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BACTERIOPHAGE POPULATIONS IN NATURAL WATER

AUTHOR:

William T. Lammers

**UNION CARBIDE CORPORATION
NUCLEAR DIVISION
OAK RIDGE GASEOUS DIFFUSION PLANT**

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BACTERIOPHAGE POPULATIONS IN NATURAL WATER

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NATURAL WATER

Author: William T. Lammers

ABSTRACT

The application of centrifugal methods of concentrating and isolating small particles has increased the sensitivity of viral assays by approximately two orders of magnitude. With these methods, developed and applied in the Biophysical Limnology Laboratory, it has been possible to investigate the numbers, seasonal variations, and correlation of bacteriophage particles contained in samples of water from Poplar Creek which receives sewage from the eastern section of the city of Oak Ridge.

Samples from two locations, 6.4 Km and 19.3 Km downstream from the sewage effluent point of entry, were centrifugally isolated and studied. This year-long study of bacteriophage populations has given results, especially statistical correlations, which may help in assessing the behavior of these particles in water and which emphasize the importance of further investigation of natural virus populations.

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BACTERIOPHAGE POPULATIONS IN NATURAL WATER

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BACTERIOPHAGE POPULATIONS IN NATURAL WATER

INTRODUCTION

As infectious diseases caused by bacterial and protozoal pathogens are brought under control, there is increasing concern with viral agents as sources of present or potential hazard (1). This is especially true where an increased population results in both an increased quantity of viral particles and an increased reuse of the media in which the viral particles are borne. The aquatic environment in urban and semi-urban areas is a typical case where the media (water) may support an increased viral population and, in addition, be reused several times between its terrestrial origin and the sea.

There are numerous reports of human viral particles present in sewage, in swimming pools, and in streams (1). Most of the reported sites are grossly polluted, but there are additional reports that viral particles will survive for long periods under certain conditions (2). Because of the limitation of sensitivity of classical methods of viral assay, there is little information about the occurrence and behavior of viral particles in streams with a low level of sewage pollution.

The application of centrifugal methods of concentration and isolation of small particles has increased the sensitivity of viral assays by about two orders of magnitude, from 1 plaque forming unit (pfu)/100 ml to 1 pfu/10⁸ (3). With this method of concentration and isolation, it is possible to investigate the numbers, seasonal variation, and correlations of selected virus particles in streams with low level sewage contamination. These techniques, developed in the Biophysical Limnology Laboratory, have produced isolated particulate fractions of relatively high purity for study. The fractionations and studies, made during the past two and one-half years, have encompassed several areas of water pollution. This report is confined to the centrifugal isolation and study of bacteriophage found in a small local stream receiving sewage waste from the eastern section of the City of Oak Ridge.

The stream investigated, Poplar Creek, empties a 65,315 hectare watershed into the Clinch River of Tennessee at Clinch River Mile 12. The creek receives primary sewage effluent from about 10,000 people from one of the sewage plants of the City of Oak Ridge. The sewage load stays about the same all year and there is no other important source of sewage known to enter the creek. A sampling site was selected 6.4 Km downstream from the effluent point of entry. The creek had an average of about 0.6 m³/sec flow at the sampling station. A second sampling site was selected at a point 19.3 Km downstream from the effluent point of entry where the creek had an average of 7.5 m³/sec flow. The 10- to 12-fold dilution between the 6.4 and the 19.3 Km stations, and the time of travel of 1 to 3 hours should allow some time-dependent behavior differences in the viral particles to be observed.

One difficulty with studies of viral populations is only those searched for in a specific assay can be identified. While culturing aquatic bacteria on nutrient broth will grow a large number of the varieties present, viral particles are so specific that they will grow only on a susceptible host. The difficult choice must be made of which species to confirm on a regular basis, and which to record as "virus-like particles" on the basis of physical characteristics. At the first stage the choice should be a strain thought to be constantly present in fairly large quantities. In this investigation three strains of bacteriophage were investigated, and two were chosen for regular confirmation. Bacteriophage of Escherichia coli j., Aerobacter aerogenes, and Proteus vulgaris were searched for. Although phage were found for all three bacteria, E. coli j. (coli-phage) and A. aerogenes (aero-phage) gave the best plaques and were chosen for regular confirmation.

SUMMARY AND CONCLUSIONS

The ability to recover a specific virus strain, in this case bacteriophage, from an open stream at a site distant from the source of the virus and utilizing centrifugal techniques, has been demonstrated. The same centrifugal method can be applied to any viral strain, including human pathogens, suspected of being in the water sample. These techniques were successfully demonstrated in a year-long study of some bacteriophage populations in water from Poplar Creek, a small stream which receives low-level sewage waste from the eastern section of the City of Oak Ridge. This study has given results, especially statistical correlations, which may help in assessing the behavior of these particles in water and which emphasize the importance of further investigation of virus population in natural water.

EXPERIMENTAL TECHNIQUES AND RESULTS

Twenty-liter water samples were collected alternately from either the 6.4 or the 19.3 Km site on Poplar Creek, stored in an iced container, and kept below 10°C until fractionation was complete. Sixteen of these samples were collected during the period from 11/28/66 to 10/16/67. The samples were collected from the upper foot of water with a plastic bucket and the water temperature, depth, flow, pH, and conductivity were recorded at the time of sampling.

In the laboratory the water sample was centrifugally fractionated following the scheme shown in figure 1. The suspended biological or organic particles and clay-minerals ($> 20,000 S$ [S = sedimentation coefficient in Svedberg units]) were removed from the water by continuous flow centrifugation at a centrifugal impulse (force $\times$ time) of $5.6 \times 10^4 \text{ g} \times \text{min}$ (4). The particles isolated by this centrifugation included organic particles larger than 0.5μ and clay-minerals larger than 0.2μ . A direct count of the number of organic particles was made using phase contrast microscopy and a Petrof Hausser counting chamber and method. A similar count was made of clay-mineral particles. The pellet from this centrifugation was thoroughly resuspended and carefully layered over a density gradient of 3,5-DP (methyl glucamine salt of 3,5-diiodo-4-pyridone-N-Acetic

FIGURE 1

FRACTIONATION SCHEME FOR A WATER SAMPLE

acid) made with a gradient preforming device in a sector-shaped centrifuge tube (5,6,7). The gradient ranged in density from about 1 to a maximum of about 1.5 g/cc. This was centrifuged until the desired impulse of $5.6 \times 10^4 \text{ g} \times \text{min}$ was obtained. Then the centrifuge was stopped and the tube contents pumped through the flow cell of an optical densimeter and the absorbance of the gradient recorded at 530 mμ. The area of the gradient peak on the chart record was measured with a polar planimeter and this value taken as one measure of the total organic material, including detritus.

For the last nine samples, a measure was made of the number of viable biological particles by a serial dilution assay in nutrient broth. The growth rate of these particles was measured by turbidity. From these data, plus the chart record area, a value "C" was calculated to represent the relation between these three variables:

$$C = V/G \times A \quad (1)$$

Where,

V = the number of viable particles,
G = the rate of growth, and
A = the chart record area.

The (a) direct microscope count of organic particles generally represents the quantity of the more abundant, intact biological particles in a sample. The (b) chart record area generally represents the total quantity of organic material in the $> 20,000 \text{ S}$ range and includes less abundant types and organic detritus. The (c) value of "C" should reflect both the quantity of total organic material present and the bio-activity of that material (4).

The clay-mineral particles from the above density gradient centrifugation pass through the gradient and form a pellet at the centrifugal end of the tube. These particles were recovered by washing them out of the tube with pH 7.2 phosphate buffer. For the last nine samples, the acetone-washed dry weight of the clay-mineral fraction was measured after drying the pellet at 105°C . The quantity of organic and inorganic material from all samples is shown in table 1.

The supernatant from the $> 20,000 \text{ S}$ continuous flow centrifugation was subjected to a second continuous flow centrifugation at the greater impulse of $1.44 \times 10^7 \text{ g} \times \text{min}$ to sediment $> 150 \text{ S}$ particles. This impulse will pellet most of the virus particles and all of the bacteriophage for which sedimentation data are available (4). The pellet was resuspended in pH 7.2 phosphate buffer + $0.005 \text{ M MgSO}_4 \cdot 7 \text{ H}_2\text{O}$ and washed by recentrifuging at an impulse to pellet $10,000 \text{ S}$ particles to remove any chance bacteria, and then the supernatant centrifugally concentrated to a 1 ml volume in a sterile tube using an impulse of $1.44 \times 10^7 \text{ g} \times \text{min}$. This final concentration was complete within 30 hours after sampling and the $> 150 \text{ S}$ fraction was stored at -20°C until a plaque assay was made. The pellet generally appeared clear, tan, and gelatinous.

TABLE I
QUANTITY OF EACH FRACTION OF A SAMPLE

Date	Station	OM Count	OM Area	"C"	IOM Count	μ mho	pH	Temp, °C	IOM Wgt.
11/28/66	19.3	3.3×10^{10}	--	--	4.4×10^{10}	336	8.46	13	--
12/12	6.4	6.7×10^8	--	--	5.1×10^8	216	8.41	11	--
12/27	6.4	1.27×10^9	--	--	1.9×10^8	246	8.42	8	--
1/30/67	19.3	1.9×10^9	--	--	2.6×10^8	142	7.3	9.5	--
2/21	6.4	1.3×10^8	--	--	8.2×10^8	176	7.52	9	--
2/27	19.3	2.5×10^7	--	--	2.3×10^8	165	7.68	6	--
3/13	6.4	5.3×10^8	--	--	4.2×10^8	219	7.69	17	--
4/17	6.4	6.4×10^8	--	--	2.7×10^8	297	7.59	20	--
4/24	19.3	9.5×10^8	--	--	5.5×10^8	227	7.7	20	--
5/15	19.3	3.8×10^9	4.74	1.62	1.9×10^9	136	7.54	16.8	3365
5/22	6.4	9.3×10^8	8.2	2.58	2.12×10^8	222	7.68	17	1454
6/12	19.3	3.3×10^8	6.95	0.35	1.63×10^9	193	7.5	23	2896
7/17	6.4	2.5×10^8	1.75	2.35	6×10^7	251	7.71	18	7378
9/19	19.3	1.75×10^8	2.55	0.01	4×10^8	290	7.95	22	4905
10/16	6.4	3.6×10^8	1.1	2.23	4.9×10^8	359	7.5	20	3792
11/7	19.3	3×10^7	0.93	0.02	1.1×10^8	195	8.43	8	3269
12/19	19.3	6×10^7	0.75	2.22	1.5×10^{10}	80	7.28	14	2398
1/17/68	19.3	3×10^7	0.6	0.04	1.8×10^8	154	7.6	8	165

OM Count = direct microscope count of suspended organic particles/ml.

OM Area = chart record area of the density gradient centrifugation.

"C" = value of $V/G \times A$.

Station = sampling site on Poplar Creek.

IOM Count = direct microscope count of $> 0.2 \mu$ inorganic particles/ml.

μ mho = conductivity of water sample in μ mho/cm at 25°C .

pH = pH of sample at time of sampling.

Temp. = temperature $^\circ\text{C}$ of sample at time of sampling.

Dry Wgt. = acetone-dry weight of clay-mineral fraction in milligrams.

The concentrated > 150 S pellet was resuspended in 1 ml pH 7.2 phosphate buffer + 0.005 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and divided into three equivolume aliquots. One was examined by electron microscopy to confirm that virus-like particles were physically present. Selected photographs of these particles are shown in figures 2 and 3.

The other two portions of the > 150 S pellet were used for a plaque assay. This was done using exponentially growing cultures of the appropriate bacteria (E. coli j or A. aerogenes) and a standard serial dilution assay. Each set was run in duplicate and incubated at 37°C for 24 hours before counting plaques. The plaques were scored on a bacterial colony counter and confirmed by electron microscopy of material from random plaques. There were usually two types of plaques in each plate and these were isolated in pure culture (figure 4). The results of the plaque assays for all samples are shown in table II.

DATA SUMMARY AND CORRELATIONS

Linear correlations were calculated for a number of combinations of data using the statement:

$$b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}} \quad (2)$$

$$\text{and } \tilde{y} = \bar{y} + b(x - \bar{x}) \quad (8) \quad (3)$$

$$\text{and } (3) = bx + (\bar{y} - b\bar{x}) \equiv bx + c \quad (4)$$

Where x = the fraction quantity,

y = the plaque assay results,

N = the number of samples,

$\bar{x}$ or $\bar{y}$ = the mean of the samples, and

$\tilde{y}$ = the true value of y .

From equations (3) and (4) the regression line can be plotted. The values of b and c are given in table III.

The regression coefficient, r , of a linear correlation was calculated for the above values of b and c from the statement:

$$r^2 = \frac{\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N} \right]^2}{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{N} \right] \left[\Sigma y^2 - \frac{(\Sigma y)^2}{N} \right]} \quad (5)$$

Figure 2
ELECTRON MICROGRAPHS OF
SELECTED VIRUS AND VIRUS-LIKE PARTICLES

1 μ

Figure 3
ELECTRON MICROGRAPHS OF
SELECTED VIRUS AND VIRUS-LIKE PARTICLES

A and B are coli-phage and C and D are aero-phage.

Figure 4
ELECTRON MICROGRAPH OF
BACTERIOPHAGE ISOLATED FROM PLAQUE ASSAYS

TABLE II
RESULTS OF PLAQUE ASSAYS

<u>Date</u>	<u>Station</u>	<u>Coli-phage/L</u>	<u>Aero-phage L</u>
11/28/66	19.3	15	--
12/12	6.4	1100	--
12/27	6.4	55	35
1/30/67	19.3	< 0.15	< 0.15
2/21	6.4	1.5	1.7
2/27	19.3	5	--
3/13	6.4	24	90
4/17	6.4	1.47	4.13
4/24	19.3	8.2	6.4
5/15	19.3	11	29
5/22	6.4	36000	1100
6/12	19.3	155	460
7/17	6.4	0.75	0.15
9/19	19.3	45000	500
10/16	6.4	7.4	434
11/7	6.4	--	--
12/19	19.3	--	--
1/17/68	19.3	--	--

TABLE III
STATISTICAL ANALYSIS

X	Y	N	b	c	t	r
Total OM Area	Total aero-phage	6	0.82	0.74	0.15	0.60
Total OM Area	Total coli-phage	6	0.16	0.66	0.46	0.23
Total C	Total aero-phage	6	0.29	0.38	0.16	0.80
Total C	Total coli-phage	6	-0.51	0.21	0.54	0.26
Total coli-phage	Total aer-phage	11	0.16	0.13	0.33	0.74
Total OM Area	Total IOM	9	-0.14	0.34	0.64	0.23
Total OM Area	Total μ mho	9	-0.80	0.32	0.62	0.24
Total C	Total OM Area	6	-0.16	0.16	0.84	0.42
Total C	Total IOM	9	0.71	0.11	0.85	0.30
6.4 OM Count	aero-phage	7	0.30	0.57	0.70	0.30
6.4 OM Count	coli-phage	8	0.13	0.28	0.96	0.37
6.4 IOM	acro-phage	7	-0.24	0.32	0.34	0.15
6.4 IOM	coli-phage	8	-0.14	0.98	0.67	0.26
6.4 μ mho	aero-phage	7	0.42	0.13	0.14	0.61
6.4 μ mho	coli-phage	8	-0.44	0.16	0.49	0.20
6.4 coli-phage	6.4 aero-phage	7	0.28	0.94	0.55	0.93
6.4 μ mho	C	3	-0.22	0.30	0.18	0.88
6.4 IOM	OM Area	3	-0.45	0.48	0.26	0.25
6.4 IOM	C	3	-0.10	0.25	0.56	0.49
6.4 OM Count	μ mho	8	-0.90	0.62	0.33	0.14
6.4 OM Count	IOM	8	-0.75	0.88	0.14	0.48
6.4 IOM	μ mho	8	-0.66	0.27	0.71	0.28

TABLE III
STATISTICAL ANALYSIS, Continued

X	Y	N	b	c	t	r
19.3 OM Count	aero-phage	5	-0.80	0.28	0.99	0.50
19.3 OM Count	coli-phage	7	-0.27	0.79	0.44	0.19
19.3 IOM	aero-phage	5	0.28	0.17	0.14	0.83
19.3 IOM	coli-phage	7	-0.19	0.78	0.41	0.18
19.3 μ mho	aero-phage	5	0.26	-0.32	0.15	0.65
19.3 μ mho	coli-phage	7	0.995	-0.15	0.11	0.45
19.3 coli-phage	aero-phage	4	0.75	0.16	0.11	0.63
19.3 C	μ mho	6	-0.11	0.26	0.25	0.78
19.3 C	IOM	6	0.14	0.27	0.30	0.83
19.3 OM Area	IOM	6	-0.12	0.31	0.55	0.27
19.3 OM Count	μ mho	10	0.88	0.13	0.24	0.65
19.3 OM Count	IOM	10	0.69	-0.62	0.76	0.94
19.3 IOM	μ mho	7	0.33	0.19	0.22	0.70

Prefix "Total" = sum of 6.4 and 19.3 Km sampling stations.

Prefix "6.4" = that sampling site.

Prefix "19.3" = that sampling site.

OM Area = chart record area of density gradient centrifugation.

OM Count = direct microscope count of suspended organic particles.

IOM Count = direct microscope count of $> 0.2 \mu$ inorganic particles.

C = value of $V/G \times A$.

μ mho = conductivity of < 150 S supernatant in μ mho/cm at 25°C .

coli-phage or aero-phage = plaque assay results in pfu/l.

The value of Student's "t" was calculated using the statement:

$$t = \sqrt{\frac{r^2(N-2)}{1-r^2}} \quad (8) \quad (6)$$

The values of r and t are shown in table III.

From the data in table III, it appears that the number of viable coli-phage present show no correlation with any of the variables used. On the other hand, aero-phage show a number of good correlations with these variables both for the individual sampling sites and for the combined results. From the degree of correlation of aero-phage with (a) the direct count of organic particles, with (b) the chart record area of total organic material, and with (c) the value of C, it appears that the aero-phage population increases with both an increase in the amount of organic material present and with increased bio-activity, but the good correlation with (c) C, may be interpreted to mean that the increased bio-activity is the more important variable. This in turn may mean that aero-phage is able to replicate in the open water. On the other hand, the low correlations of coli-phage with a, b, and c may mean that the coli-phage is not able to replicate in the open water.

The value of < 150 S material ($\mu\text{mho/cm}$ at 25°C) shows a correlation with the value of aero-phage at both sampling stations and also with the (c) value of C. While the <150 S fraction shows a correlation with (a) the direct count of organic material at the 19.3 Km station, there is no correlation with (b) the chart record area or with (a) the direct count at the 6.4 Km station. These may be interpreted to mean that an increased amount of < 150 S material means an increased nutrient level in the stream and this in turn is usually associated with an increase in the quantity and bio-activity of the organic particles.

A comparison of the total coli-phage population versus the total aero-phage population, shows a good correlation as does such a comparison within the 6.4 Km station and the 19.3 Km station populations. The decreased correlation within the 19.3 Km station against that of the 6.4 Km station may be interpreted as another indication that there is a difference in the ability of the two bacteriophage to replicate in open water.

The ability to consistently confirm the presence of strains of coli-phage and aero-phage and the high degree of host specificity of the phage mean that they may be suitable as indicator organisms of sewage contamination. Use of a specific bacteriophage as an indicator organism would reduce some of the difficulty in differentiating between human and non-human coliform bacteria.

ACKNOWLEDGMENT

I thank Dr. J. A. Roberts of Davidson College for advice in computer analysis of the results.

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UNION CARBIDE CORPORATION
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January 24, 1969

To: Holders of Union Carbide Corporation, Nuclear Division,
Report Number K-1760

Report Number K-1760, Pages 17 and 18, Table III

Data presented in the subject report have been reviewed and corrected. Specifically, the value of the Student's "t" parameter has been recalculated and corrected where necessary.

Please remove and replace the old table with the enclosed corrected table.

Yours sincerely,

J. W. Amburgey, Jr.
Biophysical Limnology Laboratory
Separation Systems Division

JWA:egc

Enclosure: Corrected Table III

TABLE III
STATISTICAL ANALYSIS

X	Y	N	b	c	t*	r
Total OM Area	Total aero-phage	6	0.82	0.74	1.5	0.60
Total OM Area	Total coli-phage	6	0.16	0.66	0.46	0.23
Total C	Total aero-phage	6	0.29	0.38	2.6	0.80
Total C	Total coli-phage	6	-0.51	0.21	0.54	0.26
Total coli-phage	Total aero-phage	11	0.16	0.13	3.3	0.74
Total OM Area	Total IOM	9	-0.14	0.34	0.64	0.23
Total OM Area	Total μ mho	9	-0.80	0.32	0.64	0.24
Total C	Total OM Area	6	-0.16	0.16	0.94	0.42
Total C	Total IOM	9	0.71	0.11	0.85	0.30
6.4 OM Count	aero-phage	7	0.30	0.57	0.70	0.30
6.4 OM Count	coli-phage	8	0.13	0.28	0.96	0.37
6.4 IOM	aero-phage	7	-0.24	0.32	0.34	0.15
6.4 IOM	coli-phage	8	-0.14	0.98	0.67	0.26
6.4 μ mho	aero-phage	7	0.42	0.13	1.7	0.61
6.4 μ mho	coli-phage	8	-0.44	0.16	0.49	0.20
6.4 coli-phage	6.4 aero-phage	7	0.28	0.94	5.5	0.93
6.4 μ mho	C	3	-0.22	0.30	1.8	0.88
6.4 IOM	OM Area	3	-0.45	0.48	0.26	0.25
6.4 IOM	C	3	-0.40	0.25	0.56	0.49
6.4 OM Count	μ mho	8	-0.90	0.62	0.33	0.14
6.4 OM Count	IOM	8	-0.75	0.88	1.4	0.48
6.4 IOM	μ mho	8	-0.66	0.27	0.71	0.28

TABLE III
STATISTICAL ANALYSIS, Continued

X	Y	N	b	c	t*	r
19.3 OM Count	aero-phage	5	-0.80	0.28	0.99	0.50
19.3 OM Count	coli-phage	7	-0.27	0.79	0.44	0.19
19.3 IOM	aero-phage	5	0.28	0.17	2.6	0.83
19.3 IOM	coli-phage	7	-0.19	0.78	0.41	0.18
19.3 μ mho	aero-phage	5	0.26	-0.32	1.5	0.65
19.3 μ mho	coli-phage	7	0.995	-0.15	1.1	0.45
19.3 coli-phage	aero-phage	4	0.75	0.16	1.1	0.63
19.3 C	μ mho	6	-0.11	0.26	2.5	0.78
19.3 C	IOM	6	0.14	0.27	3.0	0.83
19.3 OM Area	IOM	6	-0.12	0.31	0.55	0.27
19.3 OM Count	μ mho	10	0.88	0.13	2.4	0.65
19.3 OM Count	IOM	10	0.69	-0.62	7.6	0.94
19.3 IOM	μ mho	7	0.33	0.19	2.2	0.70

Prefix "Total" = sum of 6.4 and 19.3 Km sampling stations.

Prefix "6.4" = that sampling site.

Prefix "19.3" = that sampling site.

OM Area = chart record area of density gradient centrifugation.

OM Count = direct microscope count of suspended organic particles.

IOM Count = direct microscope count of $> 0.2 \mu$ inorganic particles.

C = value of $V/G \times A$.

μ mho = conductivity of < 150 S supernatant in μ mho/cm at 25°C .

coli-phage or aero-phage = plaque assay results in pfu/l.

* Corrected value based on the Student "t" equation.