Comparative reduction of Norwalk virus, poliovirus type 1, F+ RNA coliphage MS2 and Escherichia coli in miniature soil columns

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Abstract Norwalk-like viruses (NLVs) are important agents of waterborne illness and have been linked to several groundwater-related outbreaks. The presence of human enteric viruses, in particular the presence of NLVs, is difficult to detect in the environment. Consequently, surrogate organisms are typically used as indicators of viruses from faecal contamination. Whether traditional bacterial indicators are reliable indicators for viral pathogens remains uncertain. Few studies have directly compared mobility and reduction of bacterial indicators (e.g. coliforms, Escherichia coli) and other surrogate indicators (coliphages) with pathogenic human viruses in soil systems. In this study the mobility and comparative reduction of the prototype NLV, Norwalk Virus (NV), was compared to poliovirus 1 (PV1), a bacterial indicator (E. coli, EC) and a viral indicator (coliphage MS2) through miniature soil columns. Replicate, 10cm deep, miniature columns were prepared using three soils representing a range of soil textures (sand, organic muck, and clay). Columns were initially conditioned, then incubated at 10–14°C, dosed twice weekly for 8 weeks with one column pore volume of virus-seeded groundwater per dose, followed by 8 weeks of dosing with one column pore volume per dose of unseeded, simulated rainwater. Columns were allowed to drain after each dosing until an effluent volume equivalent to an applied dose was collected. Column effluents and doses were assayed for all viruses and EC. Rapid mobility with minimal reduction was observed for all organisms in the sand. Similar reductions were observed in organic muck for most organisms but NV showed a greater reduction. No organisms were shown to pass through the clay columns. Elution of viruses, in particular PV1, from the columns was gradual. After cessation of microbe dosing, E. coli was less detectable than viruses in column effluents and, therefore, unreliable as a virus indicator.

Keywords Coliphage; Escherichia coli; groundwater; Norwalk virus; soil

Introduction
Half of the US population relies on groundwater as its drinking water source. Studies have demonstrated that groundwater was frequently contaminated with enteric viruses of faecal origin, that may survive in and may be transported by groundwater, thus posing a risk to public health (Abbaszadegan et al., 1997; Sobsey et al., 1980; Sobsey et al., 1986). Outbreaks of viral disease (e.g. hepatitis A, gastroenteritis) have been attributed to groundwater sources (Craun et al., 1985). Many of the most important viral contaminants found in groundwater, including Norwalk and Hepatitis A viruses, are recalcitrant to growth in culture and may be practically detected in environmental samples only by molecular methods. Surrogate microorganisms have been proposed as indicators of viral contamination of groundwater including traditional bacterial indicators and coliphages. The proposed US groundwater disinfection rule (GWDR) seeks to address the public health problem of faecally contaminated groundwater by requiring routine monitoring for microbial indicators of faecal contamination in groundwater sources serving as public water supplies. The reduction and mobility of NV in the subsurface, in relation to bacterial and viral indicators, has not been described and is the focus of this study.
Materials and methods

Viruses

PV1 was cultivated and plaque assayed on BGMK cells (Sobsey et al., 1978). The primers used for RT-PCR amplification of PV1 are described elsewhere (Schwab et al., 1995). MS2 was cultivated by confluent lysis of E. coli F-Amp and plaque assayed by a spot-plate method using 10 µL spots on agar medium host cell lawns. Primers used for RT-PCR amplification of MS2 are described elsewhere (Meschke and Sobsey, 1998). NV, as faeces from infected volunteers, was suspended in PBS and chloroform extracted (Schwab et al., 1995). The primers used for RT-PCR amplification of NV and RT-PCR have been previously reported (Schwab et al., 1995). Reverse transcription was performed using random hexamers and conditions were 5 min at 95°C (heat release of viral RNA), followed by 20 min at 25°C (random hexamer annealing), 60 min at 42°C (reverse transcription) and 5 min at 95°C (to inactivate reverse transcriptase). RT reaction volume was divided into three samples for specific PCR of each virus type, cycling conditions being 5 min at 95°C (to activate the hot-start enzyme), followed by 40 cycles of 30 s at 95°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C for extension. Samples were quantified by end-point serial dilution. PCR products were detected by agarose gel electrophoresis and confirmed by dot blot hybridisation.

Bacteria

Escherichia coli (ATCC 1473) was cultivated in tryptic soy broth (TSB) and assayed by the spot-plate method on MaConkey agar incubated at 37°C. Overnight cultures of E. coli were grown from stocks 1 d prior to use. An aliquot of the overnight culture was regrown for 4 h prior to use in order to obtain log phase cells.

Soils

The three well characterised soils used in this study were Corolla sand, Ponzer organic muck and Cecil clay (Sobsey et al., 1980, 1986; Meschke and Sobsey, 1998). These soils presented a range of textures (clay to sand) and compositions (organic content, mineral content, and metal oxide content).

Groundwater and simulated rainwater

Groundwater, currently used as an untreated source of drinking water, was obtained from a private well in southern Orange County, NC and had a pH of 6.1, conductivity of 38 mg/L as dissolved solids and turbidity of 0.11. There was no detectable background level of any study organism. Rainwater was simulated using distilled water brought to equilibrium with ambient atmospheric conditions.

Packing of soil columns

Silane-treated glass wool fibres were placed just above the tip in sterile 60mL polypropylene syringe barrels to retain soil material. Columns were packed with one of the soils by adding dry, sieved soil into the top of the syringe barrel as groundwater was injected into the tip. Soil was added gradually to a height of ~10 cm sand and the columns conditioned with 10 successive pore volumes (13–13.5 mL) unseeded groundwater prior to virus dosing to allow a quasi-secondary pore structure to develop. Columns that clogged during conditioning (i.e. longer than 24 h for infiltration of one pore volume) were re-packed and re-conditioned prior to seeding.

Dosing and sample collection

Six replicate columns of each soil type were incubated at 15°C. Groundwaters seeded with ~10⁶ each of NV, PV1, MS2 and EC were used to dose the columns at a rate of 1 areal inch
(13.5 mL) twice weekly (Monday, Wednesday) for a period of two months. Columns were allowed to drain under gravity until equivalent effluent volumes were collected. Columns were dosed at the same rate schedule for an additional month with simulated rainwater containing no viruses or EC, followed by weekly doses for two weeks and a final bi-weekly dose. The groundwater doses, as well as the effluents from each column, were diluted serially 10-fold after each dosing for microbial assays.

Data and statistical analysis
The following comparisons were evaluated: (1) between viruses as detected by RT-PCR, (2) between infectious viruses and culturable bacteria and (3) between virus detection by infectivity and RT-PCR. Column soil types were also compared for individual organisms. Statistical comparisons for the mean percentage reduction during steady-state performance were made between variables based on independent t-test analysis. All comparisons were made at the 95% confidence level.

Results
Apart from clay columns, significant breakthrough for most organisms was observed with all columns. For the clay columns, no viruses were ever detected in column effluents. Mean microbial reductions during the steady-state, 8-week period of virus dosing are shown in Figure 1.

Virus reductions in columns at steady-state
The mean reduction of PV1 infectivity during the steady-state, 8-week period of column dosing with virus-seeded ground water ranged from 2.2 log_{10} in organic muck columns to >4.5 log_{10} in clay columns. Mean reduction of PV1 infectivity in sand columns (2.4 log_{10}) was not significantly different from reduction in organic muck columns (2.2 log_{10}). However, both sand and muck were significantly different in virus reduction from clay columns. Differences in the steady-state reductions of infectious MS2 were statistically significant between the sand (1.4 log_{10}) and organic muck (1.8 log_{10}) columns. MS2 infectivity was reduced by >5.3 log_{10} in clay columns at steady state. The mean steady-state reduction of EC ranged from 1.6 log_{10} in sand columns to >3.8 log_{10} in clay columns and in organic muck columns was 2.8 log_{10}. Reductions of infectious PV1 were significantly greater than that of infectious MS2 in all samples and generally greater than culturable EC. However, infectious PV1 was reduced significantly less than culturable EC in organic muck columns during steady-state performance. Infectious MS2 reductions at steady state were only statistically similar to EC in sand columns (p = 0.4); in organic muck columns reductions of infectious MS2 were significantly less than reductions of culturable EC.

![Figure 1](https://iwaponline.com/wst/article-pdf/47/3/85/424087/85.pdf)
Steady-state reductions of NV, ranging from $2.1 \log_{10}$ in sand columns to $>3.0 \log_{10}$ in clay columns, were not significantly different between column types at the 95% level. The mean reduction of RT-PCR detectable PV1 was not significantly different from NV in sand columns ($1.6 \log_{10}$). However, steady-state reductions of RT-PCR detectable MS2 and PV1 were significantly different from NV reductions in the remaining columns. The steady-state reduction of RT-PCR detectable PV1 was $1.3 \log_{10}$ in organic muck columns whilst for RT-PCR detectable MS2 reductions were $1.25 \log_{10}$ with sand and $1.0 \log_{10}$ with organic muck columns. The steady state reduction of infectious PV1 was significantly greater than that of RT-PCR detectable PV1 in the sand and organic muck columns. For MS2, steady-state reductions of infectious and RT-PCR detectable MS2 were not significantly different in sand columns although infectious MS2 reductions were significantly less than RT-PCR detectable MS2 in muck columns ($p = 0.0017$). No statistical comparison could be made for either PV1 or MS2 in the clay columns as no viruses were found in the effluent by either method.

Simulated rainwater dosing

Effluent concentrations of infectious PV1 and MS2 increased in sand and organic muck columns following initial doses with rainwater. Levels in all sand column effluents were above the upper 95% confidence interval of the expected concentrations for PV1 and MS2 based on steady-state conditions. Also, 5/6 organic muck columns had effluent virus concentrations above the upper 95% confidence interval of the expected, steady-state effluent concentration for PV1. All organic muck columns had elevated levels of MS2 compared to steady-state conditions following initial doses of rainwater. The concentrations of EC in the column effluents did not increase above steady-state conditions following simulated rainwater dosing. The NV response to the rainwater dosing was variable with half of sand and one third of organic muck columns having greater than expected effluent NV concentrations compared to steady-state conditions following initial rainwater doses.

After the initial spikes in effluent virus levels, detectable infectious PV1 gradually declined with additional simulated rainwater dosings. However, levels of RT-PCR detectable PV1 in column effluents of simulated rainwater dosings remained elevated relative to steady-state levels throughout the study in sand columns and for the first 5 weeks of simulated rainwater dosing in muck columns. Infectious MS2 and culturable EC concentrations in column effluents generally declined below detection limits within 1.5 weeks of cessation of seeded groundwater dosing. MS2 was detectable in column effluents for a longer period than EC and was more similar to the PV1 detection in column effluents after seeded dosing ended. Similar to PV1, RT-PCR detectable MS2 was present in the effluents of the sand and muck columns at elevated levels to the end of the study.

Discussion

Average reductions of viruses in soil columns during the steady-state period of virus dosing, and the overall reduction of viruses in soil columns in this study, were consistent with previous observations (Sobsey et al., 1986). In Corolla sand columns the mean reduction of PV1 at steady-state performance at 14°C ($2.4 \log_{10}$) was similar to the previously reported mean reductions of $1.9 \log_{10}$ at 4°C and $2.4 \log_{10}$ at 25°C (Sobsey et al., 1986). The continued persistence of PV1 elution from sand columns after cessation of virus seeding was also consistent with previous results. Lefler and Kott (1974) described continued elution of PV1 from sand after 10 successive washings. Previous studies documented virus elution from soil by application of simulated rainwater, similar to that seen in this study (Kott, 1988; Landry et al., 1979). This “rainwater elution” was also consistent with observations made during field studies on the detection of land-applied viruses in wastewater in which viruses
were detected in wells only after heavy rain events (Wellings et al., 1975). The observed extent of elution of PV1 from soil columns in this study was somewhat greater than reported before (Landry et al., 1979). However, there are differences between strains of the same virus type, suggesting that alterations of virus adsorption by slight differences in the viral capsid structure are possible (Landry et al., 1979). Furthermore, the soils, groundwater, column size and dosing schedule used in the current study were not identical to those used in Landry et al. (1979) which could also account for the observed differences in the extent of virus breakthrough in soil column effluents.

The observed differences in reduction through soil columns and rainwater elution of infectious PV1 and MS2, compared to culturable EC, supported previous evidence that traditional bacterial indicators, e.g. *E. coli*, were inadequate indicators of viral contamination in soils and groundwater and that alternatives, such as bacteriophages, may be more appropriate indicators of human enteric virus retention and mobility in soil systems. The average reduction in soil columns of infectious PV1 was greater than or equal to the reduction of infectious MS2 for all time points in all column types during the steady-state period of dosing with seeded groundwater. The reduction of EC during the steady-state period of column dosing with seeded groundwater was generally less than or equal to that of PV1 in sand columns. However, for organic muck columns, in general, and for at least one time point in sand columns EC reduction was greater than PV1 reduction. Also noteworthy was the increased MS2 and PV1 elution from columns following application of simulated rainwater. No such increased elution from columns was observed for EC following application of simulated rainwater. These results further indicated the similarities of the indicator virus MS2 and the human enteric virus PV1 in their interactions with soils and their considerable differences from the responses of the indicator bacterium EC. Although MS2 was not detected for as long as PV1 in soil column effluents following rainwater application, it was detected for a longer period than EC.

NV reduction at steady state conditions of soil column dosing with seeded groundwater was most similar to PV1 reduction being consistent with observations on adsorption of NV, MS2, and PV1 to soils suspended in groundwater (Meschke and Sobsey, 2002a in preparation). Persistence of NV detection in column effluents following rainwater application was also similar to that of PV1 and consistent with the findings of recent long-term virus persistence studies in soils and groundwater (Meschke and Sobsey, 2002b, in preparation). The present results provided further evidence that MS2 (and perhaps other F\(^+\) RNA coliphages) conservatively indicate the reduction and mobility of PV1, NV, and possibly other enteric viruses, in soil columns and perhaps generally in the subsurface environment. This may be due to MS2 being generally more mobile and retained less by soils than either PV1 or NV.

Despite consistent viral and bacterial detection in soil column effluents, the majority of viruses and bacteria (84 – >99.999%) were retained in the soil columns after the final simulated rainwater dose. This raised the issue as to whether retained viruses and bacteria were inactivated or merely retained by the soil. The greater reductions of virus infectivity compared to RT-PCR detectable virus during the steady-state period of soil column dosing with seeded groundwater suggested that either (a) some inactivation was occurring within the column or (b) that infectious viruses were preferentially retained in the columns. Further studies have investigated the retention and fate of infectious virus within soil columns (Meschke and Sobsey, 2002c in preparation).

**Conclusions**

The reduction of NV during an 8-week steady-state period of soil column dosing with seeded groundwater was similar to or greater than MS2 and PV1 for all soil types studied.
Elution of NV from the soil columns during a period of subsequent dosing with unseeded groundwater was similar to or less than the elution of both PV1 and MS2. Therefore, it appears that NV is not a “worst case virus” in regard to its retention and mobility in soils and groundwater. Additionally, the results of this study indicated that traditional bacterial indicators, such as EC, were inadequate to characterise and quantify enteric virus retention and mobility in soils and groundwater. These findings suggested that other candidate indicators, such as coliphages, should also be included in the groundwater disinfection rule now under development in the United States and should be more generally adopted as indicators of enteric virus contamination in lieu of or as supplements to the direct detection of pathogenic enteric viruses.

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References