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Evaluation of Bacteriophages Methods for Detection and Isolation of Viruses from Surface Water

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Abstract: Detection methods of F-specific bacteriophages (FRNA), for removal of viral pollution and Somatic-Phages and fate, behavior and survival of viruses in the sediment evaluated and optimized in this research. The research focused mainly on evaluation of the different steps for culturing the host strain WG49 Salmonella Typhimurium. Salmonella typhimurium WG49 used for our analysis to act as host strain. FRNA bacteriophages were enumerated by the double layer agar method described by Adams. For survival test after mixing of sediment with a sterilized spatula 50 g sub-sample have taken and mixed with a recovery buffer,and have shaken 2 h in a shaker at 4°C,after centrifuge Concentration of Somatic-Phages in the Survival Analysis on supernatant were determined according to standard methods for examination of water and wastewater. Survival test evaluated 28 days for each sub-sample. It was concluded with this optimized method, using a culture of host strain WG49 Salmonella Typhimurium, reliable results could be obtained for the enumeration of F-specific RNA phages.

Key words: Bacteriophages . FRNA . somatic-phages . method evaluation

INTRODUCTION

Although method of detection and enumeration of F-specific RNA bacteriophages was already finalized [1], Still some optimization could be applied to the method, especially when considering the host strain and applying for storm water and wastewater. The removal of indicator and pathogenic bacteria from municipal wastewater is well-documented [2]. In addition to bacteria of fecal origin, storm water may also contain pathogenic viruses and protozoa that have been given much less consideration [3].

Enteric viruses, such as Hepatitis A virus, Norwalk virus and rotavirus are major agents of waterborne disease outbreaks such as gastroenteritis and hepatitis in humans. Certainly the few virological studies that have been carried out have focused on the treatment of municipal and industrial wastewater [4, 5], have not considered storm water. The paucity of data on the fate of viruses in aquatic environments is due in part to the lack of reliable, simple and economical methods for virus detection [6].

Despite the advances in methodology that have occurred in recent years, direct assays for viruses in water are still complex, time consuming and expensive to use routinely. Direct monitoring of the virological quality of water, therefore, is impractical. Many studies, however, have shown the ability of bacteriophages (viruses that infect only bacteria) to be used as models of virus behavior in the environment. Bacteriophages offer several advantages over virus detection in that they are non-pathogenic and methods for their recovery and enumeration are simple, rapid and inexpensive [6, 7].

There are four main groups of bacteriophages that have been found to be useful for modeling virus transport/removal in the aquatic environment and treatment systems: somatic coliphages (coliphages are phages which attack the bacterium *Escherichia coli*), F-RNA coliphages, Bacteroides fragilis phages and Serratia marcescens phages. Phages of Bacteroides fragilis are usually associated with human sewage and therefore may provide evidence of sewage overflow and/or leakage into the stormwater systems [8, 9]. F-RNA coliphages are genetically very similar to human enteric viruses such as Hepatitis A and rotavirus. They are considered to be very good indicators of the transport, persistence and presence of enteric viruses but are not specific to humans [5, 10, 11]. Somatic coliphages have been found to multiply in the environment, which limits their use to a large extent. Serratia marcescens phages usually occur in low numbers but have been successfully used in seeded experiments to study virus transport [12, 13].

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A major mechanism of pollutant removal in water treatment processes is physical sedimentation of particles of sand, silt and clay to which many pollutants adsorb. Suspended particles appear to be a dominant natural vehicle and survival aid for viruses in water [14].

It is estimated that about 77% of viruses and 65% of coliphages (bacteriophages that infect E. coli) are associated with suspended particles in natural waters [15]. Storm water typically contains a much higher proportion of small particles than municipal wastewaters. A number of workers have reported that bacteria and viruses adsorb preferentially to particles less than 2 µm in size (clay particles) [16].

The study focus is evaluation of detection methods for FRNA and Somatic-Phages for the fate of viruses and survival test on the sediments and compare the effectiveness of the methods. It will allow the development of techniques for the use of bacteriophages as surrogates for human enteric viruses in the environment which will have application for a wide-range of investigations and provide a basis for attracting external funding in the future.

MATERIALS AND METHODS

Sampling

Water sampling: Discrete inflow and outflow water samples were collected weekly in sterile containers from two natural wastewater systems in Sydney-Hawkesbury (Aus), during the six months.

Sediment sampling: Sediment samples were collected by manual the first 5 cm of the sediment transferred with using a sterile spatula and placed into the cylinders. Usually Weekly and sometime fortnightly samples of inflow and outflow waters from the two treatment systems over a period of 6 months will be analyzed for the presence of bacteriophages to characterize background levels in the systems during dry weather flow. Intensive sampling (samples taken every hour) of a minimum of four storm events also was carried out.

Sediment characteristics: Sediment samples will be collected from each of the systems and used in laboratory experiments to examine: i) the adsorption of bacteriophages to particles of different sizes and ii) the persistence of the bacteriophages in the sediment using laboratory microcosms. The pipette method, which is based on the settling properties of different sized particles, will be used to separate the different particle size fractions. The use of laboratory microcosms (closed bottle systems) will allow the persistence of bacteria withdrawn periodically from the microcosms (probably once weekly) and concentrations of bacteriophages in the subsamples determined. These experiments will provide an estimation of the potential for accumulation of pathogenic viruses in the systems.

Particle size analysis: The particle size distribution of the sediment samples for each system was determined in duplicate using the pipette method that indicated in Fig. 1. Settling times for particles <2, 2-5, 5-10, 10-20, 20-62 and >62 µm were 0 s, 26 s, 4'/10s, 16'/ 40 s, 68'/ 40 s, 416'/40 s, respectively. The sediments (100 g) were mixed with distilled water and the suspensions allowed settling in 1L cylinders. At the determined settling times, 25 ml of sediment suspension was removed from a depth 10 cm below the surface and dried at 105°C for 24 h in a pre-weighed crucible (Table 1). The dried fractions were analyzed for organic material (at 550°C for 10 h). Simultaneously, the concentrations of Somatic-Phages remaining suspended in the top 10 cm were determined from an additional sub-sample at each of the sampling times [6, 17].

Bacteriophages analysis: Concentration of Somatic-Phages in the Particle Size analysis (on sub-samples) and Survival Analysis on Sediment samples were determined according to standard methods [18]. Schematic Procedure of the Somatic-Phages determination indicated in Fig. 2.

Table 1: Particle size distribution of two natural treatment systems

	Particle size distribution (%)						
Sample sites	Moisture (%)	<2	2-5	5-10	10-20	20-62	>62
Wetland inlet	57.7	10.3	6.0	11.3	11.7	30.0	30.7
Wetland middle	65.1	17.0	15.0	15.2	15.6	30.8	6.4
Wetland outlet	81.5	7.5	7.1	9.8	5.9	40.6	29.1
Pond inlet	41.5	28.7	19.5	1.2	14.6	18.6	17.4
Pond middle	74.5	18.9	10.4	13.2	10.8	42.9	3.8
Pond outlet	74.3	28.1	17.4	17.4	28.9	3.3	4.9

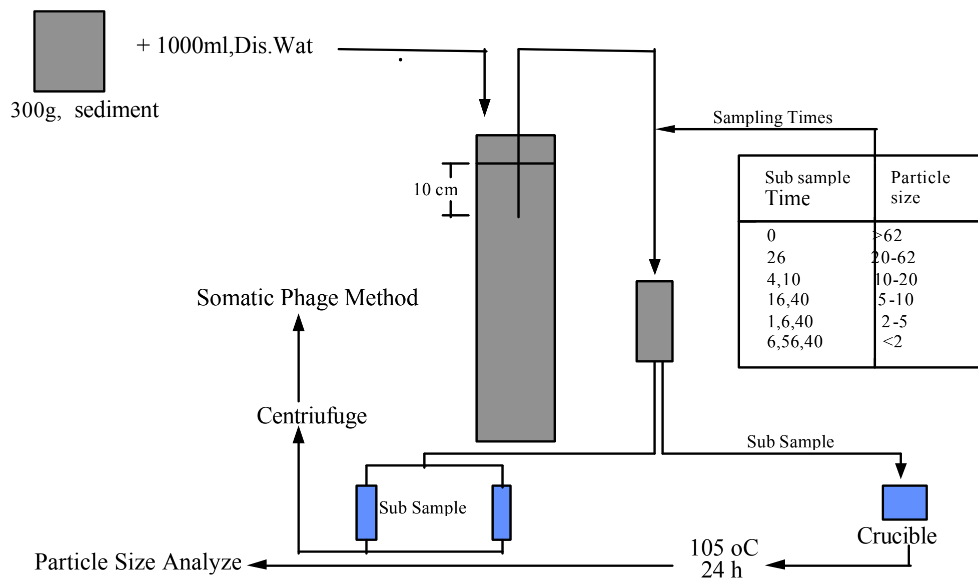

Fig. 1: Pipette method [17]

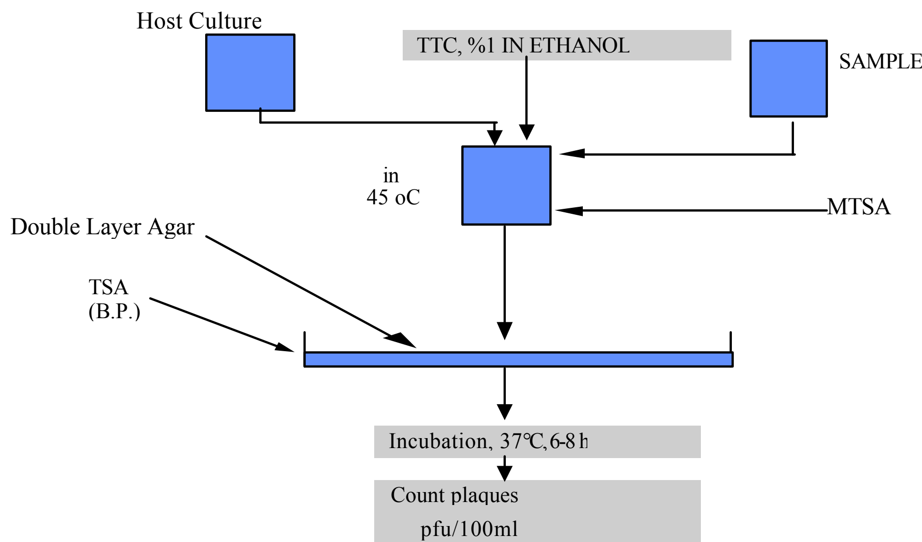

Fig. 2: Method of somatic-phage test

Survival analysis

Recovery of viruses from sediments: Sediment samples were mixed thoroughly using a sterile spatula. Three of 300 grams of sediment (Sub-sample) was weighed out into 3 bottle sterile and the sediment remaining in the microcosm was covered with 100 ml of sterilized pond or wetland water (equilibrated to 25°C). At zero time a sub-sample (50 g) of each bottle was weighed out into a 50 ml buffer sterile especial for recovery of phages and then placed in a Basket in adjacent of ice (at 4°C).These were shaken by a shaker in 100 1/min for two hours. Then with dispenser (sterile tips) the mixture was transferred into many centrifuge tube and centrifuged for 5 min at 2500 rpm.The Supernatant was transferred to a sterile bottle and used for Bacteriophages analysis (Somatic-Phages).Weekly sub-samples of sediment (50 g) were withdrawn from the microcosms by aseptically pipetting off the overlying water, taking care not to re-suspend any of sediment to determine concentration of Bacteriophages. This analysis continued in period of 28 days. This method displayed in Fig. 3.

Water analysis

Bacteriophage analysis: Salmonella typhimurium WG49 used for our analysis to act as host strain. Culture to be used for the Bacteriophages analysis were selected from those lactose-positive colonies on MacConkey agar plate (overnight cultured). These colonies by aseptically was inoculated in

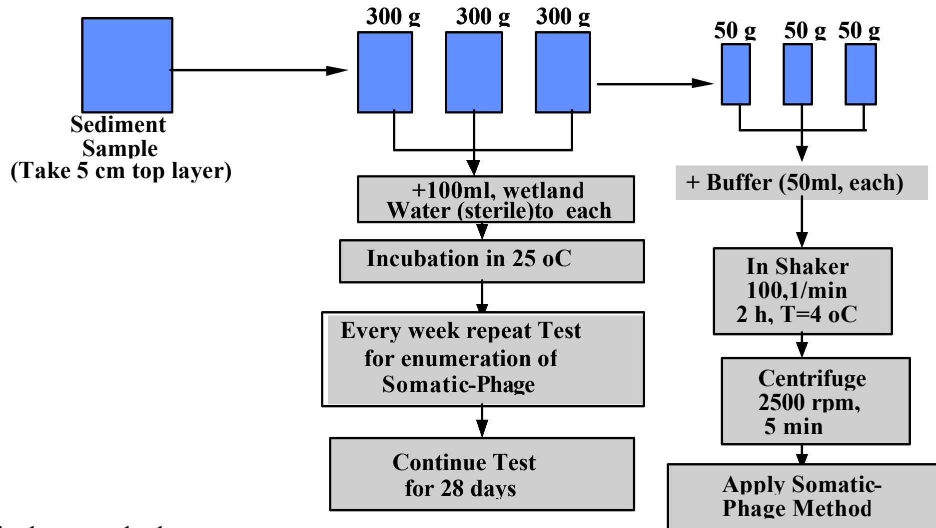

Fig. 3: Survival test method

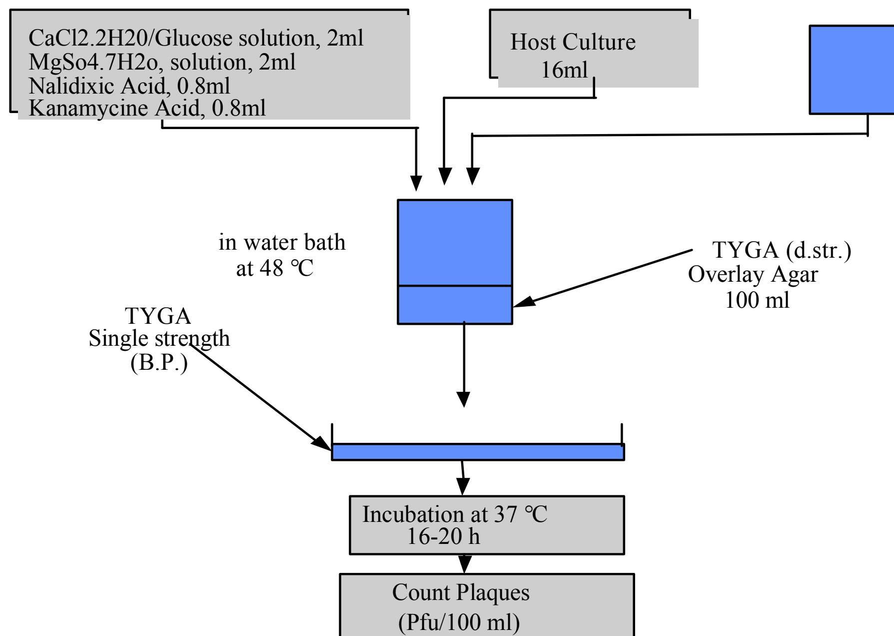

Fig. 4: Method of F-RNA test

Trypton Soy Broth(TSB) plus kanamycine and Glocuse/cacl2 solution for 3.5-6 h. After 3.5 h reading absorbance of host culture at 620 nm was performed periodically (each 0.5 h) by pipetting off 3 ml. After get the absorbency to a pleasant turbidity (in base of Pre-Test) host culture was used for Bacteriophages assay. Bacteriophages were enumerated by the double layer agar method described by Adams (Fig. 4) [6, 19].

Data analysis: Analysis of variance was performed using Excel version 4, data analysis software.

RESULTS AND DISCUSSION

Table 1 shows the particle size distribution of sediments samples taken at three different points in each system.

The difference in sediment particle size distribution in the two systems is most likely due to the different particle size inputs based on activities in catchment and presence of plants in wetland that affect the hydraulic characteristic of sediment microcosm and type of soil bed in two systems. Residential development within the wetland catchment has been established for several

years and the soil has been stabilized to some extent. In contrast, due to construction work in the catchment of the detention pond and consequently existence of large areas of disturbed and exposed clay, which may be easily mobilized and transported in storm water, are the reasons of this difference.

Concentration of somatic-phages is based on log10 PFU/100g of dry sediment. Concentration of somatic-phages in inlet point of the system varied from 4.1 to 5.4 (log10 PFU/100g) and in middle point of system varied from 4.1 to 5.3 (log10 PFU/100g) and in outlet point of the system varied from 4.6 to 5.4 (log10 PFU/100g).Also these research show that concentration of phages in fine particle size is higher than the coarse particle, that is due to more effective attachment of bacteriophages to fine particle size. These results confirm the preview researches [20, 21]. Also this research show percent of particle in range of 20-60 micron is significantly higher than the others ($p<0.05$).

Concentration of somatic-phages in inlet point of the system varied from 1.7 to 2.2(log10 PFU/100g) and in middle point of system varied from 1.6 to 2.8(log10 PFU/100g) and in outlet point of the system varied from 1.5 to 2.5(log10 PFU/100g) in A sampling point and varied from 4.3 to 5.3(log10 PFU/100g) in B sampling point. Although difference of particles in range of >10 micron is significant ($p<0.05$). Presence of particle size in range of 5-10 micron in inlet point is significantly (<0.05) different from the other sites of system. Also as before figures concentration of phages in fine particle size is higher than the coarse particle.

Monitoring of the sediment microcosms over a period of 28 days indicated stable condition throughout the two week test period, that this stability were continued at outlet point of each system. This study showed extended survival of Somatic-Phages in wetland and detention pond sediments. These results support the findings of other limit studies indicating resistant of viruses in sediment microcosms is higher than the bacteria [22, 23]. In each microcosm there was a general decline in concentration of somatic-phages with time, which indicating Die-off.

The results showed the concentration of Somatic-Phages in middle point of each system is higher than the inlet and outlet point. Also these figures show the rate of die-off of somatic-phages in inlet and middle points of each system is rapid than the outlet point. These finding is an indicators of the methods efficiency and validity of tests.

The results showed the concentration of FRNA in storm water entering to Wetland system in wet weather is different with dry condition. Concentration of FRNA in first time of raining condition or heavy raining is higher than the dry or light raining condition. The results is clearly showing that in time of a heavy raining, concentration of FRNA get up very high. So this result is a good reason for looking at to these Bacteriophages as an adequate model for the look over of the fate and transport of enteric viruses through surface water constructed wetland and detention pond systems or storm water management.

The results indicating dependency of the concentration of FRNA to dry or raining condition also show the efficiency of detention pond system is not pleasant for removal of the bacteriophages, when compared with wetland systems. So the wetland systems is preferable than the pond for water pollution control in storm water management.

It is need to the success of any phage that suitable phage can be both isolated and enriched to produce sufficient numbers for the application. Phage enrichment typically involves the inoculation of mixed environmental samples and growth media with a single host strain. Following overnight incubation, the bacteriophages produced by lytic infection of the original isolation host [7, 24].

CONCLUSIONS

It was concluded with this optimized method, using a culture of host strain WG49 Salmonella Typhimurium, reliable results could be obtained for the enumeration of F-specific RNA phages. If problems occur with the host strain it is advisable to start again with new material e.g. from a culture collection.

These finding is an indicators of the methods efficiency and validity of tests. These observations have implications in the selection of Bacteriophages as an adequate model for the look over of the fate and transport of enteric viruses through surface water constructed wetland and detention pond systems or storm water management.

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