Evaluation of F-RNA coliphages as indicators of viruses and the source of faecal pollution

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Abstract

It is a growing concern that analyses of true indicators of pathogenic viruses have not been properly established. Water treatment and catchment management strategies based on bacteriological indicators do not provide the necessary protection against viral infections because viruses are more persistent than bacteria in water environments. More than 100 types of human pathogenic viruses may be present in faecally polluted water, but only a small number can be detected by currently available methods. Analysing for enteric viruses such as the polio and hepatitis viruses is specialised and is neither practical nor commercially viable for a water utility. Human enteric viral disease is considered to be predominantly associated with human wastes, as opposed to animal wastes, and a distinction between these is desirable. Somatic coliphages are not always present whenever enteric viruses were detected and have been found to multiply in the environment outside the body. It has previously been shown that for monitoring purposes, F-specific RNA bacteriophages are model organisms and suitable indicators of the possible presence of human pathogenic enteric viruses as they behave like waterborne viruses. F-specific bacteriophages have been suggested as a useful alternative to the traditional bacterial indicators as their morphology and survival characteristics closely resemble those of some of the important human enteric viruses. F-specific RNA phages are becoming the indicators of choice for viruses in water and have been accepted as one of the rapid screening tests to determine the quality of water. Studies have shown that based on hybridisation tests, F-RNA coliphages could be associated with either a human or animal source. F-RNA coliphages have been grouped into four groups with Groups 2 and 3 being of human origin and Groups 1 and 4 originating from animals. This study aimed to fulfil the requirement of both developing a relatively simple method for F-RNA coliphage analyses in samples from the Umgeni Water catchment and further testing to elucidate whether the contamination was of animal or human origin using gene hybridisation techniques to type the F-RNA phages. The standard ISO method for the isolation of F-RNA coliphages was used and genotyping assays were performed according to methods adapted by the University of Pretoria's Medical Virology Department.

Highly specific nucleic acid probes for the detection of F-specific RNA bacteriophages were used in this study to fingerprint the origin of faecal pollution. The *E. coli* data generated for the sample sites indicated that certain sites tended to have more faecal pollution problems than others. Somatic coliphages were isolated in numbers as high as 1 100 pfu.10 ml^{-1} and were consistently isolated in higher numbers than F-RNA coliphages. The strongest positive correlation (r = 0.842) was between *E. coli* and F-RNA coliphage concentrations. The F-RNA coliphages isolated from one river sample and wastewater works effluent hybridised with the GA probe belonging to Group 2, which was of human origin.

Introduction

Sewage from human or animal sources may contain the causative organisms of many communicable diseases such as typhoid fever, amoebic dysentery or infective hepatitis. However, monitoring for the presence of specific pathogens is impracticable for routine control purposes. Reliance is therefore placed on relatively simple and more rapid bacteriological tests for the detection of intestinal bacteria, especially *Escherichia coli* and other coliforms. They are easier to isolate and characterise and are always present in the faeces of man and warm-blooded animals and hence in sewage in large numbers. Water treatment and catchment management strategies based on bacteriological indicators do not provide the necessary protection against virus infections because viruses are more persistent than bacteria in water environments (Havelaar et al., 1993). More than 100 types of human pathogenic viruses may be present in faecally polluted water, but only a small number can be detected by currently available methods (Havelaar et al., 1993). Human enteric viral diseases are considered to be predominantly associated with the ingestion of human-derived wastes because of the host-specific nature of enteric viruses (Calci et al., 1998).

Somatic coliphages have been reported as being a heterogeneous group of organisms, which could originate from faecal sources (Havelaar, 1990; Calci et al., 1998). The presence of these viruses in faecal matter means that they can serve as indicators of faecal pollution and may indicate the concurrent presence of pathogenic viruses. Somatic coliphages infect *E. coli* by adsorbing to viral receptors on the cell wall (Holmes, 1996).

F-specific bacteriophages have been suggested as a useful alternative to the traditional bacterial indicators as their morphology and survival characteristics closely resemble those of some of the important human enteric viruses (Turner and Lewis, 1995). It was shown that for monitoring purposes, F-specific RNA bacteriophages can serve as model organisms and suitable indicators to indicate the possible presence of human pathogenic enteric viruses as they behave like water-borne viruses (Havelaar et al., 1993). F-specific RNA bacteriophages enter the host cell via primary adsorption to F- or sex-pili and are a more homogeneous group that are even more resistant to UV than other micro-organisms (Seeley and Primrose, 1980 and Wiedenmann et al., 1993). The F- or sex-pili are only produced by bacteria in the logarithmic phase of growth and it has

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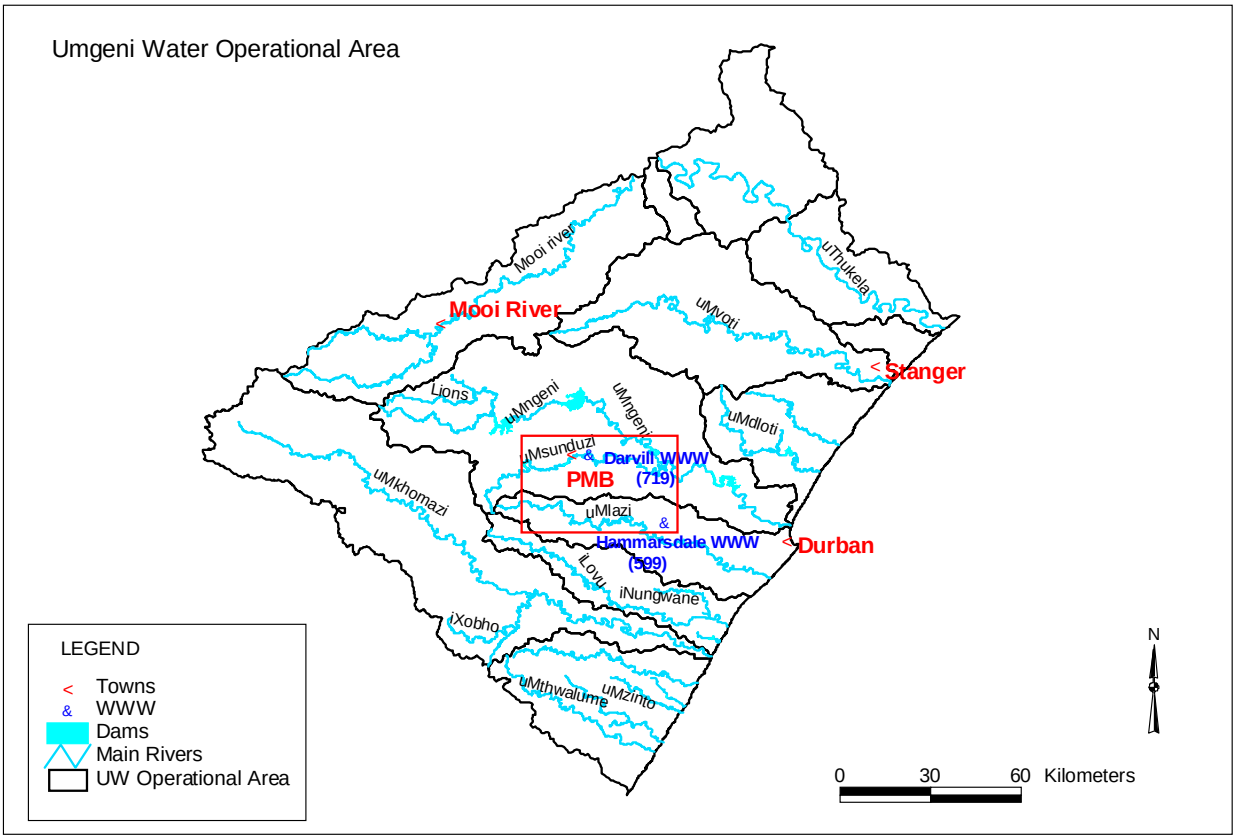

Figure 1
Umgeni water operational area

been reported that *E. coli* cells would only produce sex-pili at temperatures well above 30°C (Grabow, 2001).

F-RNA phages have been classified into 4 groups: MS-2 in Group 1, GA in Group 2, Qβ in Group 3 and SP in Group 4 (Havelaar and Hogeboom, 1984). Serotyping of F-specific RNA bacteriophages have been suggested as a method to determine the origin of faecal pollution (Schaper, 2000). Hsu et al. (1995) indicated that highly specific nucleic acid probes for the detection of F-specific RNA bacteriophages can be used as an alternative to serotyping. Havelaar et al. (1990) reported that F-specific RNA bacteriophages serogroups 1 and 4 occurred in animal wastewaters while serogroups 2 and 3 were found in wastewater from human sources. Schaper and Jofre (2000) confirmed that Genotypes 2 and 3 predominated in municipal sewage further illustrating that these genotypes were of human origin.

Enteroviruses such as polio virus and F-RNA coliphages, are morphologically similar when viewed under the electron microscope and both are excreted by humans and fail to multiply in the environment, making F-RNA coliphages ideal surrogates for human enteric viruses (Grabow, 2001). Unlike somatic coliphages, F-RNA coliphages rarely multiply in the environment making them suitable as indicator organisms (Kamiko and Ohgaki, 1993). Consistent association with faeces and infrequent isolation from stormwater support the use of F-RNA coliphages as relatively specific indicators of sewage contamination (IAWPRC, 1991, Rhodes and Kator, 1991). Studies showed that F-RNA coliphages were retained by shellfish indicating their close resemblance to food and water-borne human enteric viruses, for example, hepatitis A virus and rotaviruses (ISO, 1995). F-RNA bacteriophages have been suggested as indicators of sewage contamination based on their morphology and survival characteristics which closely resemble the important human viruses such as poliovirus (IAWPRC, 1991; Rhodes and Kator, 1991).

F-specific RNA phages are becoming the indicator of choice for viruses in water and have been accepted as one of the rapid screening tests to determine the quality of water. Studies have shown that based on hybridisation tests, F-RNA coliphages could be associated with either a human or animal source (Uys, 1999). The purpose of this study was to set up the ISO method for F-RNA coliphages and compare the results with *E. coli* and somatic coliphage results obtained for samples taken from different sample sites having different characteristics. Further to this study was to genotype the F-RNA coliphages and establish the source of pollution to be of either animal or human origin.

Materials and methods

Samples were collected in 500 ml sterile bacteriological sampling bottles, taken by Umgeni Water's Sampling Officers following sampling protocols that have been SABS accredited. All F-RNA coliphage, somatic coliphage and *E. coli* analyses were performed in Umgeni Water's accredited laboratory. All genotyping was done at the Department of Medical Virology University of Pretoria (Uys, 1999).

Sample site selection

Sample sites (blocked area around PMB in Fig. 1) were selected based on their accessibility, different catchment types and relatively high *E. coli* maximum values (see Table 1 below).

TABLE 1
The occurrence of *E. coli* detected in water samples for the period January 1999 – June 2001

UW site number	Site description	No. of results	Median *E. coli* ·100 ml^{-1}	Maximum *E. coli* ·100 ml^{-1}
22	Abattoir effluent	97	1100	12 000 000
22.2	Downstream of abattoir	104	1850	9 400 000
57	Unique catchment with both animal and human input	113	600	89 000
61	Urban with informal settlements	122	2300	960 000
64	Urban with informal settlements	118	5300	550 000
64.1	Industrial and urban	122	11300	610000
66	Upstream STW	119	1500	126 000
66.3	Downstream STW	200	2700	2 300 000
67	Further downstream STW	123	2000	160 000
599	Hammarsdale WWW effluent	586	62	340 000
719	Darvill WWW effluent	518	80	1 100 000

E. coli analyses

Ten millilitres of the appropriate dilution of the water sample was drawn by vacuum through a 0.45 µm membrane that would retain all the bacteria. The membrane was placed on an absorbent pad saturated with broth containing lactose and phenol red as an indicator of acidity. The media used was Membrane Laurel Sulphate Broth (Oxoid). The membranes were incubated for 18 ± 2 h at 44°C ± 0.25°C.

Yellow colonies were counted within a few minutes of removal from the incubator. Colonies of *E. coli* have a characteristic appearance; bright yellow in colour and more than 1 mm in diameter. Subsequent confirmation of their ability to produce gas and indole was carried out.

Somatic coliphage analyses

These analyses were done using the double agar-layer technique and any plaques (areas of clearing) that develop were counted. The double agar-layer technique was used to provide sufficient nutrients for the thin top layer and to prevent it from drying out during incubation. The host culture used is *E. coli* strain C (ATCC 700078) i.e. nalidixic acid-resistant mutant WG5.

Two and a half millilitres of top-agar containing 1% naladixic acid was pipetted into a test tube in a heating block held at 48°C. Half a millilitre (grown that day) or 0.2 ml (grown overnight) host culture was added to the top-agar. One millilitre of the test sample, or an appropriate dilution, was added to the top-agar in the test tube. The top-agar mixture was poured onto the bottom agar layer in a phage agar plate. This procedure was done in triplicate. The inverted plates were incubated overnight at 37±1°C. Most plaques were generally visible after 8 h. Plaques were clearly visible as circular disc-like clearings through the lawn of bacteria. The total number of plaques observed on all three plates for each sample were averaged and multiplied by a factor of ten to give results in pfu·10 ml^{-1}.

F-RNA coliphage analyses

Unlike plaques formed by somatic coliphages, the plaques formed by F-RNA coliphages are smaller and more opaque than clear. The host culture used was *Salmonella typhimurium* strain WG49, a naladixic acid-resistant mutant engineered to produce the sex pilus of *E. coli*. The host culture was grown up to the log phase.

Working culture

One vial of the stock organism was thawed and added to 50 ml of tryptone yeast extract glucose broth (Becton Dickinson) in a 250 ml Erlenmeyer flask. This was incubated in a shaking waterbath at 37±1°C. A 3 ml sample aliquot was taken at time 0 and used as a blank reference absorbance in a Spectrophotometer at 560 nm wavelength. The measurement of absorbance was read at 15 min intervals and once close to 0.7 absorbance, 1 ml of the host was added to a pre-warmed test tube containing 2.5 ml of top-agar and 1ml of a positive control. This host, top-agar and positive control were then poured onto the bottom agar layer in a phage agar plate. The above step was done for as many absorbances as possible, up to an absorbance of 0.8. The inverted plates were then incubated overnight at 37±1°C. Following incubation, the absorbance where the most number of plaques where observed was noted. Cultures used in future tests using this batch of host were grown up to this pre-determined absorbance to yield optimal results. Note that it was important to grow the host up to the logarithmic growth phase to ensure that pili were produced making the host susceptible to infection by the F-RNA viral particles.

Analysis

The host was grown up to the pre-determined absorbance as described above and stored on ice to prevent any more growth and loss of pili. The host was stored on ice for a maximum of 2 h and all work was completed well within this time. Two and a half millilitres of top-agar containing 1% naladixic acid was pipetted into a test tube in a heating block held at 48°C. One millilitre of the host culture was added to the top-agar, followed by addition of 1 ml of the test sample, or an appropriate dilution of the sample. The top-agar mixture was poured onto the bottom agar layer in a phage agar plate. This procedure was done in duplicate. The inverted plates were incubated overnight at 37±1°C. Plaques were opaque and much smaller than the somatic plaques. The total number of plaques observed on all plates for each sample were averaged and multiplied by a factor of ten to give results in pfu·10 ml^{-1}.

TABLE 2
Average numbers of *E. coli* and phages detected in water samples from the UW catchment area from September 2001 to January 2002

Sample site	*E. coli*· 100 ml^{-1}	Somatic pfu·10 ml^{-1}	F-RNA pfu·10 ml^{-1}
22	31855	352	25
22.2	35710	410	24
57	2475	196	2
61	73421	340	126
64	11238	198	23
64.1	14609	120	42
66	9304	197	23
66.3	7043	64	12
67	11407	141	19
599	130	11	9
719	12807	125	39

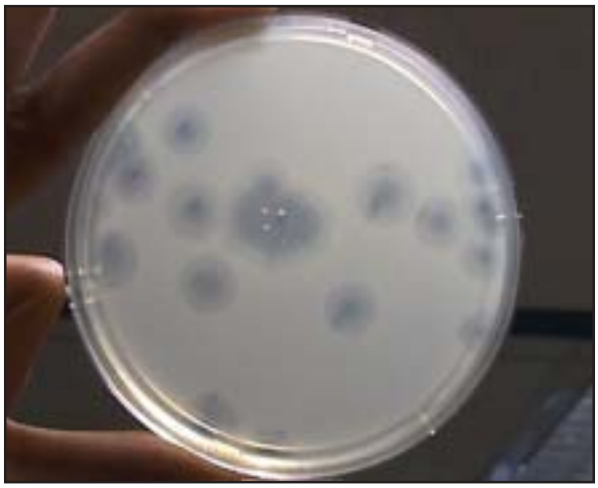

Figure 2
Somatic coliphage plaques obtained for a river water sample tested using the double agar-layer technique

Figure 3
F-RNA coliphage plaques obtained for a river water sample tested using the double agar-layer technique

Genotyping of F-RNA phages

- Plaques obtained were transferred to Behringer Mannheim (Roche Diagnostics) nylon membranes. The first membrane was adsorbed to the top layer for 1 min. Four transfers were made from each plate by increasing the adsorption time to 2 min, 3.5 min and 5 min respectively. Nucleic acids were released and denatured by fixation for 1 min using 0.05 NaOH followed by neutralisation with 0.1 M sodium acetate (pH 6) for 30 s. Nucleic acids were crosslinked to the membrane by 5 min UV irradiation of both sides with a UV transilluminator (UVP).
- Membranes were washed in a prehybridisation solution for 1 h. Probes obtained from MWG-Biotech were added to the hybridisation solution and left overnight at 37°C.
- Detection was performed at room temperature. A DIG Wash and Block Buffer kit containing washing, blocking and detection solutions was used. Membranes were incubated with fresh blocking solution containing an alkaline phosphatase-conjugated anti-DIG. After unbound antibody conjugate was removed, each membrane was equilibrated in a detection buffer. The detection substrate, CPD-star was diluted with the detection buffer and incubated with the membranes for 2.5 min. The membranes were then sealed in a new hybridisation bag, and exposed for 3 min at room temperature to X-ray film.

Results and discussion

The method used for *E. coli* analyses was accredited and validated routinely. The test was easy to perform and colonies were clearly identified. The *E. coli* data generated (Table 2) for the sample sites would indicate that certain sites tend to have more faecal pollution problems than others and the objective of the research was also partly to try and source the pollution to either be of animal or human origin. The surges in *E. coli* levels at sample Site 61 (highest concentration of 670 000 *E. coli*·100 ml^{-1}), which was in an urban/informal settlement catchment, was as a result of sewer leak problems experienced and faecal contamination was likely to be of human origin in contrast to sample Sites 22 and 22.2. These sites, situated downstream of the Cato Ridge Abattoir, probably receive most of the sometimes inadequately treated abattoir wastewater. The treated sewage effluent at Site 599 had relatively low average *E. coli* concentrations indicating fairly good operating practices.

Somatic coliphages were isolated using the accredited and validated method used routinely. Plaques were clearly visible (Fig. 2) and the test methodology was also simple to follow. These phages were isolated in high numbers (as high as 1 100 pfu·10 ml^{-1}) from sites that had high *E. coli* concentrations (Table 2) and the trendline (Fig. 4) clearly showed a correlation with both indicators which had a positive correlation "r" value of 0.512 (Table 3) indicating that increases in *E. coli* concentrations could be correlated with increases in somatic phage concentrations. This widely accepted indicator of pathogenic viruses was detected in high concentrations (520 pfu·10 ml^{-1}) when the *E. coli* levels were relatively low (5 400 *E. coli*·100 ml^{-1}). This could be attributed partly to the ability of somatic coliphages to multiply in certain water environments (Grabow, 2001).

The methodology for F-RNA coliphage analyses was more involved and required the host to be grown up to the log phase for optimum infection results. The plaques were mottled and less clear than somatic plaques (Fig. 3). These phages were isolated in higher numbers when the *E. coli* levels were higher as can be seen in the trendline (Fig. 5). The highest F-RNA coliphage result of 607 pfu·10 ml^{-1} was obtained for Site 61 which had the highest recorded *E. coli*

result of 670 000 *E. coli*·100 ml^{-1}. The high positive correlation "r" value of 0.842 (Table 3) for *E. coli* and F-RNA coliphage concentrations confirmed this strong correlation. The data (Table 2 and Fig. 5) clearly showed that the concentrations of F-RNA coliphages were lower when *E. coli* levels were relatively lower. It is well known that these phages were unable to multiple in the environment because of their specificity with respect to the receptor site which is only produced by bacteria growing under optimum growth and temperature conditions.

The genotyping assays were done following the protocol established and optimised at the Department of Medical Virology, University of Pretoria. Isolates of F-RNA coliphages were assayed using the specific probes representative of the 4 groups of F-RNA coliphages. The two plaque plates assayed were isolates from the Hammarsdale Wastewater Works and Site 61 which was the Slangspruit River known to have had pollution loads from broken and blocked sewers and informal settlements in the area. Plaques from both plates hybridised with the GA probe belonging to Group 2. This group has been reported to be of human origin (Havelaar and Hogeboom, 1984). This result would confirm that the faecal pollution was of human origin, considering that one isolate was from a wastewater works effluent having human excrement and the other site was known to have traces of human excrement because of the input from the informal settlement and broken sewers feeding into the river. Further assays of F-RNA coliphage isolates would be useful in establishing the methodology of characterising the phages and grouping them into human or animal origin.

TABLE 3
Correlation results comparing all data generated for *E. coli* and somatic and F-RNA coliphages

Correlations	No. of results	Result (r)	Critical value (95% confid.)
Somatic coliphages vs. *E. coli*	89	0.512	0.1982
F-RNA coliphages vs. *E. coli*	101	0.842	0.1921
Somatic coliphages vs. F-RNA coliphages	89	0.637	0.1982

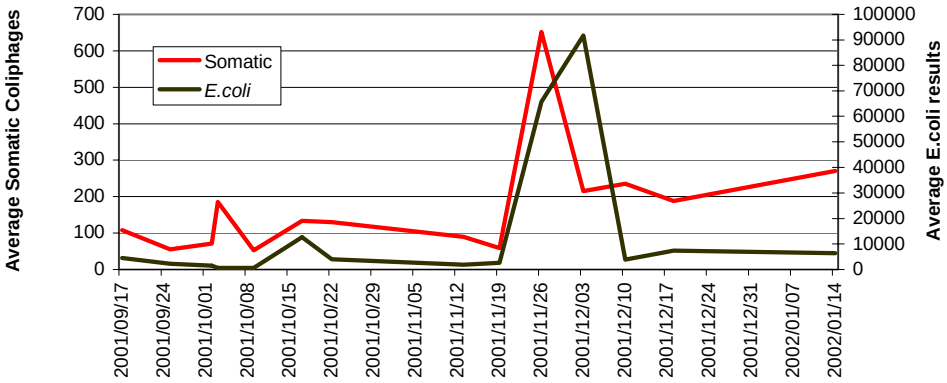

Figure 4
Trendline showing average E. coli *and somatic coliphage results obtained for river water samples in the UW catchment area*

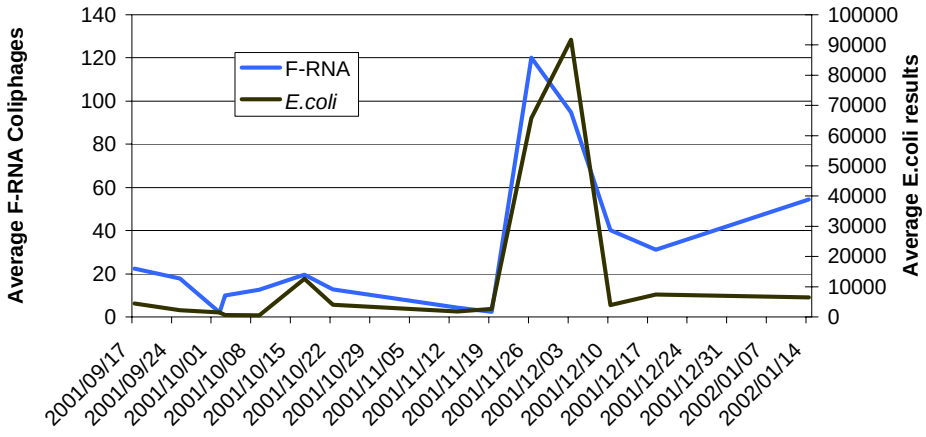

Figure 5
Trendline showing average E. coli *and F-RNA coliphage results obtained for river water samples in the UW catchment area*

Conclusions

- Certain sample sites had very high *E. coli* concentrations due to pollution problems experienced in the particular catchments.
- The average somatic coliphage counts were consistently higher than the average F-RNA coliphage counts (Fig. 6), also showing a positive correlation (Table 3).
- Somatic coliphage counts were also sometimes high when the *E. coli* concentrations were relatively lower and these coliphages had a lower correlation with *E. coli* than F-RNA coliphages
- F-RNA coliphages are of animal or human origin, unable to propagate in the general environment and highly specific with regard to the infective site on the sex-pilus, thereby making them better indicators than somatic coliphages.
- Somatic coliphages should be superseded by, or be used concurrently with, F-RNA coliphages and *E. coli* analyses to assess the presence of pathogenic viruses.
- Characterisation of the F-RNA coliphages is an additional step that could assist in tracing pollution events and contribute in helping to understand and evaluate the potential health risk involved.
- Future research is needed to genotype F-RNA coliphages detected in the different catchments in attempts to correlate the

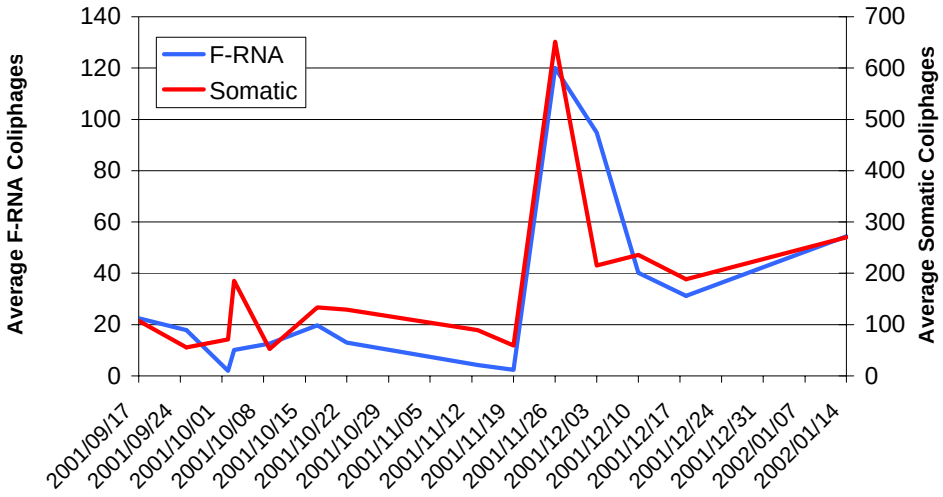

Figure 6
Trendline showing average somatic and F-RNA coliphage results obtained for river water samples in the UW catchment area

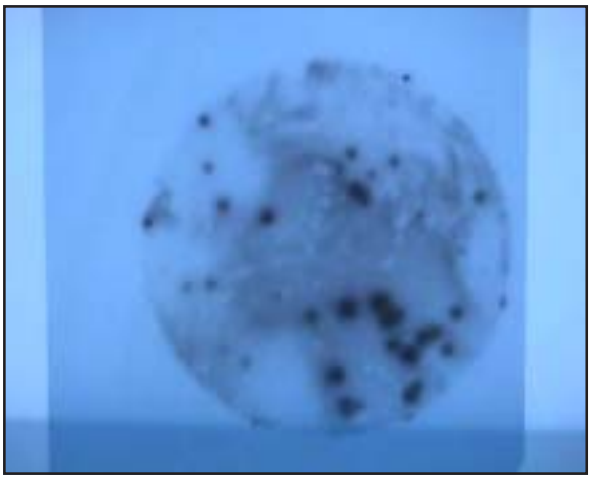

Figure 7
X-Ray Lumi-Film of a mixture of hybridised positive control F-RNA phages

data with the respective grouping, thereby assisting in tracing pollution events.

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