Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

A rapid reverse transcription-PCR assay for F+ RNA coliphages to trace fecal pollution in Table Rock Lake on the Arkansas–Missouri border

S.K. Dryden[1], B. Ramaswami[1], Z. Yuan, D.E. Giammar, L.T. Angenent*

Department of Energy, Environmental & Chemical Engineering, Washington University in St. Louis, St. Louis, Missouri 63130, USA

ARTICLE INFO

Article history:
Received 13 February 2006
Received in revised form
24 July 2006
Accepted 4 September 2006
Available online 27 October 2006

Keywords:
Bacteriophages
Fecal indicators
F+ RNA
MPN
RT-PCR

ABSTRACT

Source determination of fecal contamination is imperative to efficiently reduce the fecal material load to environmental waters. This study developed primer pairs targeting three F+ RNA bacteriophages and a simple filtration sampling method to enumerate and identify coliphages in environmental waters. Water samples were collected seasonally for one year from the watershed of Table Rock Lake on the Arkansas–Missouri border in areas predisposed to fecal contamination. Collected samples were analyzed quantitatively with most probable number and plaque assays and qualitatively with reverse transcription-PCR. We demonstrated the usefulness of F+ RNA coliphages as an indicator of fecal contamination, but were unable to distinguish between human and non-human sources. F+ coliphage numbers in Table Rock Lake showed seasonal variation with the highest level of coliphage presence during the January sampling event.

1. Introduction

Elucidating the source of a fecal contaminant is imperative to controlling pollution. A variety of methods targeting bacteria, viruses, and biological macromolecules have been used to distinguish between fecal pollution of human and non-human origin. For example, workers have developed microbiological and molecular methods that include an initial bacterial culture originating from mammalian and bird intestines. These methods include: fecal coliforms to fecal streptococci ratios (Geldreich and Kenner, 1969); *Bacteroides* sp. presence (Kreader, 1995); *Escherichia coli* ribotypes (Carson et al., 2001) and antibiotic resistance patterns (Harwood et al., 2000). Such assays, however, are time consuming, labor intensive, and require extensive culture collections.

Detection of certain host-specific markers with molecular biology assays does not require culturing of bacteria, and therefore are a more precise and rapid methodology for identifying sources of fecal pollution. Host molecular markers include specific nucleic acid sequences of bacteriophages (i.e., viruses of bacteria) infecting *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*, which are mammalian gut bacteria (Blanch et al., 2004; Puig et al., 1999; Puig et al., 2000; Tartera et al., 1989). The absence of *B. fragilis* phages in polluted waters in the United States; however, limit the usefulness of this marker (Havelaar et al., 1993; Jagals et al., 1995; Puig et al., 1999; Scott et al., 2002). Researchers identified specific RNA coliphages (i.e., bacteriophages that infect *E. coli*) from human and non-human fecal material, suggesting that these phages can also be used to distinguish between human and non-human fecal sources of pollution (Cole et al., 2003; Havelaar and Hogeboom, 1984; Luther and Fujioka, 2004).

Coliphages have been characterized into two groups: somatic, which infect through the cell wall, and male-specific

*Corresponding author. Tel.: +1 314 935 5663; fax: +1 314 935 5464.
E-mail address: angenent@seas.wustl.edu (L.T. Angenent).
[1] Contributed equally.
0043-1354/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2006.09.003

(F^+), which infect through sex pili. To discriminate between fecal sources, workers have also typed F^+ coliphages into four sub-groups that are either highly associated with humans (groups II and III) or non-humans (groups I and IV) (Scott et al., 2002). Historically, typing was performed with serotyping assays after several viral isolation steps, including concentration of phages from environmental samples, isolation and purification of phages with single or double agar layer plaque assay methods, and propagation in broth (Havelaar and Hogeboom, 1984; Havelaar et al., 1993; Sobsey et al., 1990). However, the methods of F^+ RNA coliphage serotyping have been shown to produce ambiguous results (Beekwilder et al., 1996). For this reason, researchers have developed a method that utilizes reverse transcription-polymerase chain reactions (RT-PCR) or PCR and a subsequent reverse-line blot hybridization technique for genotyping of F^+ RNA or F^+ DNA coliphages, respectively (Vinje et al., 2004). Both serotyping and genotyping suffer from the need to isolate viruses, which can be time consuming.

The overarching goal of this study was to develop a simple and reliable technique to detect and identify the source of fecal contamination in environmental waters. The study developed primers for a RT-PCR technique to differentiate between fecal sources without the need for viral isolation and membrane hybridization. A suite of three PCR primer pairs specific for F^+ RNA coliphages was designed to discriminate between human and non-human fecal pollution after a propagation step. We tested this method with samples collected from source-rich surface waters in the Table Rock Lake watershed on the Arkansas–Missouri border. The RT-PCR technique was used to identify bacteriophages, while single agar layer (SAL) Petri dish techniques and a traditional most probable number (MPN) assay were used to quantify bacteriophages. Samples gathered for microbiological analysis were collected via direct sampling with filtration to discard bacteria and debris.

2. Materials and methods

2.1. *Bacterial strains and bacteriophages*

Bacteriophages MS2, GA and SP were used as a reference in all experiments for groups I, II and IV, respectively. *E. coli* strain C-3000 (ATCC 15597) was used as a host strain and grown in minimal media (6.0-g Na_2HPO_4, 1.0-g NH_4Cl, 3.0-g KH_2PO_4, 10-mL 10% glucose in 1 L) supplemented with 1-mL 1 M $MgSO_4$, and 100-mg thiamine. *E. coli* and bacteriophage MS2 (ATCC 15597-B1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Bacteriophages GA and SP were provided by Dr. Mark Sobsey, University of North Carolina, Chapel Hill, NC.

2.2. *Sample collection*

Sample sites (see web-based supplementary data) for specific contaminants from chicken farms, private septic systems, and municipal treatment facilities, and a background site were selected through a multicriteria geospatial information systems (GIS) analysis (data not shown). Sampling locations and events for October 2004 through January 2005 were the same as described in Yuan et al. (2006). In addition to the full sample sets gathered from October 2004 to January 2005, a subset of samples was collected in May and August 2005. We sampled once per season to assess the effects of seasonal variation in source loadings and lake dynamics. During each sampling event, environmental samples were directly collected into sterile 250-mL polypropylene bottles and stored at 4 °C for subsequent laboratory analysis. An additional protocol using granular activated carbon (Jothikumar et al., 1995) was also performed during the October 2004 and January 2005 sampling events (see web-based supplementary data).

2.3. *Sample preparation*

The directly collected samples were filtered through 0.22 μm pore-size filters (Stericup, Millipore, Billerica, MA). A SAL plaque assay was used to enumerate the presence of bacteriophages. First, 100 mL of the filtered sample was mixed with tepid minimal media agar and 2 mL of host bacteria, and then plated into Petri dishes and incubated for 24 h. Translucent areas in the SAL plates were counted. A modified MPN assay (Eaton et al., 1998) was also conducted for each sample. A serial dilution of a 10-fold gradient was performed into minimal media with the bacterial host. For the October 2004 and January 2005 samples, triplicate MPN vials with 2, 20, and 200 × dilutions of the sample were assessed. Following an insufficient dilution of the January samples, five replicates of MPN vials with 2, 20, 200, and 2000 × dilutions of the sample were assessed for May and August samples.

2.4. *RT-PCR bacteriophage identification*

Positive MPN tubes were extracted using a TRI REAGENT LS protocol for RNA isolation according to the manufacturer's specifications (Sigma, St. Louis, MO). The resulting RNA pellet was suspended in 5 μL of TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), 1 μL was used for reverse transcription and the remaining solution was stored at −20 °C.

Multiple primer alignments based on the complete genomic sequences of F^+ RNA phages (MS2, GA and SP) were constructed using Clustal X Multiple Sequence Alignment Program version 1.81 (University of British Columbia Bioinformatics Centre, Vancouver, Canada). Primer pairs were designed using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA), NetPrimer (Premier Biosoft International, Palo Alto, CA), and OligoAnalyzer 3.0 (IDT-DNA, Coralville, IA) primer evaluation software. The primers's alignment and specificity were also checked with Amplify 3.1 (University of Wisconsin, Madison, WI).

Extracted environmental samples and bacteriophage standards were used to identify the bacteriophages present via a RT-PCR procedure. Extracted RNA was transcribed into cDNA with reverse transcriptase. A 10-μL solution with 1 μL of extracted sample and 1 μL of random primers was thermally denatured for 10 min at 70 °C and then chilled on ice. These templates were added to a 10-μL solution composed of 100-μM dNTP mix, 5 U of placental RNase inhibitor, 1.5 U of avian myeloblastosis virus reverse transcriptase, and reverse transcriptase reaction buffer. The suspension was transcribed at

Table 1 – Sequence of designed primers for F^+ RNA coliphages

Coliphage	Primer	Sequence	Temperature (°C)	Amplicon (bp)	Source
MS2	1F	5′-AATCTTCGTAAAACGTTCGTGTC-3′	53.7	204	Group I (non-human)
	1R	5′-GAGCCGTACCCACACCTTATAG-3′	56.8		
GA	6F	5′-CGTACTTAGCGGTATACTCAAGACC-3′	56.3	240	Group II (human)
	6R	5′-GTTTCCTGCATATAAGCATACCA-3′	52.9		
SP	2F	5′-TTAAACTAATTGGCGAGTCTGTACC-3′	54.9	236	Group IV (non-human)
	2R	5′-AACAGTGACTGCTTTATTTGAAGTG-3′	54.1		

45 °C for 30 min to produce complementary DNA (cDNA). Subsequent PCR reactions were carried out in 25-μL reaction mixtures containing 1 μL of cDNA, 1 μL of a mix of forward and reverse primers (Table 1), 1.5-mM $MgCl_2$, 100-μM dNTP mix, 1.25-U *Taq* polymerase, 25-μM betaine, and 20-μg bovine serum albumen. The complete reaction mixture was heat activated for 15 min at 95 °C followed by 40 PCR cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min) with a final extension at 72 °C for 10 min in a DNA thermocycler (Eppendorf, Mastercycler gradient, Hamburg, Germany). The amplified samples were analyzed using 2% agarose gel in 0.5 M tris-boric acid EDTA buffer.

3. Results

3.1. F^+ RNA Coliphages in water samples collected in October 2004

In October 2004, samples were collected from 12 source sites and two different drinking water taps of the domestic water supply. At each of these sites a 250-mL sample was collected for enumeration of coliphages by SAL and MPN assays. Four of 12 environmental samples were positive for F^+ coliphages during the October sampling event, while the two drinking water samples were negative (Table 2). Nucleic acid was extracted from positive MPN tubes; virus propagation amplified the number of phages originally present in the environmental sample ensuring adequate amounts of nucleic acid for RT-PCR identification techniques. The Springfield WWTP upstream, downstream and effluent (C1, A1 and S1) samples tested positive for both MS2 and SP, groups I and IV (non-human bacteriophages). The other positive sample, effluent from a septic system near Indian Point (S3C), did not test positive with the coliphage primer sets used in this study, indicating the presence of other F^+ specific phages.

3.2. F^+ RNA coliphages in water samples collected in January 2005

In January 2005, samples were collected according to the October 2004 protocol. Of the collected water samples, all of the environmental surface and source water samples were positive, while the two drinking water samples were negative for F^+ coliphages by plaque assay (Table 2). The plaque assay resulted in an abundance of plaques (>50 per plate) with complete lysis observed on some plates. Similarly, the MPN results showed 11,000 PFU/L of phages or greater from the 12 environmental samples due to an overwhelming amount of phages and an insufficient dilution range, while both drinking water samples were negative. Each of the 12 samples that were positive for F^+ coliphages were also positive for MS2 coliphage (non-human) with RT-PCR analysis. Initially, we also used a sampling method that concentrated phages in a granular activated carbon filled column. However, when only nine of the 12 concentrated samples were positive at lower levels we decided to sample directly (see web-based supplementary data).

3.3. F^+ RNA Coliphages in water samples collected in May 2005

During the May 2005 sampling event, seven of the original 14 sampling sites were tested. Since the continuation of the study focused on the seasonal effects of viable F^+ coliphages as an indication of fecal pollution, samples from sewage treatment facilities, septic tanks, and drinking water sources were eliminated from the sample set. These sites were expected to be the least affected by seasonal changes. Three of the seven samples were positive for F^+ coliphages (Table 2). RNA extraction and RT-PCR techniques identified GA coliphages, classified as human coliphages, upstream of the Springfield WWTP (C1) and MS2 coliphages, classified as non-human coliphages, in the chicken-waste impacted Kings River (A5) arm of the lake. We were unable to amplify positive samples taken downstream of the Springfield WWTP (A1) despite the indicated presence of coliphages.

3.4. F^+ RNA coliphages in water samples collected in August 2005

During the August 2005 sampling event, seven water samples were collected following the May 2005 protocol. Coliphage presence was shown both upstream and downstream of the Springfield WWTP (C1, A1), upstream of the Branson West WWTP (C2), and at the Kings River site (A5) (Table 2). RT-PCR assays revealed that upstream of the Springfield WWTP (C1), GA coliphages were present, indicating human fecal pollution in the water. The primer sets used in this study did not amplify the remaining three positive samples.

Table 2 – Enumeration of F^+ RNA coliphages by plaque assay, MPN and RT-PCR identification of samples collected during the October 2004, January 2005, May 2005 and August 2005 sampling events

Site information			Plaque assay PFU/L				MPN MPN/L				RT-PCR identification			
			October 2004	January 2005	May 2005	August 2005	October 2004	January 2005	May 2005	August 2005	October 2004	January 2005	May 2005	August 2005
1	Downstream of Springfield WWTP	A1	600	550	3	248	750	>11,000	13	79	MS2, SP	MS2	Unamplified	Unamplified
2	Upstream of Springfield WWTP	C1	380	1600	3	199	430	>11,000	22	2	MS2, SP	MS2	GA	GA
3	Downstream of Branson West WWTP	A2	0	1580	0	0	0	>11,000	0	0	—	MS2	—	—
4	Upstream of Branson West WWTP	C2	0	1500	0	147	0	>11,000	0	240	—	MS2	—	Unamplified
5	Lake Near Septic Discharge, Indian Pt.	A3	0	>4100	0	0	0	11,000	0	0	—	MS2	—	—
6	Lake at Inflow of Kings River	A5	0	>4100	5	109	0	>11,000	2	493	—	MS2	MS2	Unamplified
7	Effluent of Springfield WWTP	S1	10	790	NA	NA	40	>11,000	NA	NA	MS2, SP	MS2	NA	NA
8	Effluent of Branson West WWTP	S2	0	>6800	NA	NA	0	>11,000	NA	NA	—	MS2	NA	NA
9	Septic System near Joe Bald	S3A	0	20	NA	NA	0	>11,000	NA	NA	—	MS2	NA	NA
10	Septic System on Aunts Creek	S3B	0	2500	NA	NA	0	>11,000	NA	NA	—	MS2	NA	NA
11	Septic System on Indian Point	S3C	900	370	NA	NA	2400	>11,000	NA	NA	Unamplified	MS2	NA	NA
12	Less Developed Site, Piney Creek	B	0	1200	0	0	0	11,000	0	0	—	MS2	—	—
13	Tap water from Springfield	D1	0	0	NA	NA	0	0	NA	NA	—	—	NA	NA
14	Tap water from Indian Point	D3	0	0	NA	NA	0	0	NA	NA	—	—	NA	NA

NA, not analysed; –, not positive with MPN; Unamplified, not positive with PCR.

4. Discussion

4.1. F^+ RNA phages are useful indicators of fecal pollution

We aimed to establish the use of F^+ RNA coliphages in three areas: as indicators of fecal pollution, for phosphorous source apportionment (i.e., determination of the relative contribution of multiple sources towards phosphorous loading), and assessment of the original fecal source. The environmental samples from locations that were most likely impacted by fecal pollution yielded higher levels of F^+ RNA coliphages than the other locations. Therefore, F^+ RNA coliphages can be considered an indicator of fecal pollution in the watershed. This study did not find F^+ RNA coliphages to be a reliable target for source apportionment, since we did not find a correlation between total phosphorous levels and F^+ RNA phages (data not shown). Likewise, genotyping results did not show a correlation between the presence of human (GA) coliphages at sites likely to be impacted by human inputs (C1, C2, A3) and the presence of non-human (MS2, SP) coliphages at locations likely to be impacted by mainly non-human inputs (A5, B). Others have reported that genotyping distinguished between phages from human or non-human origin (Cole et al., 2003; Havelaar and Hogeboom, 1984; Luther and Fujioka, 2004). In this study, however, genotyping was not successful in determining the source of pollution, primarily because bacteriophages that were described by others as "non-human" were present in our sources of human fecal pollution (Cole et al., 2003; Luther and Fujioka, 2004; Vinje et al., 2004). A more recent paper has indeed shown that associations between wastewater source and F^+ RNA phages are not absolute and that caution in using genotyping to determine the source must be taken (Long et al., 2005). We used a MPN method prior to genotyping throughout the study, which may have introduced a bias towards coliphages that are better adapted to *E. coli* strain C-3000. However, the method allowed identification of multiple F^+ RNA phages in a single sample (October 2004, Table 2).

4.2. Seasonal conditions affected the levels of F^+ coliphages in the watershed

F^+ RNA coliphages have limitations as an indicator of fecal pollution, despite promising correlations to viral concentrations in environmental waters (Cole et al., 2003). For example, the effects of temperature, pH, salt concentration, photo-oxidation and chlorination on phage survival have been reported by several researchers (Maynard et al., 1999; Sinton et al., 1999). Among the factors that inactivate F^+ RNA bacteriophages, temperature and photo-oxidation are the critical inactivation factors in freshwater (Schaper et al., 2002). Thus, seasonal changes likely influence the probability of detecting viable bacteriophages in the environment. We found that in October 2004, four of 14 samples were positive; in January 2005, 12 of 14 samples were positive; in May 2005, three of seven samples were positive; and in August 2005, four of seven samples were positive for F^+ coliphages. The number of phages during the winter sampling event was the highest compared to all other seasons. In the winter, the numbers of F^+ coliphages surpassed the dilution scheme for the MPN test in each of the 12 environmental samples, while both drinking water samples were negative.

4.3. Seasonal changes affected the type of phage present

Cole et al. (2003) concluded that the resiliency of group I coliphages (such as MS2) is significantly higher than group II, III or IV coliphages. In our study, the number of F^+ RNA coliphages detected was the highest during cold weather sampling. Warm weather summer samples; however, also demonstrated high concentrations of F^+ coliphages. RT-PCR identification of the summer samples revealed that they contained substantial quantities of F^+ coliphages that were not amplified with the primer sets utilized in this study. The specificity of the host strain *E. coli* C3000 was investigated for F^+ RNA as well as somatic coliphages; results showed infectivity with both types of phages (data not shown). Therefore, the increase in detected F^+ coliphages was due to either an unknown and resilient type of F^+ RNA coliphage present in the summer, or an increase in somatic and/or F^+ DNA coliphages. Other studies have shown that somatic and F^+ DNA coliphages are more resistant to sunlight and warmer temperatures than F^+ RNA bacteriophages, making them more readily detected in summer months (Cole et al., 2003; Duran et al., 2002; Vinje et al., 2004).

4.4. F^+ RNA coliphages in high quantities were found in the background site

During the winter sampling event in particular, the unanticipated high numbers of F^+ RNA coliphages in the sampling location on the less developed site (B, the background site) coincided with unanticipated high total phosphorus levels of ~80 ppb (Yuan et al., 2006). In addition, data from the US Geological Society and the US Army Corps of Engineers showed apparent shifts in the water flow patterns in Table Rock Lake, resulting from opening and closing various dams along the water body (USACE, 2005; USGS, 2005). These flow changes produce a backward flow of water from the main body of the lake into individual lake fingers including the inlet of background site (B). Mixing of lake water may have affected phage types and levels for the lake samples (A3, A5), but they did not affect river samples (A1, A2, C1, C2). Thus, the inability to distinguish between fecal sources was not affected by the lake mixing, because it was based on river sample data.

5. Conclusions

- F^+ RNA coliphages can be used as a biological indicator for fecal pollution, but they cannot be used for phosphorous source apportionment or for distinguishing between human and non-human fecal pollution in Table Rock Lake.
- Enumeration of phages showed seasonal variation. Winter samples contained the highest concentration of coliphages while fall and spring samples had the least. Coliphages isolated during the summer of 2005 were not amplified with our methods and are thought to be somatic or F^+ DNA coliphages.

- Future studies are required to elucidate the diversity of the F^+ RNA coliphage community in the Table Rock Lake watershed. The investigation of a suitable host bacteria for specific isolation of F^+ RNA coliphages in environmental surface waters is necessary to suppress detection of somatic and F^+ DNA coliphages. In addition, the development of primers targeting all known F^+ RNA coliphages in the four sub-groups and a direct quantitative RT-PCR (qRT-PCR) assay of filtered environmental samples should have high priority.

Acknowledgements

We would like to acknowledge Dr. Stefan Falke, Washington University in St. Louis, and David Casaletto of Table Rock Lake Water Quality, Inc. for their part in planning the sampling events. We would also like to thank the WWTP operators at Springfield and Branson West, the septic tanks owners, and the assistance of James Noel and Eric Kettleson. We acknowledge NDWRCDP (Project Number WU-HT-03-00) and the National Science Foundation for support of SKD.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.watres.2006.09.003.

REFERENCES

Beekwilder, J., Nieuwenhuizen, R., Havelaar, A.H., van Duin, J., 1996. An oligonucleotide hybridization assay for the identification and enumeration of F-specific RNA phages in surface water. J. Appl. Bacteriol. 80 (2), 179–186.

Blanch, A.R., Belanche-Munoz, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kuhn, I., Moce, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G., Taylor, H.D., Wallis, J., Jofre, J., 2004. Tracking the origin of faecal pollution in surface water: an ongoing project within the European Union research programme. J. Water Health 2 (4), 249–260.

Carson, C.A., Shear, B.L., Ellersieck, M.R., Asfaw, A., 2001. Identification of fecal *E. coli* from humans and animals by ribotyping. Appl. Environ. Microbiol. 67 (4), 1503–1507.

Cole, D., Long, S.C., Sobsey, M.D., 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. Appl. Environ. Microbiol. 69 (11), 6507–6514.

Duran, A.E., Muniesa, M., Mendez, X., Valero, F., Lucena, F., Jofre, J., 2002. Removal and inactivation of indicator bacteriophages in fresh waters. J. Appl. Microbiol. 92 (2), 338–347.

Eaton, A.D., Clesceri, L.S., Greenberg, A.E., Franson, M.A.H., American Public Health Association, American Water Works Association, Water Environment Federation, 1998. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Geldreich, E.E., Kenner, B.A., 1969. Concepts of fecal streptococci in stream pollution. J. Water Pollut. Control Fed. 41 (8, Suppl.), R336.

Harwood, V.J., Whitlock, J., Withington, V., 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: Use in predicting the source of fecal contamination in subtropical waters. Appl. Environ. Microbiol. 66 (9), 3698–3704.

Havelaar, A.H., Hogeboom, W.M., 1984. A method for the enumeration of male-specific bacteriophages in sewage. J.Appl. Bacteriol. 56 (3), 439–447.

Havelaar, A.H., van Olphen, M., Drost, Y.C., 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. Appl. Environ. Microbiol. 59 (9), 2956–2962.

Jagals, P., Grabow, W.O.K., Devilliers, J.C., 1995. Evaluation of indicators for assessment of human and animal fecal pollution of surface run-off. Water Sci. Technol. 31 (5-6), 235–241.

Jothikumar, N., Khanna, P., Paulmurugan, R., Kamatchiammal, S., Padmanabhan, P., 1995. A simple device for the concentration and detection of Enterovirus, Hepatitis-E virus and rotavirus from water samples by reverse transcription-polymerase chain-reaction. J. Virol. Meth. 55 (3), 401–415.

Kreader, C.A., 1995. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. 61 (4), 1171–1179.

Long, S.C., El-Khoury, S.S., Oudejans, S.J.G., Sobsey, M.D., Vinje, J., 2005. Assessment of sources and diversity of male-specific coliphages for source tracking. Environ. Eng. Sci. 22 (3), 367–377.

Luther, K., Fujioka, R., 2004. Usefulness of monitoring tropical streams for male-specific RNA coliphages. J. Water Health 2 (3), 171–181.

Maynard, H.E., Ouki, S.K., Williams, S.C., 1999. Tertiary lagoons: a review of removal mechanisms and performance. Water Res. 33 (1), 1–13.

Puig, A., Queralt, N., Jofre, J., Araujo, R., 1999. Diversity of *Bacteroides fragilis* strains in their capacity to recover phages from human and animal wastes and from fecally polluted wastewater. Appl. Environ. Microbiol. 65 (4), 1772–1776.

Puig, M., Pina, S., Lucena, F., Jofre, J., Girones, R., 2000. Description of a DNA amplification procedure for the detection of bacteriophages of *Bacteroides fragilis* hsp40 in environmental samples. J. Virol. Meth. 89 (1-2), 159–166.

Schaper, M., Duran, A.E., Jofre, J., 2002. Comparative resistance of phage isolates of four genotypes of F-specific RNA bacteriophages to various inactivation processes. Appl. Environ. Microbiol. 68 (8), 3702–3707.

Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R., Lukasik, J., 2002. Microbial source tracking: Current methodology and future directions. Appl. Environ. Microbiol. 68 (12), 5796–5803.

Sinton, L.W., Finlay, R.K., Lynch, P.A., 1999. Sunlight inactivation of fecal bacteriophages and bacteria in sewage-polluted seawater. Appl. Environ. Microbiol. 65 (8), 3605–3613.

Sobsey, M.D., Schwab, K.J., Handzel, T.R., 1990. A simple membrane-filter method to concentrate and enumerate male-specific RNA coliphages. J. Am. Water Works Assoc. 82 (9), 52–59.

Tartera, C., Lucena, F., Jofre, J., 1989. Human-origin of *Bacteroides fragilis* bacteriophages present in the environment. Appl. Environ. Microbiol. 55 (10), 2696–2701.

USACE, 2005. Water management. 〈http://www.swl-wc.usace.army.mil/〉 November 2005.

USGS, 2005. Waterwatch—current water resources conditions. 〈http://water.usgs.gov/waterwatch/〉 December 13, 2005.

Vinje, J., Oudejans, S.J., Stewart, J.R., Sobsey, M.D., Long, S.C., 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. 70 (10), 5996–6004.

Yuan, Z., Ramaswami, B., Casaletto, D., Falke, S., Angenent, L.T., Giammar, D.E., 2006. Evaluation of chemical indicators for tracking and apportionment of phosphorus sources to a reservoir in a mixed land use watershed. Water Res., submitted for publication.