COLIPHAGE IN THE HUDSON RIVER AS AGENTS OF COLIFORM MORTALITY AND INDICATORS OF WATER QUALITY

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

This research focused on the enumeration of total and infective viruses from the Hudson River and select Hudson River tributaries. Techniques for total viral enumeration involved concentration, followed by epifluorescence and transmission electron microscopy (TEM). Detection of coliphage and coliform bacteria, organisms of concern for human health reasons, was done using United States Environmental Protection Agency (USEPA) established protocols. Water samples were collected during June, July and August, 2000, from the Saw Kill, Stony Creek, the Hudson River National Estuarine Research Reserve (HRNERR) sites at Tivoli Bays, the Institute of Ecosystems Studies (IES) mid-river site near the Kingston-Rhinecliff Bridge, and at the Hudson River near Marist College (Poughkeepsie, NY). Coliform, *E. coli,* and coliphage viruses were present at each site studied. Total bacteria were found to be 1.4×10^{-4}% coliforms and 2.4×10^{-6}% *E. coli.* Total viruses were found to contain 5.7×10^{-6} % *E. coli* strain C-infecting phage. On average, approximately 3 infective viruses existed for every *E. coli* cell present, and about 5 % of bacterial cells were infected with lytic bacteriophage. Samples from Saw Kill sites were analyzed for correlations between microbial and nutrient indicators of septic pollution, and no significant positive correlations were found. This research provides a baseline for the study of the effects of viruses on bacterial populations which are an important carbon pool in the Hudson River ecosystem. The study also adds to our knowledge of coliform bacteria and coliphage of potential human origin in the Hudson drainage.

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INTRODUCTION

Recent studies indicate that viruses contribute significantly to bacterial mortality in aquatic ecosystems, and serve as a valuable source of dissolved organic carbon in those systems (Gonzalez and Suttle 1993). Up until recently, it had been assumed that most primary production in aquatic systems remained at the herbivore level, and bacteria, protozoa and viruses were ignored in studying the fate of organic matter--they were considered too sparse and not active enough (Azam 1998). However, major fluxes of organic matter move via dissolved organic matter (DOM) into bacteria and the microbial loop (Azam *et al.* 1983, Sherr *et al.* 1987, Azam 1998). Azam (1998) indicates that previous methods had missed more than 99 % of microorganisms, and greatly underestimated their metabolism. Now it is known that DOM flux into bacteria is a major pathway in aquatic ecosystems – so much so that about one-half of primary production in some aquatic systems is estimated to be channeled through bacteria (Azam 1998).

Little is known about viruses in the Hudson River ecosystem; however, Findlay *et al.* (1996) found that bacteria are an important link in the food web of the Hudson. Bacteria are likely the major component of living biomass in the Hudson River (Findlay *et al.* 1996). While viruses have not been studied previously in the Hudson, they have been found to have a significant impact on mortality in other aquatic microbial communities. Recent studies have indicated that viruses may contribute significantly to bacterial mortality in planktonic systems, and that mortaility attributable to viruses may equal that due to protozoan grazing (Fuhrman and Noble 1995). Viruses can lyse 10 – 20 % of marine heterotrophic bacterial communities daily (Suttle 1994). Based on

electron microscopy work (in which only the viral lytic cycle is visible), it was estimated that viruses could account for about 34 % of total mortality in bacteria (Suttle 1994). Another study found that over 2.5×10^8 virus particles were present in natural lake waters, according to plaque assay methods (Bergh *et al.* 1989). In 1995, a study done on the Danube River found viral concentrations of $3.4 - 8.4 \times 10^6$ virus-like particles (VLPs) / mL (Mathias *et al.* 1995).

In light of these findings, we hypothesized that: 1) if viruses are present, they may have an impact on the bacterial communities that occur in the Hudson River ecosystem, and 2) specific viral types (*e.g.* coliphage) may act as indicators of water quality.

Specifically, this study focused on the detection of coliphage virus in the Hudson River and some of its tributaries. Coliphage represent introduced viruses that are often associated with human pathogen contamination resulting from untreated or insufficiently treated wastewater. Coliphage may also be associated with fecal waste from other mammals. Total virus densities, coliform bacterial densities, and specific activities of coliphage were tested from several sites in order to determine their abundance in the Hudson, and test for relationships with various water quality parameters.

Members of the coliform bacterial group are used as indicators of possible sewage contamination because they are commonly found in human and animal feces (USEPA 2000a). For drinking water, total coliform testing is the standard measurement because their presence indicates contamination of a water supply by outside sources such as wastewater treatment plants, on-site septic systems, domestic and wild animal manure, and storm runoff. Typically the presence of one total coliform colony forming unit (CFU) per 100 mL of water is considered unsafe to drink (Mitchell and Stapp 1994). If

coliphage viruses are present, this could affect the number of total viable coliforms and water quality measurements. Coliphage could also be indicative of water contamination.

The main question this research addressed was:

"Do coliphage exist as infectious agents in Hudson River waters?"

Along with this question, the following points were also of interest:

1) Coliphage Densities – natural densities of intracellular and extracellular coliphage in the Hudson River and its tributaries (Saw Kill and Stony Creek) and HRNERR sites.
2) Total Virus Densities – proportion of total viruses present as infectious coliphage.
3) Relationship to Nutrient Inputs – relationships between bacterial and viral indicators and nutrient (NO_3^- and PO_4^{3-}) concentrations.

In the past, the surface waters of the Saw Kill were shown to have high NO_3^- levels, mainly associated with residential land use (Nieder, unpubl. data). The only seasonally continuous sources of NO_3^- along the entire length of the Saw Kill are residential septic systems, with the exception of the Bard College sewage treatment plant (Nieder, unpubl. data).

In this study, some of the sites in the Saw Kill watershed previously sampled by Nieder were revisited and samples were analyzed for total viruses, total bacteria, total coliform, *E. coli* and coliphage numbers, as well as NO_3^- and PO_4^{3-} levels. These might be indicators of septic inputs, particularly if positive correlations exist between nutrient and microbial indicators of water contamination.

The goal of this research was to begin answering the numerous questions surrounding the activity and effects of viruses in the Hudson River ecosystem. Coliphage

density, proportion of total viruses that are infectious coliphage, total bacteria, coliform and *E. coli* densities were determined at each study site on each sampling date. We compared the numbers of these various classes of bacteria and viruses between the sites. For the Saw Kill and Stony Creek tributaries, it was expected that sites downstream of residential areas would have an increased number of coliphage and coliform as well as higher NO_3^- and PO_4^{3-} concentrations. Baseline data on coliform, coliphage, total bacteria, and total virus abundance provided one indication of how much an effect residential development has on microbial abundance. Since these tributaries lead directly to the Hudson, they may have a direct effect on main channel coliform and coliphage numbers as well.

MATERIALS AND METHODS

Description of Study Sites

A total of 10 sites were sampled in this study (Figure 1). Five of these sites are located along the Saw Kill tributary to the Hudson River (northern Dutchess County, NY). Traveling downstream, the Saw Kill sites included Rock City (RC), Echo Valley Rd. (EV), Linden Ave. (LA), Saw Kill at Bard College (SK) and Tivoli South Bay (SB), respectively. Monthly samples in June, July and August, 2000, from these sites were suspected of containing septic runoff from the town of Red Hook, NY (Nieder, unpubl. data). Monthly samples were also collected from Stony Creek (SC), which flows into Tivoli North Bay (NB). Stony Creek receives sewage and septic contamination from the village of Tivoli, located north of Red Hook. Samples from the Tivoli Bays, as well as the Saw Kill at Bard College and Stony Creek were obtained with the assistance of HRNERR personnel.

The remaining sites are located on the Hudson River main channel. The Kingston-Rhinecliff Bridge (KR) site, located midriver, 158 km north of the Battery, was sampled biweekly with the aid of staff from the Institute of Ecosystem Studies (IES, Millbrook, NY). Two other sites are located nearshore at the Marist College campus in Poughkeepsie, NY. Monthly samples were taken off the boat docks at Marist at ebbing (bP) and flowing tides (aP). This site is located just south of the Poughkeepsie Water Treatment Facility and just north of the Poughkeepsie Sewage Treatment Plant (PSTP). The Hudson, being a tidal river, flows in opposite directions depending upon the tide. At ebbing tide, it flows south, so samples collected at the Marist boat docks should contain no recently treated water from the PSTP. However, at flowing tide, the river flows

northward, so samples at this site should contain some unquantified fraction of water directly from the PSTP.

Sample Collection

Duplicate samples were collected in 1.0 L plastic grab bottles. At HRNERR sites, conductivity, salinity, and dissolved oxygen (DO) data were collected using portable Yellow Springs Instrument (YSI, Yellow Springs, OH) meters. Temperature was recorded at every sample site. Samples were kept in coolers and returned to the lab immediately and processed on the same day as collection.

Processing Samples for Infectious Coliphage Counts

All reported coliphage densities reflect data collected from plaques of 1 mL unconcentrated water sample. Coliphage assays were performed according to the USEPA-recommended plaque assay method (USEPA 1996). Using this procedure, *E. coli*-strain C and 1 mL of water sample is added to tryptone top agar and incubated for 24 h. After this time, the number of plaques formed by viral lysis in the *E. coli* lawn are counted.

Sample Preservation and Microbial Enumeration

Upon return to Marist College labs, portions of all samples were preserved in 1 % formaldehyde, 1 % glutaraldehyde and 1 % EM-grade glutaraldehyde. Replicate samples preserved in formaldehyde were filtered onto 0.02 μm Anodisc filters and stained with SYBR-Green I nucleic acid stain (Noble and Fuhrman 1998). For our epifluorescent microscopic counts, an Olympus BH-2 epifluorescence microscope was used to count total visible viral particles and bacterial cells at ×1000 magnification. At least 400 bacteria and virus-like particles were enumerated for each replicate sample using an

ocular Whipple grid. Appropriate filtering volumes were determined for each sample. Stained samples on slides were kept in the dark at 4.0 °C to prevent fading (Bettarel *et al.* 2000).

Monthly samples from RC, EV, LA, SK, and SB, preserved in EM-grade glutaraldehyde were centrifuged onto 300 mesh Cu EM grids and negative stained with uranyl acetate (Bozzola and Russell 1999) for 90 s. Virus-like particles were then enumerated using a JEOL JEM 1010 TEM at ×75K magnification.

For the determination of viable coliform and *E. coli* densities, replicate samples were filtered onto 0.45 μm membrane filters and cultured on MI agar (USEPA 2000b). Using this protocol *E. coli* colonies appear blue-green under ambient light and coliform colonies fluoresce white under long-wavelength UV light.

Nutrient Analyses

Nitrate and PO_4^{3-} analyses were performed for each replicate sample. Samples were collected in 60 mL acid-washed bottles, 0.45 μm membrane filtered and preserved with H_2SO_4 within 2 h of sample collection. Nitrate-nitrogen (NO_3^--N) values were determined using a Milton Roy Co. Spectronic 1001 at 500 nm as per Eckblad (1978). Phosphate was determined using a Beckman Coulter DU-640 spectrophotometer at 700 and 880 nm, with a long path-length cell (Eckblad 1978).

Study Sites:

Sample Sites	Identification
Tivoli South Bay	SB
Tivoli North Bay	NB
Sawkill outflow	SK
Stony Creek outflow	SC
Sawkill at Linden Avenue	LA
Sawkill at Echo Valley Rd	EV
Sawkill at Rock City	RC
Poughkeepsie Ebbing (before treatment)	bP
Poughkeepsie Flowing (after treatment)	aP
Kingston-Rhinecliff Bridge mid-river	KR

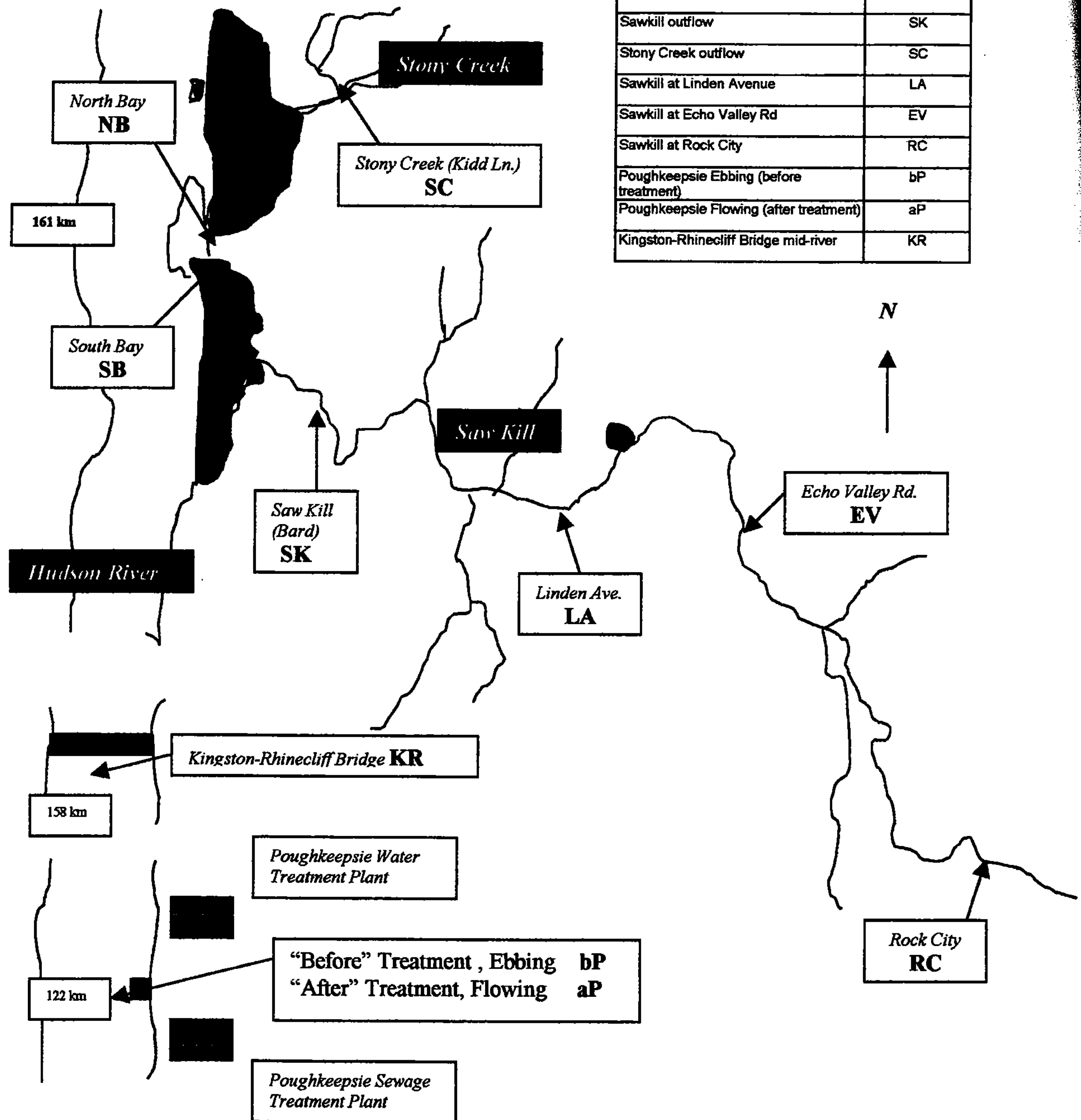

Figure 1. Map of 10 sampling sites along the Saw Kill, Stony Creek and Hudson River.

RESULT S

MI agar analyses indicated that coliform bacteria and, more specifically, *E. coli* were present at all sampling sites (Figure 2). *E. coli* and coliform numbers tended to increase downsteam in the Saw Kill and Tivoli Bays in June and July, with the exception of Rock City. Once August data was incorporated, this trend was less obvious. The main channel Hudson sites had lower total coliform and *E. coli* numbers. On average, 1 % of total coliforms were *E. coli* over all sampling sites.

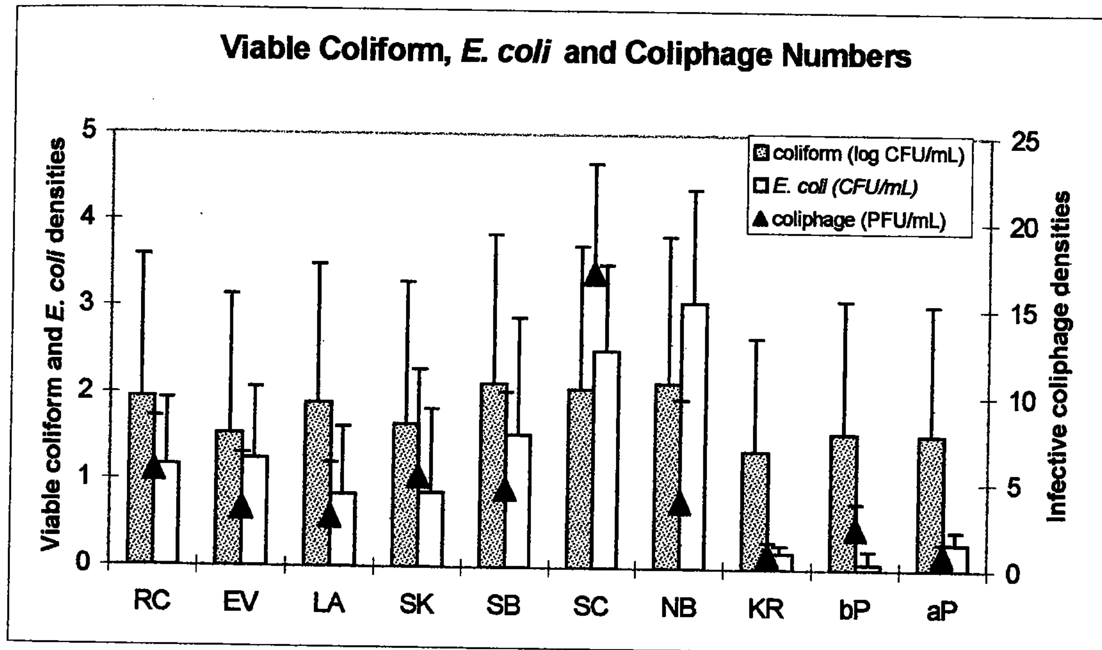

Figure 2. Viable coliform, viable *E. coli* and coliphage numbers averaged over June, July, and August samples. Coliform numbers are expressed in logarithmic format (log CFU/ mL). Error bars represent the standard deviation. Each value represents $n = 6$ samples (2 replicates / 3 months).

Coliphage numbers also displayed a similar pattern of concentration from upstream to downstream, with the exceptions of the Rock City and Stony Creek sites (Figure 2). Coliphage numbers were well correlated with *E. coli* numbers, but were not

significantly correlated with coliform numbers. Total coliform and *E. coli* numbers were significantly correlated (Table 1). However, neither viable total coliform nor viable *E. coli* numbers were significantly correlated with total bacterial numbers from SYBR Green I counts. Conversely, infective coliphage numbers were significantly correlated with total virus numbers (Table 1).

Table 1. Correlation analyses for viruses, bacteria, coliphage, coliforms and *E .coli*. Statistically determined P-values, derived from critical r-value of 0.344 and $n = 33$, except for TEM virus counts, (critical r-value of 0.532 and $n = 12$). P-values were calculated using averaged data for all replicate samples for June, July and August, 2000. Bold numbers indicate significant correlations ($P < 0.05$).

	Total Viruses (VLP/mL) by EMC	**Coliphage (CFU/mL)**	**Coliforms (CFU/mL)**	***E. coli* (CFU/mL)**
Total Bacteria (cells/mL) by EMC	**$0.02 < P < 0.05$**	**$0.02 < P < 0.05$**	$0.20 < P < 0.50$	$P > 0.50$
Total Viruses (VLP/mL) by TEM	**$0.02 < P < 0.05$**	**$0.01 < P < 0.005$**	$P > 0.50$	$P > 0.50$
Total Viruses (VLP/mL) by EMC		**$0.002 < P < 0.005$**	$0.02 < P < 0.05$	**$P < 0.001$**
Coliphage (PFU/mL)			$0.05 < P < 0.1$	**$0.02 < P < 0.05$**
Coliforms (CFU/mL)				**$P < 0.001$**

TEM total viral counts were significantly correlated with total viral counts, by epifluorescence microscopy. Similarly, TEM viral counts were significantly correlated with viable plaque forming coliphage numbers (Table 1). Coliphage and coliform densities were close to being significantly correlated ($P = 0.07$).

Attached and intracellular viruses accounted for a small proportion of the total viruses counted by TEM (Figure 3). On average, approximately 5 % of total viruses counted by TEM at ×75K magnification were attached or inside bacteria (Figure 4). The other 95 % were extracellular (sometimes referred to as “free”) viruses.

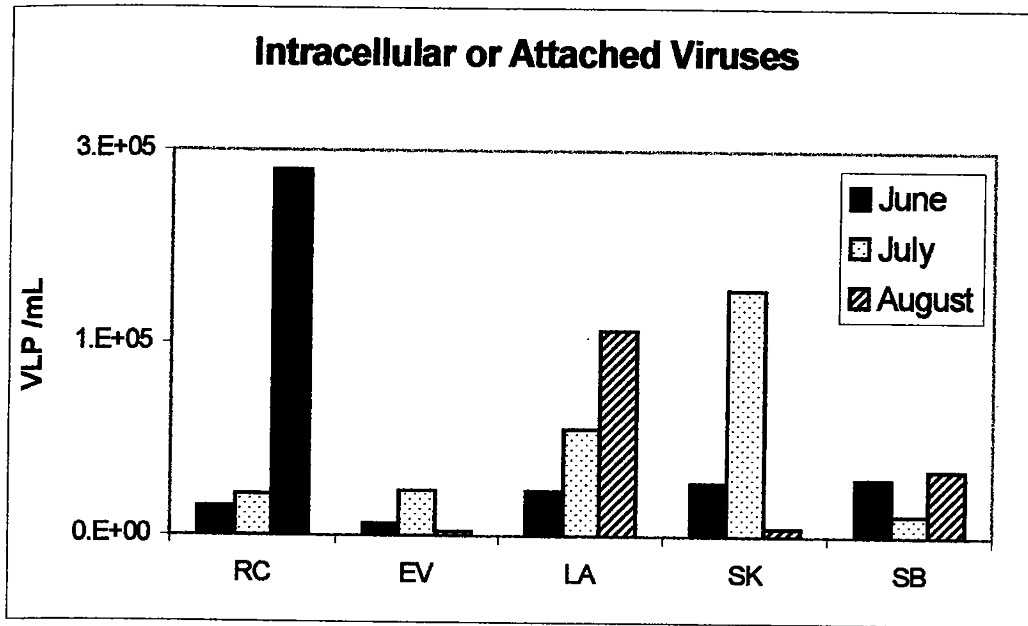

Figure 3. Intracellular or attached viruses. Counts obtained from TEM at × 75K magnification.

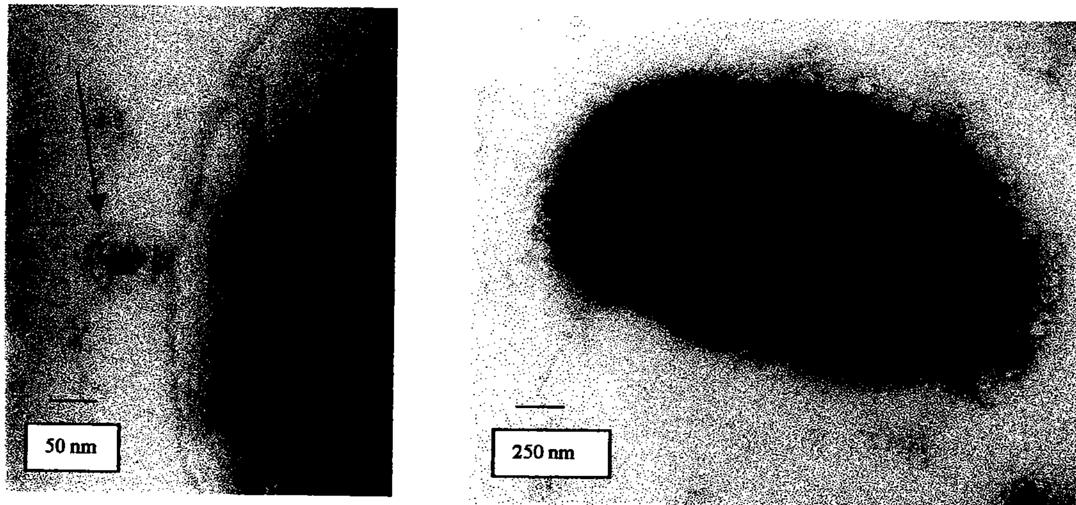

Figure 4. Viruses infecting *E. coli* strain C. (Left) Electron micrograph (×150K) of coliphage infecting *E. coli* strain C from June KR sample plaque. Arrow indicates viral capsid. (Right) Electron micrograph of coliphage infecting *E. coli* strain C cell, (×25K) from June SC sample plaque.

Table 2. Site by site and overall averages for various bacterial and viral density data. ND = no available data.

Site	RC	EV	LA	SK	SB	SC	NB	KR	bP	aP	Avg
Average Total Bacteria (cells/mL x 10^6)	29.7	27.0	47.9	35.1	83.5	63.0	54.7	51.7	39.2	62.7	49.5
Average Viable Coliforms (CFU/mL)	87.8	46.5	77.5	44.3	133.2	115.4	136.7	16.9	37.2	35.9	69.1
% Coliforms	3.0	1.7	1.6	0.11	1.6	1.8	2.5	3.3	9.5	5.7	1.4
Average Viable *E. coli* (CFU/mL)	1.2	1.2	0.8	0.9	1.5	2.5	3.1	0.2	0.1	0.3	1.2
% *E. coli*	3.9	4.6	1.7	2.4	1.8	4.0	5.6	4.0	1.7	4.8	2.4
Average Total Viruses by EMC (VLP/mL x 10^6)	44.4	47.0	75.6	55.9	43.6	321.6	117.2	22.3	50.5	38.8	81.7
Average Total Viruses by TEM (VLP/mL x 10^6)	1.0	1.1	1.0	1.8	1.6	ND	ND	ND	ND	ND	1.3
Average infective *E.coli*-C coliphage (PFU/mL)	5.5	3.3	2.8	5.2	4.5	17.0	3.8	0.8	2.3	0.8	4.6
% *E. coli*-C coliphage	1.2	7.0	3.7	9.2	10.0	5.3	3.3	3.7	4.6	2.2	5.7
Intracellular Viruses by TEM (VLP/mL x 10^4)	11.0	1.6	9.2	8.0	3.7	ND	ND	ND	ND	ND	6.8
% Intracellular Viruses	11.5	1.4	9.2	4.4	2.3	ND	ND	ND	ND	ND	5.8

Table 2 summarizes average densities and proportional findings regarding coliforms, *E. coli* and total bacteria as well as infective coliphage, intracellular viruses and total viruses. Coliforms and *E. coli* made up only a small percentage of total bacteria found at our sites. It is therefore reasonable that we found that coliphage made up only a small proportion of total viruses.

Nutrient Analyses:

Total coliform numbers at Saw Kill sites (SB, SK, LA, EV and RC) were negatively correlated with NO_3^- and uncorrelated with PO_4^{3-} (Figure 5).

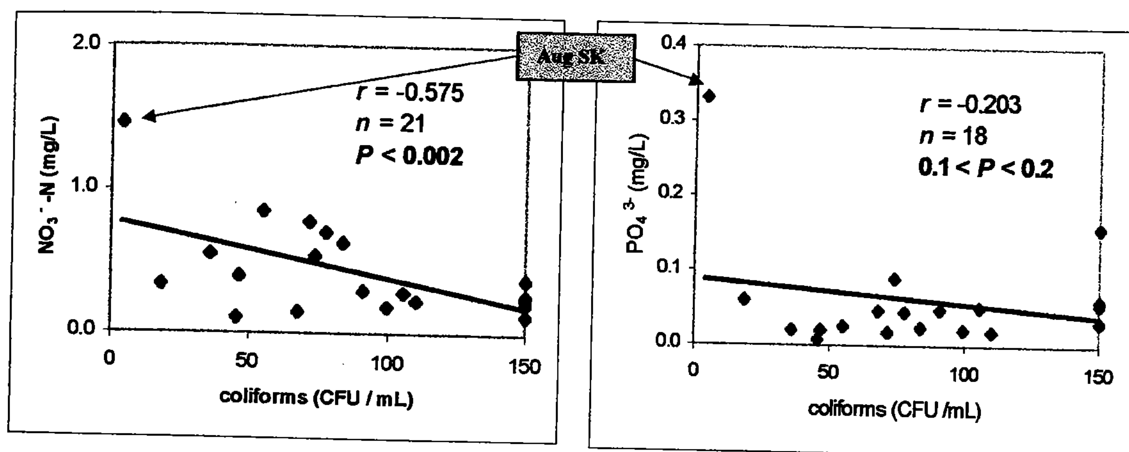

Figure 5. (Left) Nitrate-nitrogen and (Right) phosphate *vs.* total coliforms for all Saw Kill samples collected.

NO_3^--N levels in the Saw Kill were between 0.109 and 1.46 mg/L. These are considered safe levels for drinking water (Fisher 1992). Summer PO_4^{3-} levels in the Saw Kill were between 0.008 and 0.331 mg/L. Those values above 0.03 mg/L may contribute to eutrophication (Fisher 1992) under conditions where phosphate is limiting production. Unusually high NO_3^- and PO_4^{3-} levels were observed at the Saw Kill site near Bard College in August (Figure 5). Without inclusion of these outlier points, NO_3^- and PO_4^{3-} levels are both uncorrelated with total coliform numbers. No significant correlations were found between total coliform numbers and any other water quality data collected.

DISCUSSION

Our results indicate that viruses do exist as infectious agents in the Hudson River waters. In this study, only infective viruses lysing *E. coli* strain C were enumerated with plaque assays, so infectious virus counts are limited to those bacteriophage lysing this particular strain. We believe that the coliphage enumerated in this study represent only a tiny fraction of the total infective virus community in the Hudson. We found that total viral concentrations ranging as high as 8.2×10^7 VLP/mL exist in the Hudson, which is higher than viral concentrations found in many other aquatic environments. (Table 3).

Table 3. Extracellular virus abundance and virus:bacteria ratio (VBR) values obtained from previous studies of virus densities in a variety of aquatic habitats (adapted from Kepner 1997).

Mean Virus or VLP Density ($x 10^6$ /mL)	VBR	System	Reference
10.1	3.2	Chesapeake Bay	Bergh *et al.* 1989
6.1	5.5	Korsfjorden	Bergh *et al.* 1989
4.9	12.2	Faunefjorden	Bergh *et al.* 1989
14.9	49.7	North Atlantic	Bergh *et al.* 1989
0.1	3	Barents Sea	Bergh *et al.* 1989
158	37.1	Southern California Bight (nearshore stations)	Steward *et al.* 1992a
9.8	20.7	Southern California Bight (offshore stations)	Steward *et al.* 1992a
25	11.9	Chesapeake Bay	Wommack *et al.* 1992
2.5 - 36.0	10	Bering and Chukchi Seas	Steward *et al.* 1996
5.5	12	Mission Bay, Southern California	Steward *et al.* 1992b
7.9	10.8	Japanese coastal waters (Osaka and Otsuchi Bays)	Hara *et al.* 1991
5.3	3.4	Japanese offshore waters	Hara *et al.* 1991
251.5	171.2	Gulf of Mexico, nearshore at Port Aransas, Texas	Hennes and Suttle 1995
254	39	Plussee, Germany	Bergh *et al.* 1989
3.4 - 8.4	12.0 - 16.0	Danube River backwater	Mathias *et al.* 1995
10.0 - 40.0	10	Lake Constance, Germany	Hennes and Simon 1995
36	13	Lac Gilbert, Quebec	Maranger and Bird 1996
18.3	62.5	Lake Hoare, McMurdo Dry Valleys, Antarctica	Kepner *et al.* 1997
33.4	42.9	Lake Fryxell, McMurdo Dry Valleys, Anarctica	Kepner *et al.* 1997
1.3 - 81.7	0.03 - 1.7	Hudson River and Saw Kill watershed	This study

Total virus to bacteria ratios (VBR) in our samples were unusually low (Table 3). This is likely due to the high bacterial counts we obtained using SYBR Green stain. Our

counts tended to be one order of magnitude greater than bacterial counts obtained at one of the same sites in the past. For example, Findlay *et al.* (1998) found that total bacterial numbers at the KR site were in the range of 10^6 - 10^7 cells/mL, using acridine orange stain for enumeration. The difference could be due to several factors, including the different staining techniques used, sample storage time, and investigator differences in distinguishing bacteria from similarly shaped and sized inorganic or detrital particles. In any case, our higher bacterial counts account for our lower VBR ratios.

Coliforms and *E. coli* were present at each of our study sites, indicating hazardous drinking water conditions. On average, 1.4×10^{-4} % of total bacteria were calculated to be viable coliforms and 2.4×10^{-6} % of total bacteria were viable *E. coli* in the months studied (Table 2). There were poor correlations between total bacteria and both coliforms and *E. coli*. Obviously, the vast majority of bacteria in the river and its tributaries are not of human nor animal origin.

The small percentages of bacteria that were *E. coli* were similar to the proportions of total viruses that were coliphage. On average 5.7×10^{-6} % viruses enumerated by EMC were infectious coliphage as enumerated in plaque assays. Taking these percentages into account, it follows that there were, on average, three infective coliphage for every *E. coli* cell at the sites studied, which would have the potential of lysing that bacterial cell.

Coliform, *E. coli* and coliphage densities generally increased from upstream to downstream in June and July with a few exceptions. The Rock City (RC) site on the Saw Kill had consistently higher numbers than the Echo Valley Rd (EV) site located downstream. Once August data was included, this trend became less obvious. We

suspect concentrated residential coliform inputs from nearby residential sewage enter at this site. As the water flows downstream, it becomes diluted, accounting for lower numbers at downstream sites.

The main channel Hudson River sites showed consistently lower numbers than either of the tributaries studied or North or South Tivoli Bay sites. These main channel sites, being located south of the Tivoli Bays sites, receive input from these systems. However, this input becomes mixed with upriver Hudson waters, which have a diluting effect on total numbers, resulting in lower coliform, *E. coli* and coliphage concentrations at the main channel sites. As expected, there tended to be higher coliform numbers on the ebbing (bP) Pougkeepsie tide than the flowing (aP) tide, possibly owing to input from the PSTP (Figure 2).

Summer 2000 coliphage numbers were well correlated with both total viable coliform and viable *E. coli* numbers. This positive correlation appears despite the dynamic relationship between parasite and host. As coliform numbers increase, so might their coliphage parasites. Yet it is even possible that there could be some indirect viral stimulation of host cell production. On average, we observed that there were approximately three infectious viruses for every *E. coli* cell and that 5 % of total viruses were attached to bacteria. As coliphage numbers increase and the percentage of infected cells increase, coliform bacteria could increase their rate of reproduction to account for cell loss to viral lysis. Eventually, a critical level will be reached at which coliforms cannot keep up with viral production, and coliform numbers will drop. It follows that coliphage numbers will decrease as well, as hosts cells become less abundant. The interaction between coliform and coliphage depends upon whether coliform can