbial faecal sources in the New River in the United States–Mexican border region

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### ABSTRACT

Water samples were analysed to differentiate human and animal faecal contamination of the New River, Mexico/USA, by genotyping bacterial viruses detected in the samples. From 46 water samples collected from the New River, 372 plaques of male-specific coliphages were isolated and genotyped; 44% of the plaques were identified as F-RNA coliphages and further characterized into four groups. Group I was the most prevalent (56%), followed by group IV (25%), group III (10%) and group II (9%). Group III coliphages were only detected at the sampling site in the vicinity of the international boundary, indicating human faecal contamination. As the New River traverses through the US region, groups I and IV coliphages were predominantly identified, but no human-specific genotypes were detected. The study also found that water temperature influenced the prevalence of the relative proportions of F-RNA coliphages in the environmental water samples. The strategy used in this study appears to be a practical and reliable tool for monitoring and distinguishing between human and animal faecal contamination.

**Key words** | F-RNA coliphage genotyping, United States–Mexican border region

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### INTRODUCTION

Faecal pollution is a serious environmental problem that affects surface waters worldwide. Faecal contamination in surface waters can result from numerous sources, including municipal sewage, livestock operations, wildlife and urban runoff. Faecal pollution from humans and animals imposes risks to human health from exposure to pathogenic bacteria, viruses and protozoa (Rotbart 1995; Baker *et al.* 1999; Schaper *et al.* 2002; Seurinck *et al.* 2005). Waters contaminated with human faeces are generally considered a high risk to human health, as they are more likely to contain human enteric pathogens. Identifying sources of pollutants is a critical factor for controlling faecal contamination of source waters. Routine monitoring of pathogens in water is costly, and entails an enormous task for water providers. Therefore, there is a need to find reliable, rapid and economical indicators for identifying faecal contamination that also allow characterization of pollutants in surface waters.

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Faecal coliforms have traditionally been used as indicators of faecal contamination of water, but no direct correlation between faecal indicator bacteria and human enteric viruses has been shown (Le-Guyader *et al.* 1993; Abbaszadegan *et al.* 2003). Coliphages are viruses that infect coliform bacteria. They are integral flora of both raw and treated domestic sewage. A variety of domestic and feral animals shed coliphages in their faeces (Osawa *et al.* 1981; Havelaar *et al.* 1986; Goyal *et al.* 1987; Calci *et al.* 1998). Coliphage detection methods are easy and inexpensive, and coliphages are considered as indicators of the presence of enteric viruses in water. Recently, the United States Environmental Protection Agency (USEPA 2006) has suggested the use of coliphages as an indicator of viral contamination of groundwater.

Coliphages can be categorized as somatic and male-specific (F<sup>+</sup>) coliphages. Male-specific coliphages can be either F-DNA or F-RNA. Among coliphages, the F-RNA

coliphages appear to be the most appropriate surrogates for enteric viruses because in terms of composition, structure and size, the F-RNA male-specific coliphages closely resemble human enteric viruses (Havelaar *et al.* 1986; Goyal *et al.* 1987; Dore *et al.* 2000; Grabow 2001; Cole *et al.* 2003). F-RNA coliphages can be divided into four serogroups that are selectively excreted by humans or animals. Several studies have revealed that serotypes II and III are mainly isolated from human faeces, while serotypes I and IV are mainly from animal faeces (Furuse *et al.* 1978, 1981; Havelaar *et al.* 1990; Schaper & Jofre 2000; Schaper *et al.* 2002). Pigs are the exception as their faeces are reported to contain all groups except III. Studies have shown that members of each of the serogroups display genetically distinguishable genotypes. Therefore, the use of nucleic acid probes highly specific for each genotype can be used for source tracking pollution in environmental samples (Hsu *et al.* 1995; Beekwilder *et al.* 1996). Genotyping by nucleic acid recognition is an alternative to serotyping and is a new approach for identifying sources of faecal pollution.

The New River is the most polluted river in the United States (US). It originates 24 km south of the city of Mexicali, in the Mexicali Valley, Mexico. The river carries urban

runoff, untreated and partially treated municipal and industrial wastes, and agricultural runoff from the Mexicali Valley into the US (California EPA 2002). After it crosses the international boundary at Calexico, California, the river travels approximately 100 km through Imperial County, California, before discharging into the Salton Sea (Figure 1). By the time it reaches the Salton Sea, approximately 64% of its flow consists of wastewater in the form of agricultural runoff from Imperial County (California EPA 2002). The main sources of microbial contamination in the river include discharges of municipal waste from the Mexicali Valley in Mexico, treated wastewater from Imperial Valley domestic wastewater treatment plants, wastewater from confined animal feeding operations (CAFOs) and several non-point sources (California EPA 2002). The untreated waste discharges from the Mexican side of the border have long been recognized as a major source of microbial contaminants in the New River, and these microbial contaminants of faecal origin impair the entire segment of the river in the US. A microbial genotyping and surveillance plan is needed to define water quality markers in the New River, which can be used to identify and quantify the faecal contamination in this river.

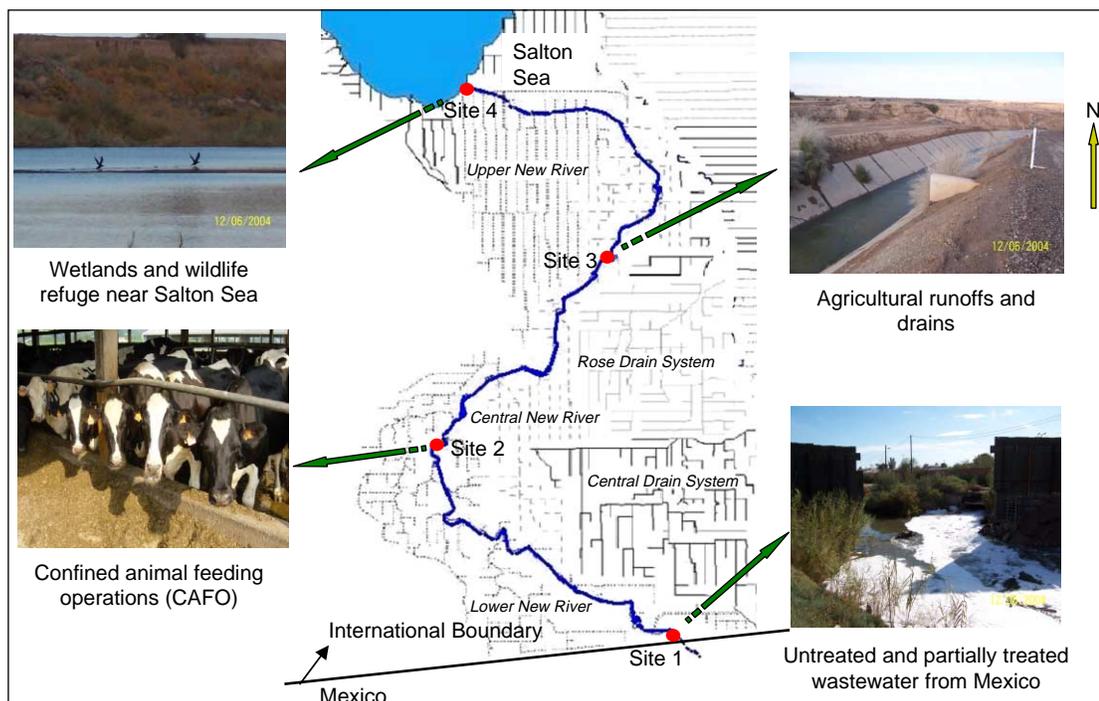


Figure 1 | Sampling sites at the New River.

The objective of this study is to identify the type and sources of faecal contamination in the New River using F-RNA coliphage genotyping. This research will help to define water quality biomarkers, which can be used for distinguishing between human and animal faecal contamination in the study area.

## MATERIALS AND METHODS

### Site selection

Four sampling sites were selected along the New River (Figure 1). Sampling site selection criteria were based on probable sources of pollution. Site 1 was located at the international boundary between the city of Calexico, California, and the city of Mexicali, Mexico. This site provided a measure of background microbial concentration originating from Mexico and entering the US. Site 2 was located downstream from a wastewater treatment plant and two major CAFOs in the city of Calexico where more than 20,000 cows are raised (California EPA 2002). Manure and wastewater from these CAFOs are diverted to manure beds near the New River for waste management purposes. Possible contamination routes to the New River include groundwater infiltration and conveyance, and storm water runoff from these manure beds (California EPA 2002). The distance between site 1 and site 2 is approximately 24 km. The average flow (velocity) of water at site 1 was  $0.55 \text{ m s}^{-1}$  (USGS 2006) and the travel time of a body mass of water from site 1 to site 2 was approximately 12 h. Site 3 is downstream of many agricultural drains and is approximately 40 km downstream from site 2. Site 4 is downstream of the discharge point from the wetland located in the Imperial Valley. This site is located approximately 32 km downstream from site 3 and prior to discharge into the Salton Sea. Birds frequent this area in large numbers. No USGS gauge stations were available at sites 2 and 3 for measuring the flow of water.

### Sample collection

Samples were collected at the four designated sites on a monthly basis for 12 months starting from January 2005. As a result of heavy rainfall, samples from sites 3 and 4

could not be collected during the months of January and October 2005, respectively. Samples were collected in sterilized 250-ml wide-mouth, high-density polyethylene bottles and shipped overnight under darkened conditions at 4°C to the Environmental Microbiology Laboratory at Arizona State University, Tempe. The samples for bacteria and coliphages were analysed within 48 h of sample collection. The temperature and pH of water were measured at each site during sample collection.

### Detection of coliphages and faecal coliforms

Water samples (10 ml) were assayed for coliphages using the double agar layer (DAL) technique (Adams 1959). *Escherichia coli* F<sub>amp</sub> (ATCC 700891) and *E. coli* CN-13 (ATCC 700609) were used as host bacteria for male-specific coliphages and somatic coliphages, respectively (USEPA 2001). Briefly, 5 ml of water sample and 1 ml of appropriate host suspension were added to 4 ml of molten top agar and then poured onto bottom agar plate. Each sample was analysed in duplicate. The plates were incubated at 37°C overnight, and plaques were counted. Positive and negative controls were included in each set of assays and for each coliphage group. Individual plaques of male-specific coliphage from the DAL assay plates were transferred, using a sterilized inoculating loop, to micro centrifuge tubes containing 0.3 ml of Tris buffer. Each of the isolated plaques was further propagated and stored at 4°C until genotyped.

Faecal coliform analysis was performed using the membrane filtration technique on mFC agar (EM Science, Gibbstown, New Jersey) (Standard Methods 1995). Samples were filtered using 47-mm diameter cellulose acetate membranes with 0.45- $\mu\text{m}$  pore size (Pall Gelman Laboratory, Ann Arbor, Michigan). The colony forming units of faecal coliforms were enumerated after overnight incubation at 44.5°C.

### Propagation and stock preparation of F-RNA coliphages for procedural controls

MS2, GA, Q $\beta$  and SP were selected as representative of F-RNA coliphage groups I, II, III and IV, respectively. MS2, Q $\beta$  and SP F-RNA coliphages were obtained from the American Type Culture Collection (ATCC, Rockville,

Maryland). The coliphage GA was kindly provided by Dr John S. Meschke at University of Washington, Seattle. The host bacteria were obtained from the ATCC. Information on F-RNA coliphages, their bacterial hosts and the nutrient media used for their propagation is provided in Table 1. The coliphages and bacterial stocks were propagated as recommended by ATCC.

### Recovery efficiency and survivability of F-RNA coliphages in water

To determine the recovery efficiency of the detection method, MS2, GA, Q $\beta$  and SP F-RNA coliphages were spiked in the New River water samples at a concentration of  $\sim 10^3$  pfu ml $^{-1}$ . The water samples were autoclaved prior to spiking to eliminate background microbial activity. Spiked coliphages were enumerated before and after each procedure using the DAL method as described above. Procedural recovery efficiency evaluations were performed, and mean percentage recoveries and relative standard deviations (RSD) were calculated (Montgomery & Hines 1990). The RSD was defined as the standard deviation  $\times$  100 divided by the mean.

To study the survival of the four groups of F-RNA coliphages in environmental waters, the spiked coliphage samples were stored at ambient temperature. The concentrations of coliphages were determined at 0, 24, 48 and 72 h. Survival of the coliphages in the New River over time was modelled using Equation 1:

$$\text{Log}\left(\frac{C}{C_0}\right) = -kt \quad (1)$$

Here,  $C_0$  and  $C$  are the initial concentration and the concentration of F-RNA coliphages at any time  $t$ , respectively, and  $k$  is the decay constant. For quality assurance two

replicates were performed for the recovery efficiency and survival experiments with duplicate assays.

### Hybridization conditions and detection

The environmental isolates of male-specific coliphage samples were denatured by heating at 98°C for 10 min, and then 100- $\mu$ l of each sample were filtered through a positively charged nylon membrane (Zeta Probe Blotting Membrane Bio-Rad, California) using Dot-Blot apparatus (Bio-Rad, California). The membrane was removed and air dried at ambient temperature. The exposed nucleic acid strands were fixed to nylon membrane by UV crosslinking (under UV $_{254}$  for 2 min). Fixed membrane was pre-hybridized for 30 min at 56°C in DIG hybridization solution (20% sodium dodecyl sulfate, pH 7.2). Hybridization was performed in the same solution by adding 5 pmol ml $^{-1}$  of tailed oligonucleotide probe and incubated overnight (16 h) at 56°C. Following hybridization, the membrane was washed twice in each of the two washing solutions, 2X SSC (0.3 M NaCl, 30 mM sodium citrate [pH 7.5])/0.1% SDS and 0.5X SSC/0.1% SDS, to remove excess unbound probes. After washing, the membrane was blocked using blocking solution to prevent nonspecific binding, and then incubated with alkaline phosphatase-conjugated anti-Digoxigenin antibodies for 30 min at room temperature. The antibody conjugate bound to the target DNA-RNA hybrids, and the unbound antibody conjugate was removed by washing the membrane with wash buffer solution (0.1 M maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20) for 30 min at room temperature. After the washing step, a chemiluminescent substrate (CSPD) was added to the membrane which was incubated for 15 min at 37°C. Chemiluminescent substrate bound with the antibody conjugate and emitted a light signal that was detected

**Table 1** | F-RNA coliphages, bacterial host strains and media used for the propagation of procedural controls

F-RNA coliphages	Bacterial host	Nutrient media
MS2 (I) (ATCC 15597-B1)	<i>E. coli</i> EC3000 (ATCC 15597)	Trypticase soy agar/broth
GA (II)	<i>E. coli</i> Famp (ATCC 700891)	Trypticase soy agar/broth
Q $\beta$ (III) (ATCC 23631-B1)	<i>E. coli</i> (ATCC 23631)	Trypticase soy agar/broth
SP (IV) (ATCC 23059-B1)	<i>Bacillus subtilis</i> (ATCC 23059)	Nutrient agar/broth

by exposing the membrane to X-ray film for 1 to 15 min. The autoradiograph thus obtained is a permanent record of a successful hybridization reaction.

### Labelling of oligonucleotide probes

The oligonucleotide probe sequences for F-RNA coliphage groups are shown in Table 2 (Hsu *et al.* 1995; Beekwilder *et al.* 1996). The probes were synthesized by GeneTech (Tempe, Arizona). Two non-radioactive labelling kits, DIG Oligonucleotide Tailing kit, 2nd Generation (Roche Diagnostics, Indianapolis, Indiana) and HybQUEST Complete DNP system (Mirus Bio Corporation, Madison, Wisconsin), were evaluated for labelling efficiency. The protocols provided by the manufacturers were followed for the labelling. For DIG (digoxigenin) labelling, the oligonucleotide probes were tailed with DIG-dUTP and dATP at an average tail length of 50 nucleotides at the 3' end, while for the DNP (dinitrophenyl) system, the DNP was tagged directly onto the probes. The labelled oligonucleotide probes were stored at  $-20^{\circ}\text{C}$  until used in the hybridization procedure.

### Data analysis

SPSS version 11.0 (SPSS Inc., Chicago, Illinois) was used for statistical analysis. The average concentrations of coliphages and faecal coliform bacteria for each sampling site were compared using analysis of variance (ANOVA), and a Levene's test was performed to determine equality of variances prior to ANOVA. Also, for the prevalence of different groups of F-RNA coliphages, the ratios of each group among all four groups of F-RNA coliphages were compared. The analyses were performed at a significance level ( $\alpha$ ) of 0.05. If significant differences were observed,

**Table 2** | Oligonucleotide probes specific for four groups of F-RNA coliphages

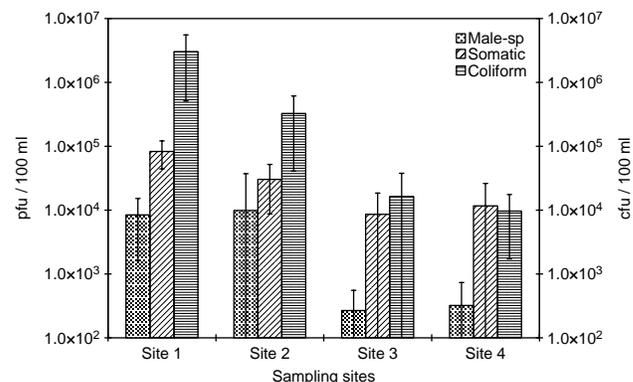
Group	Probe sequence
I	5' CGCATCTAAGGTATGGACCATCGAGA 3'
II	5' AATCGTTCAGGAAGTGAGATTCAAAAACC 3'
III	5' AGCCAGAGATTACCAGCAGTAGC 3'
IV	5' GGCATAGATTCTCCTCTGTAGTGCG 3'

Duncan's post hoc test was performed to determine which values differed from all other values.

## RESULTS AND DISCUSSION

### Occurrences of coliphages and coliform bacteria in environmental samples

Every water sample collected from the four sampling sites was positive for somatic and/or male-specific coliphages. At all sites, the average concentration of somatic coliphages in the samples was one to two orders of magnitude higher than for male-specific coliphages (Figure 2). The average concentrations of male-specific coliphages were similar in sites 1 and 2 ( $P > 0.1$ ), and were much higher compared with their concentrations at sites 3 and 4. The average concentrations of somatic coliphages were the highest at site 1 ( $P < 0.05$ ), and a steady decrease in average concentration from site 1 to site 3 followed by a slight increase at site 4 was observed (Figure 2). This increase in concentration of both male-specific and somatic coliphages can be attributed to the impact of the wildlife refuge (birds) near the wetland area at site 4. The level of faecal coliform bacteria was one to two orders of magnitude higher than coliphage concentration throughout the year. The highest numbers of faecal coliform bacteria were detected in the water samples from site 1 followed by sites 2, 3, and 4 ( $P < 0.05$ ). Because of very high background levels of coliform bacteria at sites 1 and 2, the



**Figure 2** | Occurrences of indicator microorganisms in the New River (geographical variation). All error bars in the figure represent one standard deviation, and all the data points are an average of 12 sampling events with duplicate assays.

impact of the wildlife refuge (birds) could not be observed at site 4 as it was for both male-specific and somatic coliphages. The results indicate that the majority of the faecal contamination in the New River may originate from raw and partially treated wastewater from Mexico and the waste streams from the CAFOs located on the US side of the border.

Consistent with previous reports regarding the seasonality of coliphage occurrence (Cole *et al.* 2003), this study documented that the prevalence of F-DNA and F-RNA coliphages in the New River was impacted by season (Figure 3). During the winter months (below 20°C), the proportion of F-RNA coliphages was relatively high at all sampling sites, whereas an inverse trend was observed for F-DNA coliphages which were more abundant during the summer months (warmer water temperature, 20 ~ 32°C). This result indicates that water temperature may influence the relative proportions of F-DNA and F-RNA coliphages in the samples (i.e. the F-RNA coliphages are inactivated at a higher rate when warmer water temperatures occur).

### Recovery efficiency of the detection method and survival of F-RNA coliphages

In order to determine the efficiency of the double agar layer (DAL) method for the detection of coliphages, composite water samples from all sampling sites were spiked with F-RNA coliphages: MS2, GA, Q $\beta$  and SP. The average recovery efficiency of the DAL method for MS2, GA, Q $\beta$  and SP was 81%, 80%, 55% and 64%, respectively (Table 3).

In this study, the average holding time between sample collection and sample analyses was 48 h. To investigate the impact of the sample holding time on the detectability of male-specific coliphages, the survival of F-RNA coliphages in the New River water was studied. Water samples were spiked with the control F-RNA coliphages and stored at 4°C and analysed after 48 h. MS2, GA, Q $\beta$  and SP coliphage concentration decreased by 1.3, 0.81, 0.74 and 0.57 log, respectively (Figure 4). Despite the decrease in the control

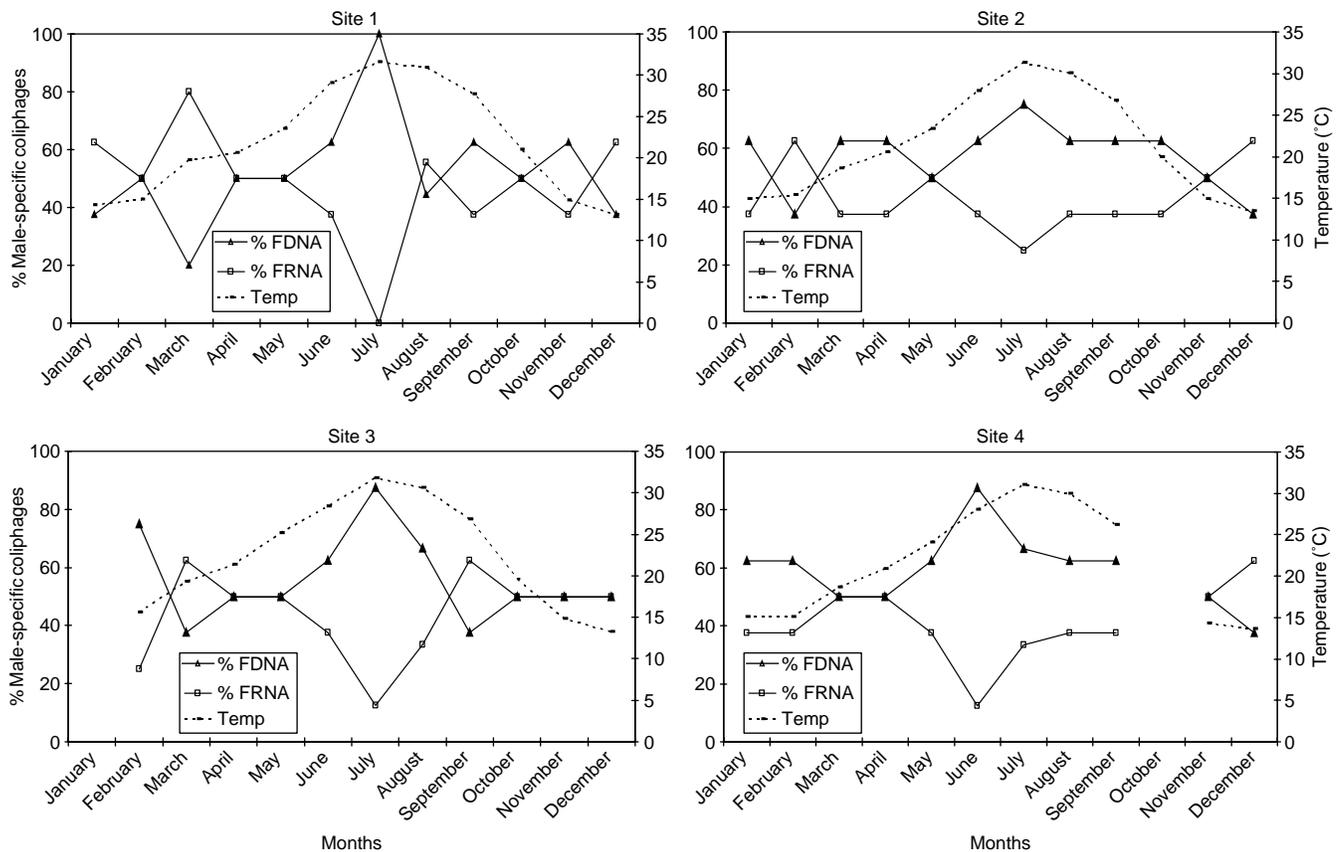


Figure 3 | Year-round variation in the occurrence of F-RNA and F-DNA coliphages in the New River.

**Table 3** | Average recovery efficiency of the DAL method and pseudo first-order equations for calculating the decay rate of F-RNA coliphages in the New River

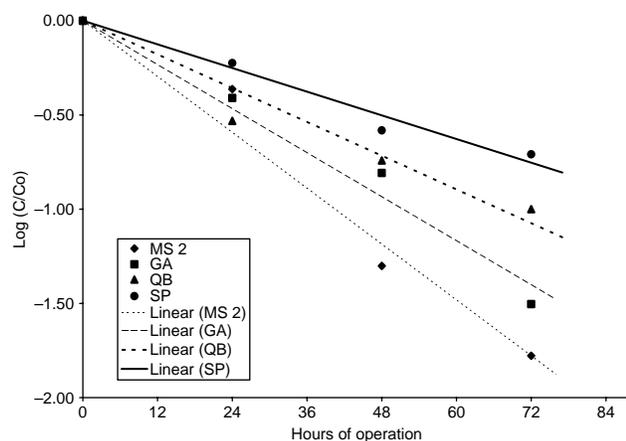
Male-specific coliphages	Recovery [mean $\pm$ RSD (%)]	Equation [ $y = \text{Log}(C/C_0) = -kt$ ]	$R^2$
MS2	81 $\pm$ 4	$y = -0.0247 t$	0.97
GA	80 $\pm$ 5	$y = -0.0195 t$	0.98
Q $\beta$	55 $\pm$ 23	$y = -0.0149 t$	0.93
SP	64 $\pm$ 18	$y = -0.0105 t$	0.97
Average	70 $\pm$ 6		

coliphages' concentrations, significant numbers of F-RNA coliphages could still be detected in the New River water by the DAL method.

F-RNA coliphage inactivation (decay rate) followed the first order kinetic model. The equations are summarized in Table 3. The equations are expressed as decay rate constants ( $k$ ), with higher decay rates indicating a more rapid decline in the number of organisms enumerated. The decay rate of SP coliphages in the New River water was the lowest among the entire group representing F-RNA coliphages followed by Q $\beta$ , GA and MS2. The decay rates for Q $\beta$ , GA and MS2 in surface water were consistent with previously reported works (Brion *et al.* 2002).

### Sensitivity and efficacy of commercial kits for probe labelling and RNA:DNA hybridization

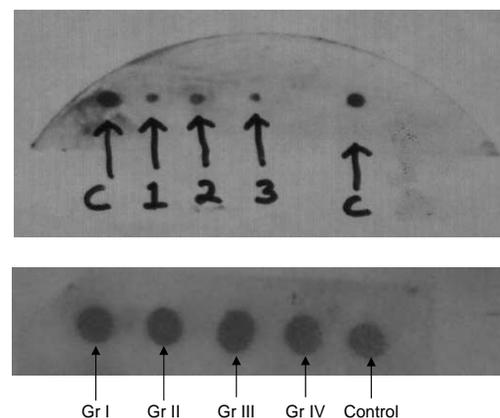
In order to evaluate the labelling efficiency, group-specific oligonucleotide probes, specific for each genotype, were



**Figure 4** | Regression model to calculate the survival of male-specific coliphages in the New River.

labelled by two commercially available non-radioactive DNA labelling kits. The autoradiograph showed that the DIG Oligonucleotide Tailing kit (Roche Diagnostics) successfully labelled all four group-specific oligonucleotide probes, whereas the HybQUEST Complete DNP system kit (Mirus Bio Corporation) could label only group I, II and III oligonucleotide probes (Figure 5).

The coliphages MS2, GA, Q $\beta$  and SP were denatured and were separately hybridized using DNP and DIG kits. The autoradiograph of DIG-labelled probes showed a strong hybridization signal from the RNA:DNA hybrids. However, the autoradiograph of DNP-labelled probes showed no hybridization signal, suggesting that either the DNP-labelled probes did not hybridize with the control male-specific F-RNA coliphages, or the signal intensity produced by this kit was not high enough to be detected by autoradiograph. Genomic DNA or RNA is normally entangled, and hybridization of oligonucleotide probes with genomic RNA targets is achieved with greater efficiency when labelled probes are capable of steric adjustment. In DNP-labelled probes, the DNP tags onto the backbone of the oligonucleotide probes, and this rigid chemistry minimizes the steric adjustability of labelled probes, which could be the reason for reduced efficacy. DIG-labelled probes have an average tail (DIG-dUTP and dATP) length of 50 nucleotides tagged at the 3' end rather than onto the backbone of the oligonucleotide probes, which makes the probes better capable of steric adjustment. The results obtained using DIG-labelled probes also showed that there was no cross-reactivity between the group-specific probes of different F-RNA coliphage genotypes. Based on the probe



**Figure 5** | Group-specific probes labeled with HybQUEST complete DNP system kit (top) and DIG Oligonucleotide Tailing kit (bottom).

labelling efficiency and procedural evaluation of RNA:DNA hybridization, the DIG Oligonucleotide Tailing kit was selected for performing hybridization experiments with environmental samples.

### Prevalence of different groups of male-specific coliphages

From 46 water samples collected from the New River, 372 plaques of male-specific coliphages were isolated to characterize the sources of faecal pollution; 44% (163/372) of the plaques were identified as F-RNA coliphages, which were further genotyped and characterized into four groups (Table 4). Group I was the most prevalent among all the F-RNA coliphages (56%), followed by group IV (25%), group III (10%) and group II (9%), suggesting that animal faeces are the biggest source of pollution in the study area ( $P < 0.001$ ).

At site 1, groups II and III were detected in 7 and 9 of the 12-month samples and represented 17% (8/48) and 29% (14/48) of detected F-RNA coliphages, respectively. This result suggested that site 1 was impacted by human faecal pollution possibly because of untreated or poorly treated discharges into the river from Mexican cities. At site 2, group I represented 61% (25/41) of the detected F-RNA coliphages ( $P < 0.001$ ), followed by group IV (19%), group II (15%) and group III (5%). Group II coliphages were detected in 6 of the 12-month samples, suggesting that the possible sources of faecal contamination are either humans or pigs. As pigs are not raised in the CAFOs in the vicinity of site 2, the source of group II coliphages is most likely human. Moreover, two isolates of group III coliphages were detected at site 2, confirming human faecal pollution. The possible sources for groups II and III coliphages are the treated discharges from the wastewater treatment facility at Calexico and/or the isolates transported

from site 1. The distance between site 1 and site 2 is approximately 24 km and with an average flow rate of  $0.55 \text{ m s}^{-1}$  (USEPA 2006) the estimated travel time of water is 12 h. The survival study suggests that 65% of group II and III F-RNA coliphages survived longer than the 12 h travel time (Table 3), so we can assume that these coliphages could have been transported from site 1. However, further study is needed to characterize and identify the faecal origin of groups II and III coliphages at site 2. At sites 3 and 4, no F-RNA coliphages in groups II or III were detected. Agricultural runoff and the wildlife refuge near the wetlands and the Salton Sea are the possible sources of animal faecal contamination.

The genotyping of F-RNA coliphages used in this study allowed characterization of faecal pollution and appears to be a practical and reliable tool for monitoring and distinguishing between human and animal faecal contamination in the New River.

### CONCLUSIONS

This study was performed to identify the type and sources of faecal contamination in the New River using F-RNA coliphage genotyping. The proportion of predominant F-RNA coliphages present in the New River water samples provided information regarding the most important sources of faecal pollution affecting water quality. The study showed that animal faeces were the biggest source of pollution at all sites. Site 1 at the international boundary was impacted by human faecal pollution coming from Mexico, but no human faecal pollution was identified downstream of the international boundary in the New River. Further study is required to identify and characterize the actual sources of group II and III F-RNA coliphages at site 2 that point to human faecal pollution. Thus, the results of the present study establish that F-RNA coliphages are a valid biomarker for the assessment of microbial quality of New River water.

**Table 4** | Genotypes of male-specific coliphages isolated from different segments of the New River

Sampling location	Isolates	Geno-groups of F-RNA coliphages				Total
		Group I	Group II	Group III	Group IV	
Site 1	100	22	8	14	4	48
Site 2	96	25	6	2	8	41
Site 3	87	26	0	0	12	38
Site 4	89	19	0	0	17	36
Total	372	92	14	16	41	163

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