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***SMALL SCALE WASTE MANAGEMENT PROJECT***

# FRNA Coliphages For Monitoring Groundwater Near On-site Systems

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1994

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# FRNA COLIPHAGES FOR MONITORING GROUNDWATER NEAR ON-SITE SYSTEMS

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Written for Presentation at the
Seventh National Symposium on Individual
and Small Community Sewage Systems
Sponsored by ASAE

Atlanta, Georgia
December 11-13, 1994

**Summary:** Groundwater may be contaminated by on-site wastewater treatment. The coliform test assesses the bacteriological safety of groundwater, but does not address the possibility of viral contamination. "FRNA coliphages" (small, round viruses that infect *Escherichia coli*) have some favorable and unfavorable features as indicators of viral contamination of groundwater.

**Keywords:** FRNA coliphages, groundwater contamination, monitoring, on-site wastewater

# FRNA COLIPHAGES FOR MONITORING GROUNDWATER NEAR ON-SITE SYSTEMS

Mary Alice Woody and Dean O. Cliver[1]

ABSTRACT

Improper subsurface disposal of wastewater after on-site treatment may sometimes lead to groundwater contamination. Bacterial indicators such as coliforms and fecal coliforms are adequate to monitor bacterial contamination, and groundwater contamination with parasite cysts or eggs is relatively unlikely. However, practical methods to test groundwater for possible virus contamination have been lacking. Coliphages are bacteriophages (viruses) that infect the bacterium, *Escherichia coli*. FRNA coliphages are small, round RNA-containing viruses that use the F-pilus (if present) of the *E. coli* cell as their point of attachment to initiate infection. They resemble human enteric viruses such as poliovirus and hepatitis A virus in size, shape, and composition. They can be recovered from groundwater samples by adsorption-elution concentration methods and detected in appropriate *E. coli* cultures. Plaques appear in as little as 4-6 hr at 37°C but are conveniently observed after overnight incubation. Human enteric viruses vary widely in transport and die-off properties in soil and groundwater; the FRNA coliphages are generally in the enteric virus range in these respects. Though seldom detected in individual fecal specimens, they are often present in septic tanks and always in raw urban sewage. Indicators should not multiply in the environment: the ability of FRNA coliphages to multiply in the environment is limited by inadequate temperatures, lack of susceptible host cells, etc. Despite some possible deficiencies, these coliphages appear to offer a rapid and inexpensive method of groundwater monitoring.

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## COLIPHAGES AND GROUNDWATER MONITORING

On-site treatment of wastewater typically ends with subsurface disposal of the treated effluent. If the treatment is improperly done or the disposal site is inappropriate, microbial - contamination of groundwater--which later becomes someone's drinking water--may result (Keswick and Gerba, 1980). The coliform test has long been the standard method for monitoring drinking water for microbial contamination, but the absence of coliform bacteria does not ensure that viruses are not present in the water (Snowdon and Cliver, 1989). Viruses transmitted to people via water are shed from the human intestines in feces. Because the feces of all warm-blooded animals contain the bacterium, *Escherichia coli*, coliphages (viruses that infect *E. coli*) have been suggested as indicators of viral contamination of water (Gerba, 1987; Havelaar, 1987; Snowdon and Cliver, 1989).

FRNA coliphages are a special group that are similar in size (approximately spherical, diameter ~25 nm) and composition (single, plus-strand of RNA coated with protein) to the human enteric viruses (hepatitis A virus and the small, round, structured viruses of gastroenteritis) that are most often transmitted via water (Snowdon and Cliver, 1989). Therefore, it seemed likely that the FRNA coliphages would resemble the human enteric viruses in transport through soil and in inactivation properties.

Potential limitations of the FRNA coliphages in the role of indicators of groundwater contamination by human viruses include: *E. coli* is present in the intestines of all warm-blooded animals, so the presence of FRNA coliphages might be unrelated to presence of human fecal contamination and of human viruses. *E. coli* persists and may multiply in the environment; if the FRNA coliphages can replicate in *E. coli* in the environment, their presence in groundwater may be irrelevant to fecal contamination and the incidence of enteric viruses Havelaar and Pot-Hogeboom, 1988).

The objectives of this study were: (1) to develop a practical

procedure for routine detection of FRNA coliphages in groundwater; (2) to study the incidence of FRNA coliphages in wastewater being treated on-site and in associated groundwater; and (3) to determine whether FRNA coliphages can multiply in *E. coli* outside the intestines, or perhaps the septic tank.

## MATERIALS AND METHODS

### Assays for coliphages and coliforms

Plaque assays were done by the agar overlay method (Adams, 1959). *Escherichia coli* A($F^+$)/λ (supplied by Dr. K. Furuse, Shimane Medical University, Izumo, Japan) was the host strain, unless otherwise noted. Host cells were grown to mid-logarithmic phase at 37°C, and plates were counted within 24 hr of preparation.

To enumerate FRNA coliphages specifically, the assay procedure was modified. Because FRNA coliphages can be distinguished from other coliphages by their sensitivity to RNase, 100 μg of RNase was added per plate to a duplicate set of plates (Furuse et al., 1978). This amount of RNase completely inhibits plaque formation by FRNA coliphages, and only non-FRNA coliphages, which include somatic phages, will form plaques. The difference between the two sets of plates is inferred to be the FRNA coliphage concentration. Student's t test was applied when it was not clear that the mean plaque counts on the two sets of plates were significantly different.

Environmental samples were assayed for coliphages as follows. Generally, a trial assay was done with freshly filter-sterilized samples of wastewater, solids-extracts, or groundwater concentrates. Five plates were prepared of the undiluted material--and of 10-fold dilutions (in L.B. broth), if high coliphage counts were anticipated. If total phage concentrations exceeded 150 PFU/mL in the trial plaque assay, then a second plaque assay was done to enumerate the FRNA coliphages. Two sets of 10 plates were prepared with freshly filter-sterilized samples, one set of which was prepared with 100 μg RNase per plate.

If total phage counts were low (<15 per plate), individual plaques were sampled and tested for RNase sensitivity in a spot

test. Agar plates were overlaid with 2 mL soft agar to which 100 $\mu$g RNase and 0.4 mL of a mid-logarithmic host cell culture had been added. When the soft agar had solidified, a drop of phage suspension was placed on the plate using either a sterile inoculating loop or Pasteur pipette. After a few minutes, the plates were inverted and incubated at 37°C.

Total and fecal coliforms were determined in groundwater samples according to the membrane filter technique (Clesceri et al., 1989). The analyses were performed by Kris Lund, Department of Soil Science, UW-Madison campus.

Assays for enteroviruses

Poliovirus and other enteroviruses will form plaques on BGM and FRhK-4 monolayers. Poliovirus samples were diluted serially as necessary to yield 10-50 plaques in a 25-$cm^2$ culture flask (Cliver and Herrmann, 1969). Maintenance medium was decanted from the flasks, and 1-mL samples of diluted virus were split between two 25-$cm^2$ flasks with confluent monolayers (0.5 mL/flask). The flasks were rocked for 1.5 to 2 hr at 37°C, inocula were decanted, and the monolayers were overlaid with agar medium. When the medium had solidified, the flasks were inverted and incubated at 37°C for 4-5 days. Plaques were made visible in live monolayers with neutral red, or were stained with crystal violet after fixing the cells with formalin.

Preparation of environmental samples

Sample extraction or concentration methods were developed or modified in this laboratory by inoculating dechlorinated tap water with FRNA coliphages, and then performing the procedures. Madison municipal water is obtained from wells, and was used as a groundwater model when unsoftened. Water was dechlorinated either with $Na_2S_2O_3$ (0.1 g/L) or by storing the water overnight in an uncovered flask. Triplicate plaque assay plates were generally prepared of sample dilutions when methods were being developed.

Wastewater samples, solids extracts, and groundwater concentrates were prepared for plaque assays by filtration of the samples through 0.45- and 0.2-$\mu$m porosity Acrodisks (Gelman) into sterile glass tubes. Serial 10-fold dilutions in nutrient broth or L.B.

broth were made as necessary to obtain plaque counts of 30-300 per plate for one of the dilutions. Samples were assayed within a day after filtration, as coliphage titers would decrease after filtration and storage in the glass tubes. As much as possible, wastewater samples were assayed within 2-3 days after receipt.

To test for coliphages adsorbed to solids (from wastewater or groundwater), solids were extracted with 10% phosphate-buffered beef extract, pH 5.6 (USEPA, 1989). Sludge and fecal specimens were extracted with volumes (in milliliters) of the buffered beef extract equal to the weight of the sample in grams. Solids in some wastewater samples were collected by centrifugation of a well-mixed portion of the sample (10,000 × *g*, 30 min, 4°C), and were extracted with a volume of beef extract equal to the volume of the sample which had been centrifuged.

Concentration of coliphages from water samples was done by adsorption to 1 MDS filters (Sobsey and Glass, 1980). Groundwater samples were concentrated and assayed within 36 hr of collection. The concentration procedure was not performed aseptically, but filter holders, vacuum flasks, etc., were decontaminated between samples with boiling water. Fresh filters were used for each sample. Sample turbidity was dealt with either by allowing particulates to settle, and then carefully decanting the sample, or by filtration through a 47 mm diameter AP25 prefilter (Millipore). Soil or clay collected on the prefilters was occasionally used for adsorption-extraction procedures in which a FRNA coliphage (Qß or MS2) was inoculated into dechlorinated tap water, adsorbed to the soil particulates, and then extracted with 10% buffered beef extract. Groundwater samples were filtered through a double layer of 47 mm diameter 1 MDS Virosorb filters (Cuno, Meriden, CT), according to modifications of a published procedure (Goyal et al., 1980; Sobsey and Glass, 1980). Adsorbed coliphages were eluted from the filters by step-wise addition of four 5 mL portions of 0.3% beef extract + 1.0% NaCl, pH 9.5. Eluates were collected in sterile plastic tubes. The pH of each sample was adjusted to 6.5-7.5 with 10-20 µL additions of 0.1 N HCl, and the concentrates were filter-sterilized for assay. Some relatively clear wastewater samples were also concentrated by the filter adsorption-elution method.

The ammonium sulfate-beef extract flocculation method was used to

concentrate coliphages from groundwater samples <400 mL (Shields and Farrah, 1986). Beef extract and NaCl were added to levels of 0.3% and 1%, respectively. Solid ammonium sulfate was added to make 67% saturation. The sample was stored 1-3 days at 4°C, and then the precipitate was collected by centrifugation (10,000 × g, 20 min, 4°C), resuspended in 0.1 original sample volume of sterile distilled water, and filter-sterilized for assay.

Collection of environmental samples

Wastewater samples were collected from the Madison Metropolitan Sewerage District (MMSD) wastewater treatment plant and from several on-site systems from in Wisconsin. Influent sewage samples from the MMSD plant were 24-hr composites. Samples of lagoon-stored sludge and gravity belt-thickened sludge were also obtained from the MMSD plant. When possible, samples of septic tank effluent or dosing chambers were also obtained from on-site systems where groundwater had been obtained. Samples were transported on ice and stored at 4°C at the laboratory.

Ten human fecal specimens were obtained from the Virology Division of the State Laboratory of Hygiene, UW-Madison campus. These were negative for viruses and other pathogens.

Groundwater samples were collected from monitoring wells in the vicinity of Stevens Point, WI. Each site contained four nested wells, with bores spaced about 1.5 ft in depth; depth of groundwater ranged from 24 in to 60 in. The monitoring wells were located within 25 ft of pressurized on-site systems that served one to five households, in the plume of contaminated water. Wells were disinfected with hypochlorite and pumped until hypochlorite was undetectable.

With the help of the Wisconsin Geological and Natural History Survey, households near Sturgeon Bay, WI, were identified with wells in which coliforms had occasionally been found. Thirteen households were contacted, and five water samples were eventually collected. Other groundwater and wastewater samples were obtained from an on-site system near Merrimac, WI. Because Lake Wisconsin was located approximately 25 ft from the system, a 1-L sample was obtained from the lake. A 5-gal sample of municipal water (originally groundwater) from Waunakee, WI, was submitted for alleged association with consumer illness. Groundwater

samples were obtained from six monitoring wells in the vicinity of the cluster system serving the Wisconsin Heights High School. The wells are probably in the plume of contaminated water from the portion of the infiltrative field which is failing. Water samples from monitoring wells in the vicinity of several other sites in Wisconsin were also collected.

Replication conditions for FRNA coliphage

To determine how environmental conditions constrain replication of FRNA coliphages, several variables were examined experimentally. These included the temperatures at which the bacterial host cells were grown and at which the host cell population was challenged with FRNA coliphage, the concentration and growth stage of the host cells, the concentration of nutrients in the system and the competition of other bacteria for the nutrients, and the competition of non-FRNA coliphages for potential host cells. Other significant variables were identified in the course of the experiments and were investigated when possible. Details of these experiments are not provided here, due to space constraints.

## RESULTS

Detection of FRNA coliphages

The methods described for processing samples and detecting the FRNA coliphages are the result of extensive comparative trials. Bacterial host strains were compared for sensitivity to wild type FRNA coliphages in samples from this area. Different filters, methods of elution, and other concentration techniques were evaluated. Data will not be presented here, but it will be noted that the methods used throughout the rest of the study were carefully selected and adapted.

Incidence of FRNA coliphages in the environment

Samples of Madison Metropolitan Sewerage District Treatment Plant influent contained FRNA coliphages at levels greater than 1000 PFU/mL, as had been reported earlier (Buyong et al., 1993). Roughly half of samples from septic tanks in various areas of Wisconsin yielded FRNA coliphages, at levels of hundreds to

thousands of PFU per milliliter. The rest had no FRNA coliphages and in some instances no detectable coliphages of any description. It is noteworthy that where an aeration step was included in the on-site treatment system, coliphages that had been present in the septic tank effluent generally were no longer detectable after the aerator treatment.

No FRNA coliphages were detected in the 10 individual human fecal specimens that were tested. Substances extracted from several of these samples inhibited or neutralized the model FRNA coliphage, Qß. In limited tests, FRNA coliphages were detected in association with swine manure, but not with poultry manure. Serogrouping of phage isolates from swine manure and from the septic tanks that received only human waste should probably be attempted.

Incidence of FRNA coliphages in groundwater samples

Although these phages have been found in influent sewage to the Madison wastewater treatment plant and in some septic tank effluents, we have yet to find FRNA coliphages in groundwater. Because no groundwater samples tested to date have contained FRNA coliphages, soil samples from beneath the infiltration beds of septic systems were extracted to test for adsorbed coliphages. None have been detected.

Constraints on replication of FRNA coliphages

Results of our studies on the conditions permitting Qß (a model FRNA coliphage) replication challenge an assertion (Havelaar and Pot-Hogeboom, 1988) that these coliphages can replicate freely in wastewater and groundwater. FRNA coliphages initiate infection of *E. coli* and related bacteria by attachment to the F pilus, which is expressed only under appropriate conditions of temperature and of host cell growth phase and nutrition (Tomoeda et al., 1975) that are unlikely to occur in wastewater and groundwater.

Most recently, the following factors were examined singly and in combination, to determine their effects on replication of Qß, a model FRNA coliphage: 1) host growth rate and growth phase; 2) temperature; 3) competing, insusceptible bacteria; and 4) non-FRNA coliphages in wastewater. On the basis of studies of Qß

replication in wastewater, potential host cells in wastewater are thought to be in a state like late logarithmic or stationary phase. Recently, these studies were repeated, substituting influent urban sewage for the septic tank effluents used previously. Results were very similar to those observed in earlier experiments: there was a net decrease in Qß replication, and little growth of the host cells. Diminished Qß replication in late logarithmic phase host cultures was shown to be a consequence of fewer infected cells as cultures approach stationary phase, rather than a reduction in the number of infective phage particles released by a single host cell. Qß replication is reduced in anaerobic cultures. Not only did these experiments indicate that FRNA coliphage replication would be diminished under the anaerobic conditions of a septic tank, but also that FRNA coliphage replication is reduced when the doubling time of the host cells is prolonged. Qß replication decreases as temperature is reduced from 37° to 25°C, again as a consequence of fewer cells infected at the lower temperature.

As neither wastewater nor groundwater is a nutrient-rich environment, competing, insusceptible bacteria and non-FRNA coliphages were expected to interfere with the replication of phage Qß. *Enterococcus faecalis* and *Pseudomonas fluorescens*, present with the *E. coli* phage host, reduced Qß replication. The effects of the two organisms were not additive: the enterococcus with the phage host resulted in higher phage yields than the control with the phage and its host only. Presence of a non-FRNA coliphage in the culture diminished the extent of Qß replication slightly. A recent experiment suggests that FRNA coliphages select against the $F^+$ phenotype. The extent of phage replication on *E. coli* Famp was reduced slightly in cultures in which ampicillin was omitted. Presumably, this occurred because the Famp plasmid could be lost in the cultures without ampicillin.

Environmental persistence and transport of FRNA coliphages

The stability of model FRNA coliphage Qß is significantly greater at pH 7 than at pH 5 or 9, especially at 20°C as compared to 4°C. However, the pH effect is strongly dependent on the buffer system. This suggests that FRNA coliphages are relatively persistent in wastewater, which is typically near pH 7, but may be less stable when the wastewater enters soils that are alkaline or acid. Laboratory soil (Plainfield sand) adsorption studies

and column studies have been done with mixtures of poliovirus 1 and coliphage Qß, suspended in groundwater or septic tank effluent. Both the poliovirus and the coliphage were held by the soil particles, but could be eluted with beef extract, sometimes with yields that were inexplicably higher than 100%. Many more such studies could be done, but for the time being it appears that the soil adsorption properties of the model human enterovirus and the model FRNA coliphage are similar.

## DISCUSSION

FRNA coliphages have been isolated from some on-site wastewater treatment systems receiving only human waste, as well as from urban wastewater and from swine manure. Serological methods might permit differentiation of the coliphage from swine from the coliphage from humans (Furuse, 1987). No FRNA coliphage was detectable in 10 human fecal specimens, but some of these contained substances that may have masked the presence of coliphages. Whereas FRNA coliphages are consistently present at levels above 1000 PFU/mL in raw urban wastewater, on-site systems serving single families may not contain these coliphages. Levels of coliphages in septic tank effluent are significantly reduced by aeration treatment, which may explain the reductions observed during treatment of urban wastewater that includes aeration in the secondary stage (Buyong et al., 1993).

A principal constraint on replication of FRNA coliphages in the environment is temperature--genetypically competent bacteria are unlikely to form F pili at temperatures that occur in Wisconsin groundwater. In areas where groundwater temperatures exceed 25°C, other constraints, such as inadequate numbers of host cells (given limited nutrient levels and competing bacterial flora), seem likely to prevent replication of FRNA coliphages. Other coliphages are able to replicate over a wider range of temperatures and perhaps a broader range of environmental conditions.

The FRNA coliphages are apparently in the stability range of the human enteric viruses that may be transmitted via groundwater, and show similar properties of association with soil. We suppose that the absence of FRNA coliphages from groundwater samples collected in Wisconsin means that human enteric viruses were also absent at these sampling sites. However, rigorous testing for

human enteric viruses in groundwater samples in which FRNA coliphages were not detected would have been prohibitively expensive and could well have yielded nothing. Therefore, we surmise that FRNA coliphages may well be valid indicators of probable groundwater contamination by human enteric viruses, but validation awaits the identification of sites where groundwater is so contaminated. It may well be best to pursue further studies in areas where on-site wastewater treatment has been less carefully regulated than in Wisconsin.

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