A comparison of the survival of F+RNA and F+DNA coliphages in lake water microcosms

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ABSTRACT

The survival of seven F+RNA phages (MS2 Group I ATCC type strain, two Group I environmental isolates, a Group II environmental isolate, a Group III environmental isolate, and two Group IV environmental isolates) and six F+DNA phages (M13, fd, f1, and ZJ/2 ATCC type strains, and two environmental isolates) were examined in microcosms using a surface drinking water source. Phages were spiked into replicate aliquots of a source water at about 20,000 pfu/ml. Replicate spikes were incubated at 4 and 20°C and monitored for 110 days. At 4°C, Groups I and II F+ RNA phages were detectable through 110 days, with reductions of about 1 and 3 $\log_{10}$, respectively. The Group III F+RNA phage demonstrated 5 $\log_{10}$ reduction after 3 weeks, and the Group IV F+RNA phages were reduced to detection limits (5 $\log_{10}$ reduction) within 10 days. Of the F+DNA phages, all four type strains were detectable with about 2.5 $\log_{10}$ reduction after 110 days at 4°C. The F+DNA environmental isolates were detectable with about a 4 $\log_{10}$ reduction after 110 days at 4°C. All phages demonstrated faster decay at 20°C. These results suggest that differences in F+ phage survival may influence their prevalence in environmental waters and the ability to attribute their prevalence to specific human and animal sources of faecal contamination.

Key words | coliphage, drinking water, indicator, survival

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INTRODUCTION

Microbial contamination of drinking water has been of primary interest to the public health community, drinking water providers, and sanitary engineers since the 1800s. Including the provisions of the Surface Water Treatment Rule, recent legislation has re-emphasized the importance of watershed management activities and source water protection (US EPA website 2000). While waterborne pathogens are known with either human-to-human or zoonotic or both transmission pathways, knowledge of the source of microbial inputs (source tracking) to source waters can allow water managers to develop and apply mitigation strategies in a cost-effective and targeted manner. Traditional bacterial indicator organisms such as coliforms, both total and faecal, provide information as to the microbial safety of drinking waters and are used for assessing regulatory compliance. However, coliforms as tested do no provide any information as to source.

One promising group of organisms for the indication of viral pathogens that also has the potential for providing source tracking information are the F-specific or male-specific coliphages (IAWPRC 1991). Coliphages are viruses that infect coliform bacteria, but are non-pathogenic to humans. F-specific coliphages belong to two morphologically defined families, the *Leviviridae* and the *Inoviridae* (Sobsey *et al*. 1995). The family *Leviviridae* consists of small icosahedral viruses that contain single-stranded RNA as their genetic material. The family *Inoviridae* consists of filamentous viruses that contain single-stranded DNA as their genetic material. The F-specific coliphages infect coliform bacterial hosts through attachment to F-pili. F-specific coliphages are easily measured in drinking waters using the enrichment method, the single agar layer method, or the filter-concentration/elution method with enumeration using *E. coli* Famp or *E. coli* C3000

hosts (coliform strains which possess the F-sex factor; Sobsey *et al*. 1990; US EPA 2000*a*, 2000*b*).

Coliphages are consistently present in domestic raw sewage and have been reported to occur in concentrations ranging from 10^5 to 10^7 plaque-forming units (pfu)/l (Furuse *et al.* 1983; Havelaar *et al.* 1986; Calci *et al*. 1998). It has also been demonstrated that a variety of animals also shed coliphages in their faeces (Owasa *et al*. 1981; Goyal *et al*. 1987; Havelaar *et al*. 1990; Chung 1993). Neither F-specific coliphages nor faecal coliforms were detected in a number of environmental soil and grass samples from protected buffer strips around a drinking water reservoir (Long 1999). Thus, F-specific coliphages appear to be present in faeces and sewage, the sources of pathogens, and seem to be generally present at low levels in uncontaminated environmental media.

A number of researchers have demonstrated that subgroups of F + RNA coliphages via serotyping or genotyping can distinguish between inputs from human and warm-blooded animal/non-human sources (Havelaar *et al*. 1986; Gerba 1987; Havelaar 1987; Hsu *et al.* 1995; Sobsey *et al*. 1995; Schaper *et al*. 2002; Oudejans *et al.* 2003). These subgroups are:

Group I. Non-human animals (prototype MS2);
Group II. Primarily human faeces and occasionally pig faeces (prototype GA);
Group III. Exclusively human (prototype Qβ); and
Group IV. Primarily non-human origin with rare human associations (prototypes FI and SP; after Gerba 1987).

F + coliphages are enumerated from samples, and individual isolates can be subject to serotyping or genotyping in order to discriminate between human and non-human microbial sources.

Little information regarding the environmental survival of F-specific coliphages can be found in the scientific literature. It has been demonstrated that high temperature coliphages, F + RNA coliphages that replicate at 25–45°C, can replicate in raw sewage, while their multiplication in environmental waters is unlikely (Seeley & Primrose 1980). It has been demonstrated that bacteriophages (coliphages and phages of *B. fragilis*) with different morphologies may demonstrate different rates of persistence in the environment (Lasobras *et al*. 1997). However, studies which assess the different survivals of individual types of F-specific coliphages have not been reported. This information is important for proper interpretation of the data from this source-tracking tool in environmental samples potentially impacted by a number of microbial input sources. Therefore, this study was aimed at investigating the survival of different strains of F-specific coliphages in drinking water microcosms as a first step in understanding the environmental survival of the different subtypes of F-specific coliphages.

MATERIALS AND METHODS

Coliphage preparation

A summary of the coliphages used in the lake water survival studies is presented in Table 1. Type strains of MS2, an F + RNA coliphage, and M13, fd, f1 and ZJ/2, F + DNA coliphages, were obtained from the American Type Culture Collection (Manassas, VA). These phages were grown by preparing a 10-fold dilution series of the freeze-dried stock culture in phosphate buffered saline (PBS) and plating them with *E. coli* Famp (ATCC 700891) host using the double agar layer method on 150 mm petri dishes (Adams 1959; Wait pers. comm.). After 18–22 h incubation, plates that demonstrated near confluent plaque formation were harvested by floating the phages off the plate using PBS/20% glycerol (v/v). Ten millilitres were added to the top of the plates and swirled gently. The plates were allowed to sit at room temperature for 15 min. The phage suspension was collected using a sterile pipette and transferred to a sterile centrifuge tube. The phage suspension was filtered through a 0.45 μm syringe filter (Pall Acrodisc, Ann Arbor, MI) to separate the coliphage from host cells and 0.5 ml volumes were aliquoted into sterile 1.5 ml microfuge tubes. The filtered suspensions of coliphages were stored at −80°C for 2 weeks and then titres were determined.

Environmental phage isolates were collected from environmental samples (faeces or sewage) that were enumerated using the double agar layer method. A 10-fold

Table 1 | Summary of coliphages used in lake water survival experiments

Phage ID	RNA or DNA	Source
MS2	RNA group I	ATCC
Go1	RNA group I	Goose guano
SG1	RNA group I	Seagull guano composite
Sp	RNA group II	Springfield raw sewage
Dm	RNA group III	Deerfield raw sewage
SG4	RNA group IV	Seagull guano composite
SG42	RNA group IV	Seagull guano composite
M13	DNA	ATCC
fd	DNA	ATCC
f1	DNA	ATCC
ZJ/2	DNA	ATCC
OW	DNA	OWASA raw sewage
SD	DNA	South Durham raw sewage

dilution series was made using PBS, and replicate aliquots were plated with *E. coli* Famp host for enumeration. Single plaques were harvested using sterile 1,000 μl pipette tips into microfuge tubes containing 300 μl PBS/20% glycerol (v/v). The environmental isolates were screened and typed as to whether they were F + RNA or F + DNA phages by preparing a 100-fold dilution series. Six microlitres of the three dilutions (undiluted, 10^{-2}, and 10^{-4} dilutions) were replicate spotted onto peptone-yeast extract agar plates containing *E. coli* Famp host, one containing RNAse and one without. The coliphages that were neutralized in the presence of RNAse are F + RNA coliphages and those that retained infectivity in the presence of RNAse are F + DNA coliphages. The F + RNA coliphages were further typed into one of four serogroups using spot plate methods (Havelaar *et al.* 1986; Gerba 1987; Sobsey *et al.* 1995; Alderisio *et al.* 1996). The three 100-fold dilutions were replicate spotted onto six different peptone-yeast extract agar plates. One plate containing no antiserum (control plate), and one each containing antisera against MS2 (group I), GA (group II), Qβ (group III), SP (group IV), or FI (group IV) coliphages. The isolates that were neutralized in the presence of a particular antisera were of the same group as the antigen coliphage. High titre stocks used for spiking lake water were prepared in a similar manner as described above for the ATCC coliphage strains.

The coliphage stocks were titred using a 10-fold dilution series (10^0 through 10^{-9}) in spot tests. Six microlitres of each dilution were spotted onto peptone-yeast extract agar containing *E. coli* Famp host. Dilutions which developed individual and countable plaques were used to approximate phage density in pfu/μl. Surviving coliphage levels in microcosms were enumerated by plating at least three different dilutions/volumes in triplicate using the single agar layer method and *E. coli* Famp host.

Spiked lake water microcosms

University Lake water, one of two drinking water reservoirs that serves Chapel Hill, NC, was collected in sterile 4-l polypropylene bottles (Nalgene, Nalge Nunc International, Rochester, NY) at the tap in the laboratory at the Orange Water and Sewer POTW on two separate days for preparation of the microcosms, owing to labour constraints. All F + RNA coliphage spiked microcosms were prepared on one day, and all the F + DNA coliphages were prepared on the other. The containers of lake water were transported in coolers containing blue ice packs to the laboratory. In the laboratory, five 4-l bottles were composited into a sterile 20-l carboy and thoroughly mixed. One litre aliquots were aseptically dispensed into replicate sterile 1-l polypropylene bottles (Nalgene, Nalge Nunc International, Rochester, NY). These aliquots were spiked with an individual phage to a target concentration of 20,000 pfu/ml. Each individual bottle was rapidly mixed, and a subsample collected for $t = 0$ enumeration. Replicate sets of bottles spiked with a complete set of test coliphages were incubated in the dark at either 4 or 20°C, and subsampled for enumeration over time. Samples were enumerated using the single agar layer method, as mentioned above. Unspiked lake water samples were also incubated and enumerated. Water quality parameters including turbidity, total and faecal coliform levels, and pH were measured and are summarized in Table 2.

Table 2 | University Lake water quality measurements

	RNA coliphage experiment	DNA coliphage experiment
pH	6.9	7.1
Turbidity	12.6	5.3
TSS (mg/l)	5.2	4.4
THPCs (cfu/100 ml)	9.7×10^4	3.6×10^5
Total coliforms (cfu/100 ml)	835	90
Faecal coliforms (cfu/100 ml)	2	2

TSS—total suspended solids.
THPCs—total heterotrophic plate counts.

Data analysis

Data for coliphage survival in lake water are expressed as the $\log_{10}$ surviving fraction ($\log_{10}(N_t/N_0)$), where N_t is the coliphage concentration at time $= t$ and N_0 is the coliphage concentration at time $= 0$. Linear regression was used to compare the survival of each single coliphage among all coliphages tested. The simple linear model was used to calculate slope or decay factors (k) and estimations of T_{99} reductions. The model took the form of $\log_{10}(N_t/N_0) = kt$. Tailing of the survival curves was noted for a number of phages, and only time points on the linear portion of each $\log_{10}$ survival curve were utilized in these analyses.

Decay (slope) factors were compared using the separate linear regression fits in hypothesis testing, where the null hypothesis states that $k_1 = k_2$ (Kleinbaum *et al*. 1988). Calculating a Z-statistic when $Z \geq |z_{1-\alpha/2}|$ then the slope factors are not equal at the selected α value, and the survival of the different coliphages are not equal. An α value of 0.1 or 90% confidence was used for these analyses.

RESULTS AND DISCUSSION

The survival curves for the F + RNA coliphages tested are presented in Figures 1a and 1b for samples held at 4 and 20°C, respectively. The survival curves for F + DNA coliphages tested are presented in Figures 2a and 2b for samples held at 4 and 20°C, respectively. A summary of calculated slope factors (k) and T_{99} reduction times are summarized in Table 3. For all coliphage isolates tested, decay was less rapid in 4°C spiked water samples than for samples held at 20°C. The differences observed between incubation temperatures were not statistically different for the Group I F + RNA coliphages or for the Group III F + RNA coliphage tested owing to relatively large variances in the slope. The differences in survival for the Group II and IV coliphages tested were statistically

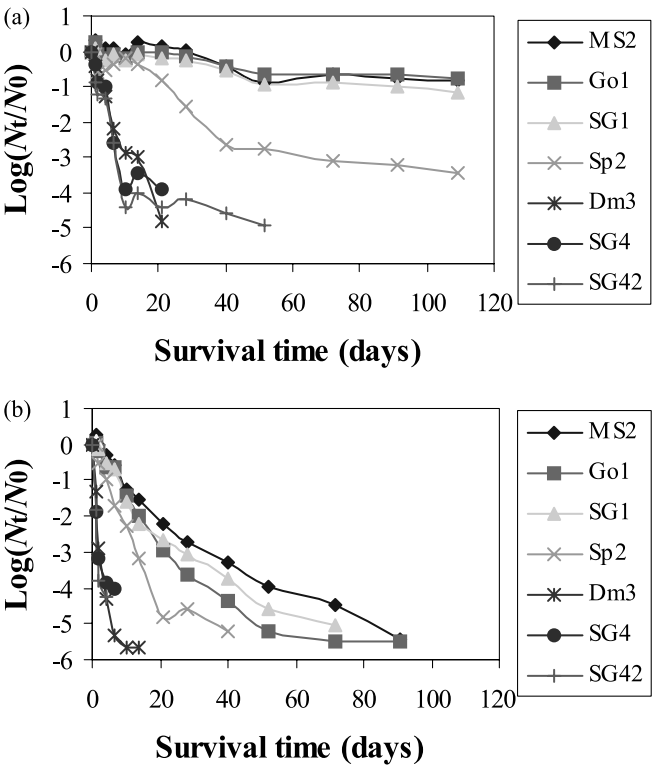

Figure 1 | (a) The survival of F+RNA coliphages in 4°C lake water microcosms. (b) The survival of F+RNA coliphages in 20°C lake water microcosms.

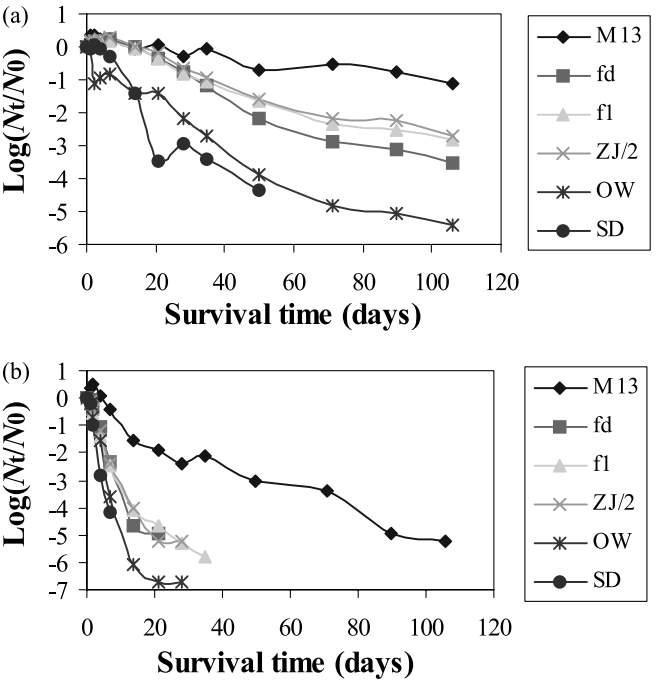

Figure 2 | (a) The survival of F+DNA coliphages in 4°C lake water microcosms. (b) The survival of F+DNA coliphages in 20°C lake water microcosms.

significant for temperature. The temperature effects resulted in statistically different survival rates for the F + DNA coliphages between 4 and 20°C, with the observed differences in decay rate being highly statistically significant at ($\alpha = 0.0001$) for all F + DNA strains tested. These results indicate that subgroups of F-specific coliphages demonstrate differential effects resulting from temperature on their survival in surface waters.

The microcosm survival characteristics of the F-specific RNA coliphages appeared to vary with subtype. The Group I F + RNA coliphages demonstrated the longest survival times, followed by the Group II F + RNA coliphage. The Group III and IV F + RNA coliphages demonstrated similar survival times, and the shortest overall for the groups of F + RNA coliphages tested. Hypothesis testing demonstrated that the decay factors for the Group I phages (MS2, Go, SG1) were statistically similar when tested at each incubation temperature separately. The decay factors for the Group III and IV phages (Dm, SG4, SG42) were similar for incubation at 4°C only. The survival of the Group III coliphage was statistically different from the Group I and II coliphages for incubation at both incubation temperatures. The survival of the Group III coliphage was statistically different from the Group IV coliphages at 20°C. The survival of the Group IV coliphages was highly statistically different from the Group I and II phages for the 20°C incubation.

All F + DNA phages incubated at 4°C demonstrated statistically similar decay rates in this study with the exception of M13 which survived longer. At 20°C M13 also decayed slower than all other phages. Statistically, the sewage isolate, SD, decayed at a different rate than all the other F + DNA coliphages tested. Based on GenBank sequences (NCBI website 2000) and literature information (Horiuchi *et al*. 1978), the F + DNA coliphages are more highly related to each other genetically as a group than are the F + RNA coliphages as a group. Sequencing of PCR products from a hypervariable region of these phages' genome demonstrated 100% homology among f1, ZJ/2 and SD isolate (Long unpublished data). The PCR product

Table 3 | Comparison of coliphage decay rates

Temp.	Phage	Slope factor (k)	R^2	T_{99} (days)	Temp.	Phage	Slope factor (k)	R^2	T_{99} (days)
4°C	MS2	− 0.0083	0.703	240	20°C	MS2	− 0.0682	0.922	29
	Go1	− 0.0080	0.806	250		Go1	− 0.0803	0.815	25
	SG1	− 0.0117	0.892	171		SG1	− 0.0856	0.881	23
	Sp2	− 0.0388	0.855	51.5		Sp2	− 0.1636	0.850	12
	Dm3	− 0.2415	0.952	8.3		Dm3	− 0.6880	0.708	2.9
	SG4	− 0.2416	0.770	8.3		SG4	− 1.1456	0.844	1.7
	SG42	− 0.2748	0.738	7.3		SG42	− 1.2716	0.820	1.6
	M13	− 0.0091	0.755	220		M13	− 0.0568	0.911	35
	fd	− 0.0349	0.947	57		fd	− 0.2706	0.940	7.4
	f1	− 0.0284	0.958	70		f1	− 0.1960	0.878	10
	ZJ/2	− 0.0262	0.938	76		ZJ/2	− 0.2274	0.916	8.8
	OW	− 0.0603	0.891	33		OW	− 0.3694	0.937	5.4
	SD	− 0.0991	0.905	20		SD	− 0.6185	0.973	3.2

for fd and OW clustered together and were 84% similar to the f1, ZJ/2 and SD cluster (Oudejans *et al.* 2003). Only the M13 PCR product was in a cluster by itself. The F + DNA phages also appear to be comprised of two antigenic groups, M13 type coliphages and all other F + DNA coliphages. Screening of 79 F + DNA isolates from six wastewater treatment facilities and six positive animal faecal samples (out of 40 animal samples) indicated that the antigenic characteristics of these phages are quite similar (Long unpublished data). Only the M13 type strain was not neutralized by polyclonal sera produced against fd, f1, ZJ/2 or an environmental isolate. Owing to this similarity, the few differences in survival observed for the F + DNA coliphages tested are consistent with the similarities among the F + DNA coliphage subtypes.

Overall, it appears the MS2 and M13 subtypes of F-specific coliphages may demonstrate longer survival in environmental waters than the other F-specific coliphage subtypes. These experiments are just the first step in understanding the survival of the different subgroups of F-specific coliphages in environmental waters. Additional factors, such as sunlight and predation, would need to be studied to fully understand differences in coliphage subtype environmental survival characteristics. These differences in turn are especially important for interpreting coliphage typing results for source tracking. The results indicate that season and ambient water temperature may affect the usefulness of subtyping F-specific coliphage isolates for microbial source delineation in natural waters. These phages may demonstrate longer environmental survival at winter temperatures than at moderate temperatures. The data also indicate that differences in survival among strains of F-specific coliphages are greater at moderate environmental temperatures, potentially making

interpretation of source tracking information more complex. It is during summer months when many utilities observe increases in coliform levels, and theoretically pollutant microorganism inputs. It is thus recommended that land-use information and evaluation of potential microbial pollutant sources be collected in conjunction with F-specific coliphage monitoring and typing in order to provide adequate information for watershed management decision-making.

CONCLUSIONS

The various strains of F + RNA (7) and F + DNA (6) demonstrated statistically significant differences in survival in source water microcosms when maintained at 4 and 20°C in the dark. All coliphage strains tested decayed more rapidly at 20°C than at 4°C, although differences were not always statistically significant at the 90% confidence level. For the F + RNA coliphages, the Group I phages survived over 110 days in source water microcosms with little decay while the Group II phages survived for 110 days with a 3 $\log_{10}$ reduction in numbers. The Group III and IV F + RNA phages reached detection limits of the assay (5 $\log_{10}$ reduction) in 2 to 3 weeks. For the F + DNA coliphages, M13 survived statistically longer than all the other F + DNA phages tested at a 90% confidence level. To provide guidance for interpreting varying densities of each subtype of F-specific coliphage in source tracking applications, further studies that more accurately mimic true environmental conditions, such as studies in dialysis bags, need to be conducted.

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ABBREVIATIONS AND NOTATION

cfu	colony-forming unit
EPA	United States Environmental Protection Agency
ml	millilitre
pfu	plaque-forming unit

REFERENCES

Adams, M. H. 1959 *Bacteriophages*. Interscience, New York, NY.

Alderisio, K. A., Wait, D. & Sobsey, M. D. 1996 Detection and characterization of male-specific RNA coliphages in a New York City reservoir to distinguish between human and non-human sources of contamination. In: *Watersheds Restoration Management: Physical, Chemical, and Biological Considerations*. American Water Resources Association, pp. 133–142.

Calci, K. R., Burkhardt, W., Watkins, W. D. & Rippey, S. R. 1998 Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters. *Appl. Environ. Microbiol*. **64**, 5027–5029.

Chung, H. 1993 F-specific coliphages and their serogroups, and *Bacteroides fragilis* phages as indicators of estuarine water and shellfish quality. Doctoral Dissertation, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC.

Furuse, K., Osawa, S., Kawashiro, J., Tanaka, R., Osawa, Z., Sawamura, S., Yanageawa, Y., Nagao, T. & Wantanabe, I. 1983 Bacteriophage distribution in human faeces: Continuous survey of healthy subjects and patients with internal leukemia diseases. *J. Gen. Virol.* **64**, 2039–2043.

Gerba, C. P. 1987 Phage as indicators of fecal pollution. In: *Phage Ecology* (ed. S. M. Goyal, C. P. Gerba & G. Bitton). John Wiley & Sons, New York, NY.

Goyal, S. M., Gerba, C. P. & Bitton, B. 1987 *Phage Ecology*. John Wiley & Sons, New York, NY.

Havelaar, A. H.1987 Bacteriophages as model organisms in water treatment. *Microbiol. Sci.* **4**(12), 362–364.

Havelaar, A. H., Furuse, K. & Hogeboom, W. M. 1986 Bacteriophages and indicator bacteria in human and animal faeces. *J. Appl. Bacteriol.* **60**, 255–262.

Havelaar, A.H., Hogeboom, W. M., Furuse, K., Pot, R. & Hormann, M. P. 1990 F-specific RNA bacteriophages and sensitive host

strains in feces and wastewater of human and animal origin. *J. Appl. Bacteriol.* **69**, 30–37.

Horiuchi, K., Vovis, G. F. & Model, P. 1978 The filamentous phage genome: genes, physical structure, and protein products. In: *The Single-Stranded DNA Phages* (ed. D. T. Denhardt, D. Dressler & D.S. Ray). Cold Spring Harbor Laboratory.

Hsu, F.-C., Shieh, Y.-S., van Duin, J., Beekwilder, M. J. & Sobsey, M. D. 1995 Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. *Appl. Environ. Microbiol.* **61**, 3960–3966.

IAWPRC Study Group 1991 Bacteriophages as model viruses in water quality control. *Wat. Res.* **25**, 529–545.

Kleinbaum, D. G., Kupper, L. L. & Muller, V. 1988 *Applied Regression Analysis and Other Multivariable Methods*. Duxbury Press, Belmont, CA.

Lasobras, J., Muniesa, M., Frias, J., Lucena, F. & Jofre, J. 1997 Relationship between the morphology of bacteriophages and their persistence in the environment. *Wat. Sci. Technol.* **35**, 129–132.

Long, S. C. 1999 Project Report—Project Continuation: Development of methods to differentiate microorganisms in MDC reservoir watersheds. Department of Civil and Environmental Engineering, University of Massachusetts, Amherst, MA.

Long, S. C. Unpublished data. Civil and Environmental Engineering, University of Massachusetts, Amherst, MA.

National Center for Biotechnology Information website 2000 *http://www.ncbi.nlm.nih.gov/*, accessed June 22, 2000.

Oudejans, S. J. G., Long, S. C. & Vinjé, J. 2003 Simultaneous detection and genotyping of male-specific coliphages by reverse line blot hybridization. Presented at the 103rd General Meeting of the American Society for Microbiology, May 18–22 (2003), Washington, DC.

Owasa, S., Furuse, K. & Watanabe, I. 1981 Distribution of ribonucleic acid coliphages in animals. *Appl. Environ. Microbiol.* **41**, 164–168.

Schaper, M., Jofre, J., Uys, M. & Grabow, W. O. K. 2002 Distribution of genotypes of F-specific RNA bacteriophages in human and non-human sources of fecal pollution in South Africa and Spain. *J. Appl. Microbiol.* **92**(4), 657–667.

Seeley, N. D. & Primrose, S. B. 1980 The effect of temperature on the ecology of aquatic bacteriophages. *J. Gen. Virol.* **46**, 87–95.

Sobsey, M. D., Schwab, K. J. & Handzel, T. R. 1990 A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. *J. AWWA* **Sept**., 52–59.

Sobsey, M. D., Battigelli, D. A., Handzel, T. R. & Schwab, K. J. 1995 Male-specific coliphages as indicators of viral contamination of drinking water. AWWA Research Foundation, Denver, CO.

US EPA 2000a *Draft Method 1601: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure*. EPA 821-R-00-009, April (2000).

US EPA 2000b *Draft Method 1602: Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure*. EPA 821-R-00-0010, April (2000).

US EPA website 2000 *http://www.epa.gov/ogwdw000/smallsys/ndwac/surface.html*, accessed on November 22, 2000.

Wait, D. Personal communications. UNC Environmental Virology Laboratory, Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, NC.