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PAPER

Leaching of viruses and other microorganisms naturally occurring in pig slurry to tile drains on a well-structured loamy field in Denmark

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Abstract The amount of animal manure used in modern agriculture is increasing due to the increase in global animal production. Pig slurry is known to contain zoonotic bacteria such as *E. coli*, *Salmonella* spp. and *Campylobacter* spp., and viruses such as hepatitis E virus and group A rotavirus. Coliform bacteria, present in manure, have previously been shown to leach into tile drains. This poses a potential threat to aquatic environments and may also influence the quality of drinking water. As knowledge is especially scarce about the fate of viruses when applied to fields in natural settings, this project sets out to investigate the leaching potential of six different microorganisms: *E. coli* and *Enterococcus* spp. (detected by colony assay), somatic coliphages (using plaque assays), and hepatitis E virus, porcine circovirus type 2, and group A rotavirus (by real-time polymerase chain reaction). All six microorganisms leached through the soil entering the tile drains situated at 1-m depth the first day following pig slurry application. The leaching pattern of group A rotavirus differed substantially from the pattern for somatic coliphages, which are otherwise used as indicators for virus contamination. Furthermore, group A rotavirus was detected in monitoring wells at 3.5-m depth up to 2 months after pig slurry application. The detection of viral genomic material in drainage water and shallow groundwater signifies a potential hazard to human health that needs to be investigated further, as water reservoirs used for recreational use and drinking water are potentially contaminated with zoonotic pathogens.

Keywords Health · Pathogen · Solute transport · Agriculture · Groundwater monitoring

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Introduction

While the threat of contamination by nutrients leaching from manure-treated fields is well recognized, the threat by leaching of zoonotic pathogens from the manure has received much less attention. Livestock manure is commonly used in modern agriculture as fertilizer. Millions of tons of manure are excreted from livestock and applied to farmland annually. In the United States, livestock excrete approximately 500 million tons of manure annually (USEPA 2003). In Europe, the entire manure production is estimated to be 1.4 billion tons per year (Foged et al. 2011). An estimated 26 million tons of livestock manure was spread on Danish farmland in 2011 (Danish Agriculture and Food Council 2012).

Livestock manure contains nutrients and organic matter used to enhance soil properties and thus crop production, but may also contain a variety of zoonotic pathogens (Cole et al. 1998; Sobsey et al. 2001; Ziemer et al. 2010). Animal pathogens with potential negative impact on human health (zoonosis) include, rotavirus group A (RV-A), hepatitis E virus

(HEV), *Salmonella* spp., *E. coli* O157:H7 and *Cryptosporidium parvum* (Ziemer et al. 2010). With the emergence of avian and swine influenza, there has been an increased surveillance and focus on zoonotic viruses. The transmission of viruses between mammals through environmental reservoirs is, however, poorly understood. In the non-industrialized part of the world, hepatic viruses such as hepatitis A virus (HAV) and HEV cause many waterborne epidemics (Naik et al. 1992). In the western world, HEV was previously regarded as a travel-related illness (Hsieh et al. 1999); however, HEV genotype 3 has since been discovered in pigs worldwide (Meng et al. 1997), and is now considered endemic in pigs in many European countries and North America and as the main reservoir for locally acquired HEV. The prevalence of anti-HEV antibodies in humans ranges between 2 and 53% (Bouwknegt et al. 2008; Christensen et al. 2008; Mansuy et al. 2011; Purcell and Emerson 2008). Detection of HEV in wastewater from urban areas has been reported in European and North American cities (Clemente-Casares et al. 2003), indicating that HEV may be present in the water environment. Another virus with zoonotic potential is RV-A. RV-A mainly infects younger animals and children, and is the primary cause of hospitalization of children due to gastroenteritis (Martella et al. 2010). RV-A has proved to be very persistent in pig slurry storage tanks, with a reduction in infectivity of only one log-unit found after 6 months (Pesaro et al. 1995).

Enteric viruses and bacteria have been isolated from and linked to disease outbreaks associated with contaminated drinking-water sources, recreational waters and rivers exposed to fecal contaminated water (Croci et al. 2008; Fong and Lipp 2005; Harris et al. 2003; Lipp and Rose 1997; Reynolds et al. 2008; Sair et al. 2002). Therefore, detailed knowledge on the transport of microorganisms through soil is important when measures to protect groundwater reservoirs from contamination are to be established. *E. coli* O157:H7 and *Campylobacter jejuni* originating from manure resulted in a large waterborne disease outbreak when people in Walkerton, Canada, consumed contaminated drinking water (Hrudey et al. 2002). The most plausible route of contamination of the city's water reservoir was rapid horizontal transport in fractured bedrock. Similarly, drinking well water in a restaurant in Wisconsin (USA) was associated with illness caused by NoV (Borchardt et al. 2011). Zoonotic viruses such as HEV originating from pig slurry could pose a similar public health risk if transported to water bodies, including drinking water reservoirs; therefore, it is necessary to determine the travel distances and survival times of viruses in soils and use such data for risk assessments and the establishment of measures to manage contamination of drinking water sources and public health protection (Azadpour-Keeley et al. 2003).

Macropores (earthworm channels, cracks, fractures, old root canals) are present in structured loamy and clayey soils (Jacobsen and Kjaer 2007). Preferential flow through macropores can take place when the soil is nearly saturated and at some point above the water-entry pressure of the soil. Differences in the hydraulic conductivity between macropores and soil matrix can then cause a non-equilibrated flow, where the water in the macropores moves faster than the wetting front in the matrix (Jarvis 2007).

Since water-dispersible colloids have surface charge and high specific surface area, they can effectively adsorb weakly soluble, strongly sorbing contaminants (Kretzschmar et al. 1999; de Jonge et al. 2004). Thereby colloids can convey adsorbed compounds such as phosphorus (de Jonge et al. 2004; Norgaard et al. 2012) and pesticides (Flury 1996; Gjettermann et al. 2009; Kjær et al. 2011) and pathogens (Bradford et al. 2013), from the surface to deeper soil layers through preferential pathways. Transport of microorganisms and colloids through soil depends on soil type and texture, the presence of macropores, precipitation and antecedent moisture content of the soil, manure constituents and chemical composition as well as the size and surface properties of the colloids and microorganisms. Preferential water movement is probably the primary route for rapid transport of microorganisms through soil and thereby has a major impact on the microbial leaching (Abu-Ashour et al. 1994; Forslund et al. 2011a; Guber et al. 2007; Jarvis 2007; Nicosia et al. 2001; Walshe et al. 2010). Increased transport of microorganisms has been observed in soil with high clay content because water flow in clay-rich soils is usually concentrated in the fractures (Abu-Abu-Ashou et al. 1998; Beven and Germann 1982). The ability of different sized microorganisms e.g. viruses, bacteria or protozoan parasites, to travel fast through soil fractures has been recognized (Bradford et al. 2013). The ability of the microorganisms to survive in the soil environment depends on factors such as type of microorganism, temperature, pH, moisture and composition of the indigenous microflora (Azadpour-Keeley et al. 2003; Vinten et al. 2002). Field experiments are an advantage compared to simulated laboratory experiments with soil cores. In field experiments, microorganisms are exposed to natural weather conditions, e.g. fluctuating temperatures and humidity, wind and precipitation influencing the transport and survival of microorganisms in soil. Further, both the variation in soil structure and the spatial distribution of connective preferential flow paths are intact in field experiments, while soil cores only represent a small fraction of the field and excavation could affect the soil architecture. Conversely, the enhanced level of complexity in field experiments also makes it difficult to estimate the dominant processes involved in the microbial migration (Bradford et al. 2013).

Field studies have shown that transport of slurry constituents through soil to tile drains is possible and can occur shortly after slurry application (Evans and Owens 1972; Fleming and Bradshaw 1992; Kjær et al. 2007; McLellan et al. 1993; Naden et al. 2010). Field studies have mainly focused on fecal indicator organisms, e.g. *E. coli* and enterococci as well as

bacteriophages used as model organisms for viruses (DeBorde et al. 1998; Oliver et al. 2005; Pappas et al. 2008; Schijven et al. 1999), while studies on waste-associated human viruses, providing valuable information on the transport of these pathogens through the vadose zone (Borchardt et al. 2011; Jansons et al. 1989), are limited. Due to the potential contamination associated with applying zoonotic viruses in environmental studies, bacteriophages have been used as a model for leaching of zoonotic viruses through soil (Forslund et al. 2011b; Havelaar 1991; Hijnen et al. 2005; Mesquita and Emelko 2012).

Many countries assess the microbiological quality of water based on bacterial indicators such as enumeration of enterococci and fecal coliform and total coliform bacteria, but such bacteria are often poor indicators of viruses (Gibson and Schwab 2011; Jiang et al. 2001). Enteric viruses have been recognized as the causative agents in gastroenteritis outbreaks caused by water that have met bacteriological standards (Bosch 1998). Over 100 types of pathogenic viruses have been described to occur in water that has been contaminated with fecal material (Pillai 2006); therefore, the use of non-pathogenic viral indicators of fecal contamination, e.g. coliphages, is an important tool in public health studies, when tracing sources of groundwater contamination (Snowdon et al. 1989). With the vast amounts of livestock manure spread on agricultural fields worldwide, there is a particular need for studies that are designed to measure the leaching of zoonotic viruses normally present in animal slurry. There are currently no regulations in place on national or European Union level regarding limiting the content of microorganisms in manure allowed to be applied to fields. The regulations regarding application of manure that are enforced in Denmark are primarily to prevent bothersome odor to nearby residential areas and to limit field run-off into nearby water bodies.

The main objective of the present study was to assess the potential of viruses from different families, such as HEV and RV-A, leaching into the aquatic environment when manure from an typical Danish pig producing facility is applied to a field under conditions used by Danish farmers. In addition, the purpose was to compare the leaching capabilities of *E. coli*, *Enterococcus* spp., somatic coliphages, HEV, PCV2 and RV-A, and lastly, to evaluate if somatic coliphages are appropriate model organisms for viruses originating from pigs under natural field conditions.

Materials and methods

Test field site

The experimental site was located at Silstrup, south of Thisted in northwestern Jutland, Denmark (56° 56′ N, 8° 39′ E). The field is a part of the Danish Pesticide Leaching Assessment Program (Lindhardt et al. 2001). The field was 17,100 m^2 (1.71 ha) and the terrain sloped gently 1–2°. The site was located on a glacial moraine of Late Weichselian age and has been exposed to weathering, erosion, leaching, and other geomorphologic processes for about 16,000 years (Lindhardt et al. 2001). The soil was a sandy clay loam (14.6% clay, 11.6% silt, 67.7% sand and 4.1% organic matter) with pH 7.1 and a porosity of 0.42 cm^3 cm^{-3} (Lindhardt et al. 2001). The soil was prone to preferential transport as it is heavily fractured and bioturbated with 400 biopores per m^2 found 0.6 m below ground surface (bgs). These observations was done in a 5 m deep 10 × 10 m test pit excavated nearby the north eastern corner of the field, following the methodology of Klint and Gravesen (1999). These observations were in line with previous studies conducted in other similar soil types in Denmark (Ernstsen 2004). The drainage system in the field consisted of five parallel field drains running from south to the north (Fig. 1). The five drains were connected to a transverse collector drain from which drainage water samples were collected. The tile drains were installed at an average depth of 1.1 m and an interspacing of approximately 17–18 m. Conventional agriculture with ploughing depths of around 22–24 cm had been practiced at the site during the previous 27 years and red fescue (*Festuca ruba* L.) was grown on the field during the study period. The slurry was surface applied with trailer hoses on 5 October 2011. There was no tillage in connection with the application of the slurry, but after desiccation of the grass with glyphosate on 10 September 2012, the field was ploughed to 24 cm depth, i.e. nearly a year after the use of slurry.

The field was encircled by a grass-covered buffer zone being 18 m wide to the north and west, and 10 m to the east, and to the south, a 7-m grass-covered buffer zone was supplemented by a 3-m paved road. The site had equipment installed to record water-table depth, the minimum and maximum air temperature, and soil temperature—30 cm below ground surface (bgs)—on an hourly basis. Soil temperature was measured hourly by means of a platinum resistance thermometer (Pt-100) at two locations (Fig. 1). Precipitation was measured at the site using a tipping bucket rain gauge system.

Sampling of pig slurry and drainage water

The pig (*Sus scrofa domesticus*) slurry was supplied by a local farmer. On the 5 October 2011, the pig slurry was homogenized in the storage tank for approximately 1 h, using a slurry agitator (Kimidan Multimixer, Denmark). A total of 49 tons (29 tons ha^{-1}) of homogenized pig slurry was applied in bands on the soil surface by trailer hosing. The pig slurry was tested for the presence of somatic- and F-RNA coliphages, *E. coli* and *Enterococcus* spp., *Salmonella* spp. and swine influenza virus (SIV), porcine parvovirus (PPV), HEV, RV-A, and PCV2 as described below. Initial analysis showed that

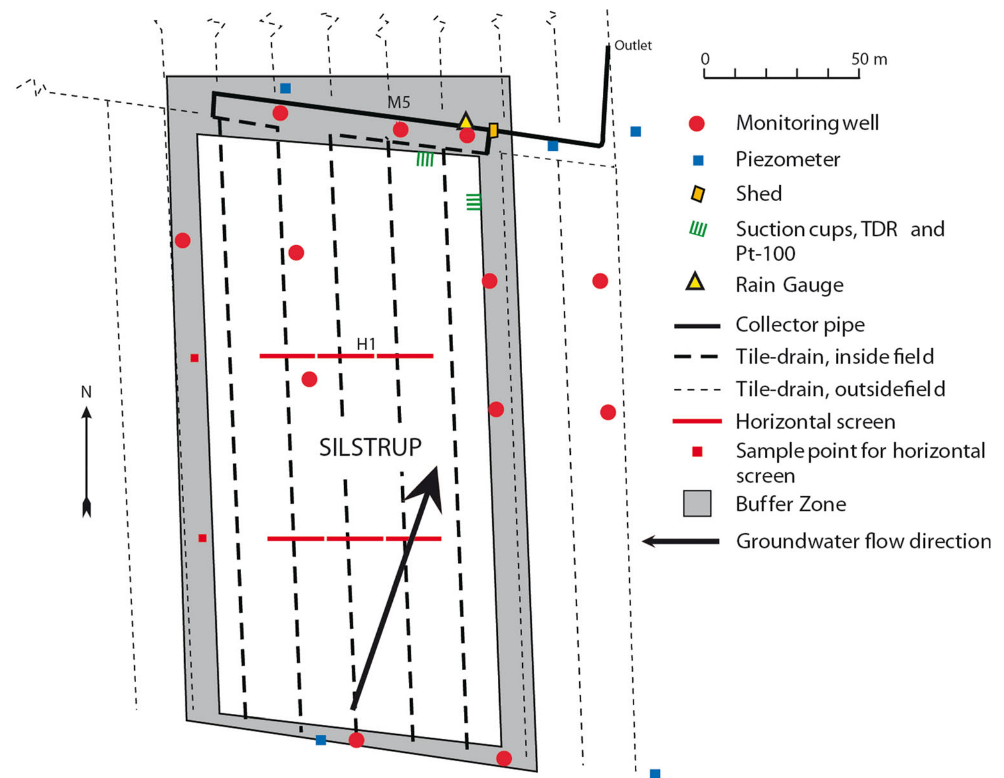

Fig. 1 Schematic drawing of the test site located near Silstrup, Denmark

E. coli, *Enterococcus* spp., somatic coliphages, HEV, PCV2 and RV-A were present in the pig slurry and they were therefore all selected for analysis in the leaching study (Table 1). Porcine circovirus type 2 (PCV2) was included as this virus is ubiquitous in swineherds and highly persistent in the farm environment (Kristensen et al. 2013). Drainage water was sampled flow-proportionally (Plauborg et al. 2003; ISCO 6700 sampler, Teledyne Isco Inc., US). For weekly samples, the microbiological analysis was performed on pooled water samples containing all the subsamples collected during the past week to obtain a weighted average concentration. Following the onset of heavy rainfall events, drainage water was sampled flow-proportionally for approximately 1 day. To obtain weighted average concentrations for each heavy rain event, the microbiological analysis was performed on pooled water samples containing all the subsamples collected during the heavy rain event. The heavy rain events were defined as events causing the water level and accumulated flow rate within the preceding 12-h period to exceed predefined levels that depended on the month of the year. The pre-defined level for triggering of sampling depended on the season of the year. An amount of 200-ml subsamples was taken for every 3,000 L of drainage flow during the winter season (September–May) and for every 1,500 L during the summer period (June–August; Plauborg et al. 2003); additionally, groundwater samples were collected monthly from both the vertical well M5 and the horizontal monitoring well H1 (Fig. 1). The vertical monitoring well, installed in the surrounding buffer zone, consists of four 1-m screens, covering the upper approx. 4 m of the saturated zone. The screens were made from high-density polyethylene (HDPE) with an outside diameter of 63 mm and a wall thickness of 5.8 mm. Samples were collected from the upper-most filter located 1.5–2.5 m bgs. In addition, horizontal monitoring wells were installed 3.5 m beneath the test sites. The horizontal screens were installed by drilling from the buffer zone on the one side of the field to the buffer zone on the opposite side, without causing any disturbance to the topsoil within the cultivated area. Each horizontal monitoring well consists of three 18-m screens providing integrated water samples characterizing groundwater quality just beneath the test site (Fig. 1). Samples were collected from the middle filter of the three filters (Fig. 1). The horizontal screens were made of HDPE with an outer diameter of 125 mm and a wall thickness of 5.8 mm. Three individual screens were installed in each borehole separated by 1-m bentonite seals. An inner pipe (outer diameter of 63 mm), used for outlet tubes from each of the three screens, transversed the entire installation (Lindhardt et al. 2001). The day before a sampling, the wells were purged to ensure that fresh groundwater was sampled. Additional information about sampling methods and monitoring design is available in Lindhardt et al. (2001) and Rosenbom et al. (2015).

The collection of water samples was conducted from 22 September until 5 January 2012. All samples were kept in a cooling box and transported to the laboratory where analysis for somatic coliphages and viable indicator bacteria were

Table 1 Physical, chemical and microbiological properties of pig slurry

Dry matter %	pH	Total-N kg ton^{-1}	NH_4+−N kg ton^{-1}	P kg ton^{-1}	Mg kg ton^{-1}	HEV (RT-)PCRu ml^{-1}	PCV2	RV-A	Somatic coliphages PFU ml^{-1}	*E. coli* CFU ml^{-1}	*Enterococcus* spp.
3.97	6.8	4.21	2.51	1.04	0.51	1.2×10^4	5.4×10^4	3.8×10^5	$2.2 \times 10^5 \pm 8.1 \times 10^3$	$6.0 \times 10^4 \pm 4.3 \times 10^3$	$3.6 \times 10^4 \pm 3.3 \times 10^3$

initiated within 12 h. Water samples for virus analysis were immediately frozen at −80 °C in 50-ml tubes.

Chemical analysis

Chemical analysis of slurry and drainage water samples was initiated within 24 h after sampling. Weekly collected drainage water samples were analyzed for the content of dissolved organic carbon (DOC; Danish Standard 1997), total dissolved phosphorus (Danish Standard 2004) and total phosphorus (Danish Standard 2004) which include total dissolved- and particle-associated phosphorus. In addition, pH was measured in water samples and slurry using a pH meter (PHM220; Radiometer, Denmark). Slurry samples were analyzed for dry matter, total-N, NH_4-N, phosphorus and magnesium at the OK Laboratory for Agriculture, Viborg, Denmark.

For the analysis of DOC, water samples were immediately filtered through a Whatman glass fiber prefilter (Whatman GmbH., Germany) and a 0.45-μm cellulose membrane filter (Whatman GmbH., Germany) and 15–20 ml of sample was transferred to a vial and adjusted to pH 2.5 using an Metrohm 848 Titrino Plus titrator (Metrohm AG, Switzerland). Measurement of DOC in triplicates was done using a Shimadzu TOC-VCPH analyzer (Shimadzu Scientific Instruments, Columbia, US). Drainage water to be analyzed for total dissolved phosphorus (Danish Standard 2004) was filtered through a Whatman glass fiber prefilter (Whatman GmbH., Germany) and a 0.45-μm cellulose membrane filter (Whatman GmbH., Germany) followed by acidification with addition of 1 ml of 4 M H_2SO_4 per 100-ml water sample. In the sulfuric acid solution, orthophosphate (PO_4^{3-}) together with molybdate and antimony (III) forms heteropoly-molybdenum blue and this is reduced by ascorbic acid into the complex antimony-phospho-molybdate. The absorbance of the complex, being proportional to the orthophosphate content, was measured at 880 nm using a Perkin Elmer Lambda 20 UV/Vis Spectrophotometer (Perkin Elmer, USA). The water sample for detection of particle-associated phosphorus (Danish Standard 2004) was processed likewise but without the filtration step.

Microbial analysis

Fecal bacterial indicators

In both slurry and water samples, the fecal indicator organisms *E. coli* and *Enterococcus* spp. were enumerated by direct plating in triplicate on selective agar plates with a detection limit of 1 CFU ml^{-1}. Water and slurry samples were 10-fold diluted in Maximum Recovery Diluent (Oxoid, Hampshire, United Kingdom). Concentration of *E. coli* was determined on Brilliance *E. coli*/coliform Selective Agar (Oxoid), where colonies appear as typical indigo blue colonies after incubation at

37 °C for 21 ± 3 h (Wohlsen 2011; Wuton et al. 2009). The concentration of *Enterococcus* spp. was determined as the number of typical red-maroon colonies on Slanetz and Bartley medium (Oxoid) following incubation at 44 °C for 48 ± 4 h (Danish Standard 1999). Since the concentration of both *E. coli* and *Enterococcus* spp. were determined from triplicate diluted samples, the average concentration reported can be less than 1 CFU ml^{-1}.

Somatic coliphages

Somatic coliphages is a group of bacteriophages with the ability of infecting *E. coli* via the cell wall and belongs to four different families (Lee 2009). Somatic coliphages were analyzed in triplicates with a detection limit of 1 PFU ml^{-1} by plaque assay according to ISO 10705–2 (ISO 2001). The acceptable range of error of the plaque assay is ±20% (Chu et al. 2001). Briefly, slurry and water samples were 10-fold serially diluted in Maximum Recovery Diluent (Oxoid) and enumerated by the double-agar layer method. The host strain *E. coli* ATCC 13706 was grown in nutrient broth (Oxoid) at 37 °C for 4 h. From the 10-fold diluted samples, 1 ml was mixed with 1-ml broth culture of the host strain and 3-ml soft agar consisting of 70% blood agar base (Oxoid) and 30% nutrient broth (Oxoid). The mixture was gently mixed and spread on a well-dried blood agar base plate (Oxoid). Plates were incubated at 37 °C for 18 h and clear zones (plaques; PFU) were counted. Slurry was filtered through 0.45-μm pore size filters (Sartorius, Goettingen, Germany) before mixed with the soft agar when high bacterial background flora was expected. Concentration of somatic coliphages was determined from triplicate diluted samples and the average concentration reported can be less than 1 PFU ml^{-1}.

Viruses

Prior to precipitation of viruses, the pH of slurry and water samples was adjusted to pH 7 using NaOH and then clarified from debris by centrifugation at 4,000 rpm for 30 min at 4 °C. To precipitate viruses; 40 ml of the supernatant was transferred to tubes containing 0.7 g NaCl (Sigma-Aldrich, Brøndby, Denmark) and 3.2-g polyethylene glycol (PEG 8000 Fischer Scientific, Slangerup, Denmark). The samples were placed on a shaking bed over night at 4 °C followed by centrifugation at 10,000 rpm for 90 min at 4 °C. The supernatant was discarded and viral nucleic acid was purified from the pellet using NucliSENSE reagents and the miniMag platform (bioMérieux, Herlev, Denmark) according to the manufacturer's protocol. The nucleic acid was eluted in 100-μl RNase free water. The efficiency of viral concentration and viral nucleic acid extraction inherent to the procedure for virus recovery were quality assessed using an internal process control (IPC). For this mengovirus (MC_0; strain ATCC VR-1957; Costafreda et al. 2006), approximately 10^4 plaque forming units was added to each water sample before the initial step of viral precipitation, and to a non-matrix sample before nucleic acid extraction. After extraction of samples seeded with the MC_0, the Ct (Cycle threshold) value of the water samples was compared to the Ct value of the non-matrix sample used in the extraction series and to a standard curve obtained by endpoint dilution with one real-time reverse transcriptase polymerase chain reaction (rt-RT-PCR) unit defined as the lowest possible detectable dilution. The difference (ΔCt) was used to determine the extraction efficiency, using $100e^{-0.6978\Delta Ct}$ (Costafreda et al. 2006); as negative process control, clean water was added in a parallel sample.

For each rt-PCR run, a positive amplification control (PAC; nucleic acid extract from feces samples previously tested positive for the three target viruses, PCV-2, HEV and RV-A) and a no template control (NTC) was included. Samples and controls were analyzed in duplicates. The requirement for successful extraction and rt-PCR run was that the negative controls tested negative and that the individual positive controls met the set Ct requirements established under the validation of the assays. The process control MC_0 were detected using the RNA Ultrasense One-Step qRT-PCR System (Invitrogen, Nærum, Denmark) and the primers, probe and reaction conditions described by Pintó et al. (2009). HEV was detected by a one-step rt-RT-PCR assay using primer probe energy transfer (PriProET) chemistry (Breum et al. 2010) but modified to a lower final primer and probe concentration of 500 nM for HEV2-R and HEV2-P and 100 nM for HEV2-F. The target for the assay is the ORF2-encoded capsid protein, whereas the standard curve was prepared from plasmids containing the target gene of the assay. The amplification efficiency of the assay was 88% with a slope of −3.64. The detection of PCV2 was accomplished with the assay which targets ORF1 and utilizes the PriProET chemistry (Hjulsager et al. 2009). The standard curve used to assess viral load was made by spiking negative fecal samples with plasmid, while the amplification efficiency of the assay was 82% and the slope −3.86. For detection of RV-A, the primer and probes used in the assay along as well as the PCR cycling conditions were adopted from Pang et al. (2004) and the assay was modified by the use of the RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen) and rt-RT-PCR analysis was performed on the Rotorgene Q real time PCR cycler (QIAGEN, Hilden, Germany). The primers and probe targets the NSP3 segment. Homologous sequences of the NSP3 target region are present in bovine, simian, porcine and human derived RV-A viruses (Pang et al. 2004). The standard curve was made from a serial dilution of RNA extracted from RV-A grown in the MA104 cell line and the amplification efficiency of the assay was 92% and the slope −3.54. The standard curves were not used for absolute quantitation but to compare concentrations of each microorganism separately. Based on the dilution series made

for the standard curve, a detection limit at Ct 38 for HEV and PCV2 and 40 for RV-A was applied. The amount of detected target genomes in (RT)-PCR units (u) were measured by interpolation of the detected Ct-values of the respective viruses to their corresponding standard curves, with one unit defined as the lowest possible detectable dilution. In this study, no comparison was done on the exact number of viruses detected by the different assays; instead the (RT-)PCRu of each virus found in the drainage water was normalized against the (RT-)PCRu detected in the applied slurry. These are comparable as they are detected by the same assay and corrected against the same PCR controls in the Rotorgene software. These normalized concentrations of the viruses were then used in relation to leaching pattern, recovery and log-reduction in depth; a similar approach was applied to the other microorganisms.

Data analysis and statistical methods

Calculation of concentration and standard deviation of replicate samples for *E. coli*, *Enterococcus* spp. and somatic coliphages was done according to Niemela (1983). Due to the limited drainage runoff available, further reduction of the detection limit, thereby yielding higher colony counts and reducing uncertainty of data, was therefore not possible (Emelko et al. 2008).

The removal rate λ (unit: $\log_{10}\ m^{-1}$) which defines the amount of microorganisms removed by passing through 1 m of soil was calculated using Eq. (1). The leaching of all microorganisms was normalized with the initial concentration detected in pig slurry (C_0). The depth (d) was set to the location of the tile drain, i.e. 1.1 m bgs, and the removal rate was calculated based on the highest concentration (C_{max}) recorded in the event samples as proposed by Pang et al. (2009).

$$\lambda = -\frac{\log_{10}\left(\frac{C_{max}}{C_0}\right)}{d} \quad (1)$$

Recovery of microorganisms from pig slurry was calculated in three different ways, based on the maximum concentration detected in drainage water samples (C_{max}), in all event samples—i.e. where the amount of microorganisms found in each event sample was summed, and all weekly samples, i.e. total amount of microorganisms detected in all weekly samples collected during the study period.

The statistical analysis was performed on log-transformed normalized data by a permutation test with the main effect on leaching differences of microorganisms and on days of sampling. The simulated P-values for the corresponding permutation tests on F-test values were calculated using R statistical software suite version 3.0.0 (R Core Team 2013) with the lmPerm package version 1.1.2 (Wheeler 2010). The significance level was set at $P = 0.05$. Pearson product–moment correlation coefficients were derived to assess the association between microbiological and environmental variables such as DOC, total dissolved- and particle-associated phosphorus. The Pearson coefficient was calculated using Excel version 15.

Results

Climate conditions

In the study period, running from 5 October 2011 to 5 January 2012, the total precipitation amounted to 286 mm. During October, drainage runoff only occurred during four precipitation events. The month of November was relatively dry with 49.5-mm precipitation compared to 108 mm for the average of November recorded in 1961–1990 on site. This resulted in an entire month devoid of drainage runoff. At the end of November and start of December heavier rain resumed drainage runoff. The experiment ended in January with large amounts of precipitation and drainage runoff (Fig. 2e). The air temperature in the study period varied between −2.6 and 15.4 °C and was relatively high for the season with the three primary months having only a total of three subzero days (Fig. 2f). Soil temperature at 30 cm bgs was below 15 °C for the entirety of the study. Prior to the study period, the site was monitored for 2 weeks. Because precipitation was scarce, insufficient drainage runoff limited microorganism analysis.

Leaching of microorganisms

Initial analysis of water, collected from drains (bacteria and somatic coliphages) 14 days prior to pig slurry application, and groundwater monitoring wells (bacteria, somatic coliphages and viruses) 1 day prior to pig slurry application, showed no presence of *E. coli*, *Enterococcus* spp., and somatic coliphages, nor of the viruses HEV, PCV2 and RV-A (Fig. 2c,d). The leaching of microorganisms is illustrated in the breakthrough curve (BTC) during the study period with the concentration detected in drainage water normalized against the initial concentration found in the pig slurry (Fig. 2). The initial breakthrough and relative concentrations of microorganisms is shown for weekly and event samples collected from the drains (Fig. 2b,c).

E. coli and *Enterococcus* spp. was detected in the first of three event samples caused by intensive rainfall during the first week (Fig. 2c). This rain event happened on the day after the pig slurry was applied and the concentration of *E. coli* and *Enterococcus* spp. in drainage water was 30 CFU $ml^{-1} \pm 3.6$ CFU m^{-1} and 27 CFU $ml^{-1} \pm 3.4$ CFU ml^{-1}, respectively. *Enterococcus* spp. was detected again in the event samples collected

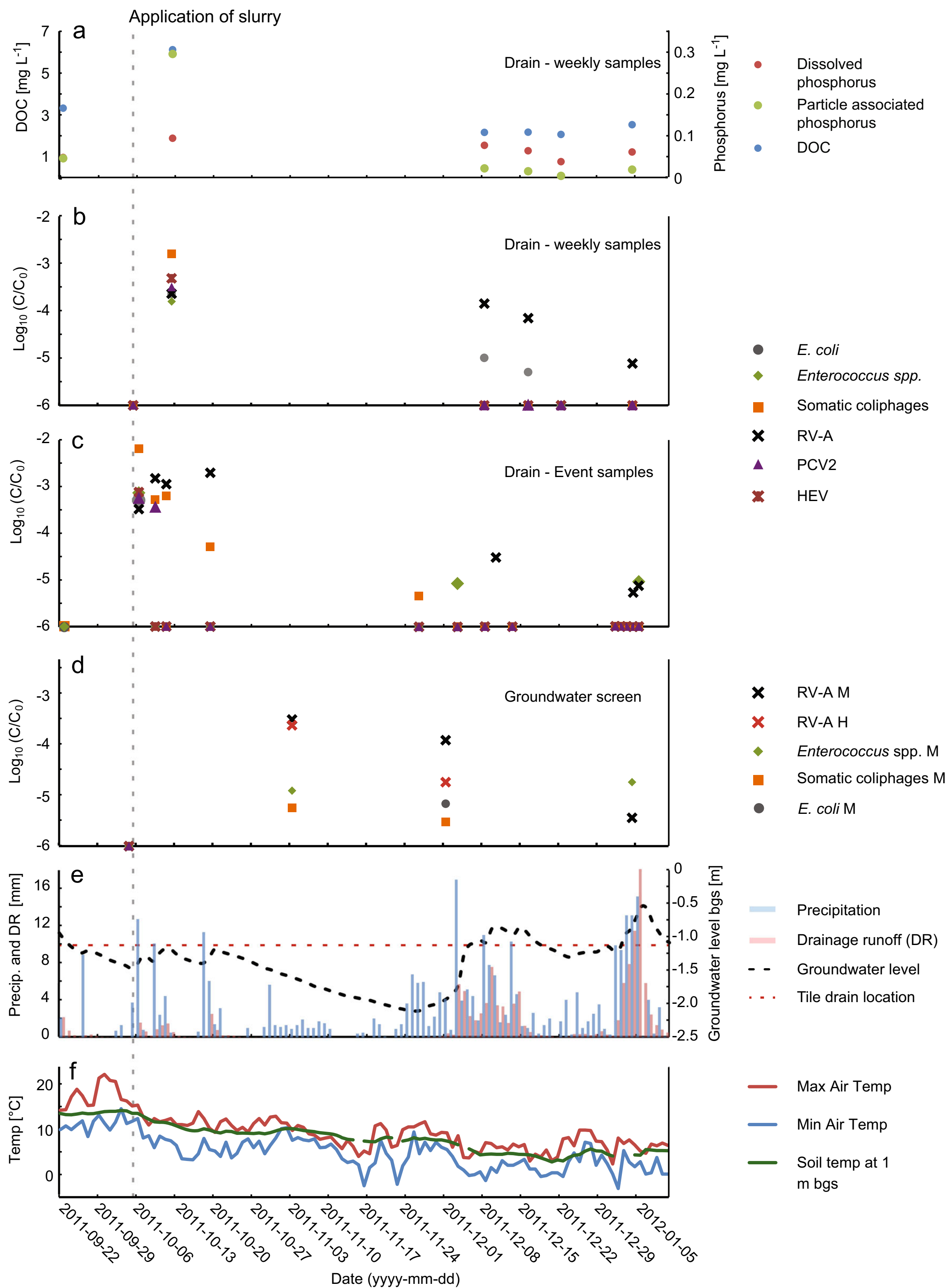

Fig. 2 Meteorological conditions and breakthrough curves for microorganisms and chemical constituents in drainage water in tile drains: **a** leaching of phosphorus and DOC; **b** breakthrough curves of the assayed microorganisms in the weekly drainage water samples; **c** the presence of microorganisms in tile drain at heavy rain events; **d** water samples collected monthly from both the vertical monitoring (M) and horizontal (H) well and microorganisms shown on the x-axis correspond to microorganisms below detection limit; **e** precipitation and drainage runoff (DR) together with the water-table depth; **f** temperature variation in air and soil. All samples below detection limit were set to −6 log units

59 days (3 December 2011) and 92 days (5 January 2012) after application of pig slurry. These event samples were both collected during heavy rainfall (16.8 and 15 mm day^{-1}, respectively) with a concentration of 0.3 CFU ml^{-1} ± 0.3 CFU ml^{-1}, essentially at the detection limit of the assay. *E. coli* and *Enterococcus* spp. was also found in the first weekly sample with a mean concentration of 13.5 CFU ml^{-1} ± 2.4 CFU ml^{-1} and 5.7 CFU ml^{-1} ± 1.6 CFU ml^{-1}, respectively (Fig. 2b). In the second and third weekly sample collected during the first and second week of December, *E. coli* was detected and the concentrations decreased to 0.6 CFU ml^{-1} ± 0.4 CFU ml^{-1} and 0.3 CFU ml^{-1} ± 0.3 CFU ml^{-1}, respectively, which is nearly a 5-log-unit reduction compared to the concentration in the pig slurry added to the soil surface 2 months earlier. *Enterococcus* spp. was only detected in the first weekly water sample while all subsequent weekly samples tested negative for *Enterococcus* spp. (Fig. 2b). The removal rates of *Enterococcus* spp. and *E. coli* were comparable at 3.1 and 3.3 log m^{-1}, respectively.

The somatic coliphages were present in high numbers in drainage water immediately after application of slurry and was detected in the first five consecutive event samples from 6 October to 26 November. The removal rate of only 2.2 log m^{-1} was calculated based on the first event sample that had the highest concentration of somatic coliphages at 1.4 × 10^{3} PFU ml^{-1} ± 6.6 × 10^{1} PFU ml^{-1}. This was the lowest removal rate of any microorganism assayed (Table 2). The somatic coliphages reached the detection limit of the assay at the fifth event sample at the end of November. Somatic coliphages had a concentration of 345 PFU ml^{-1} ± 32 PFU ml^{-1} in the first weekly sample, but was subsequently not detected in weekly samples.

To evaluate the success of virus concentration and nucleic acid extraction, the recovery efficiency of MC_0 for each individual sample was calculated. This resulted in a mean recovery of 48.3% ± 19.4 in the range 11–96%, with only one sample at each extreme. Thus, the analysis meets the criteria for successful extraction efficiency of 1% applied in the ISO/CEN method for virus detection in food and bottled water (ISO 2013).

HEV was detected only in the first event sample and correspondingly in the first weekly sample with both measurements close to the detection limit at 5 and 9 RT-PCRu ml^{-1}, respectively. The removal rate calculated based on the event sample was 3.1 log m^{-1}. Like HEV, the detection of PCV2 in water samples was low, ranging between 17 and 30 PCRu ml^{-1}. PCV2 was detected in the first and second event sample along with the first weekly sample. The removal rate was 3.3 log m^{-1} which was similar to that of HEV.

RV-A was by far the most abundant of the viruses detected in the pig slurry with 3.81 × 10^{5} RT-PCRu ml^{-1} (Table 1). RV-A was detected in the four first consecutive event samples and the concentration of RV-A increased in the drainage water over these four events (Fig. 2c). A removal rate of 2.7 log m^{-1} was calculated based on the fourth event where the highest concentration of RV-A was detected. RV-A was also detected in weekly samples collected between 12 October 2011 and 16 December 2011, but absent in the following sample collected on 22 December 2011, then reappeared in the next sample collected during very heavy rainfall in the start of January, yielding high flow in the tile drains and a significant rise in groundwater levels. The same phenomenon was observed for enterococci. The RV-A concentration of the later samples were essentially at detection limit with 2 RT-PCRu ml^{-1}. For all PCR runs, all controls met their set criteria. An attempt to sequence all three viruses were made, but the viral load of the samples were too small to allow extract of sufficient genetic material.

Table 2 Microbial removal rates λ determined from peak concentration (C_{max}) for the studied microorganisms in drain water event samples and well water samples

Microorganisms	Removal rate λ ($\log_{10}$ m^{-1})		
	Drain - event	Vertical well M5	Horizontal well H
E. coli	3.3	3.4	ND
Enterococcus spp.	3.1	3.3	ND
Somatic coliphages	2.2	3.5	ND
HEV	3.1	ND	ND
PCV2	3.3	ND	ND
RV-A	2.7	2.4	1.0

ND not detected

Well samples and groundwater

At application of slurry, the groundwater level was close to drain level for two weeks followed by a slow decrease over the next 6 weeks. Hereafter the groundwater level again fluctuated at drain level (Fig. 2e). None of the microorganisms were detected in the water samples obtained from the vertical monitoring and the horizontal wells before application of pig slurry (Fig. 2d). Very low concentrations of *E. coli* (0.4 CFU ml^{-1} ± 0.4 CFU ml^{-1}) were detected in the vertical monitoring well at the start of December (day 57) while *Enterococcus* spp. was found at the start of November (day 29) and start of January (day 91) at similar low concentrations of 0.4 CFU ml^{-1} ± 0.4 CFU ml^{-1} and 0.7 CFU ml^{-1} ± 0.5 CFU ml^{-1}, respectively, corresponding to a 5-log-unit reduction compared to the measured concentration in pig slurry (Table 1). Somatic coliphages were detected in the water sample collected in November (day 29) and December (day 57) from the vertical monitoring well, both at

low concentrations, 1.2 PFU ml^{-1} ± 0.6 PFU ml^{-1} and 0.7 PFU ml^{-1} ± 0.5 PFU ml^{-1}, corresponding to more than a 5-log-unit reduction (Fig. 2d) as compared to the pig slurry. RV-A was detected in both the vertical monitoring well and the deeper horizontal well at the first sampling on 3 November. The following month, a small decrease in the concentration of RV-A in the vertical monitoring well and an above tenfold reduction in the horizontal well was observed. In January, RV-A was no longer detected in the horizontal well and barely detected in the vertical monitoring well, balancing on the detection limit of the assay (1.3 RT-PCR u ml^{-1}). Neither HEV nor PCV2 were found in any of the wells. The removal rate of *E. coli*, *Enterococcus* spp. and RV-A in the vertical well was similar to the rate detected in the drains while somatic coliphages increased by distance. In contrast, the removal rate of RV-A had decreased in the horizontal well indicating the possibility of extended transport distance for this virus.

Microorganisms and slurry constituents

All six microorganisms were detected in water samples from the tile drains in the first event sample, and correspondingly also in the first weekly sample (Fig. 2) while different leaching profiles between the microorganisms ($P = 0.04$) were observed during the study period. The leaching profiles of HEV, PCV2, somatic coliphages and *Enterococcus* spp. were very similar ($P = 0.31$) and showed a steep decline in concentration after the first week. Similar leaching profiles were observed between *E. coli* and RV-A ($P = 0.07$) and grouping them against the other four microorganisms showed that they were significantly different ($P = 0.01$).

During the study period, pH of the individual water samples, DOC, total dissolved phosphorus and particle-associated phosphorus content was also monitored in the weekly drainage water samples (Fig. 2a). A strong correlation of all analyzed microorganisms except RV-A to particle-associated phosphorus and DOC was found (Table 3). Conversely, RV-A was the only microorganism correlating strongly to the dissolved phosphorus (Table 3). No correlation between the six microorganisms and the pH of the drainage water was observed.

The recovery of the microorganisms in the tile drains depended on the time of sampling and was associated with rain events (Table 4). The recovery was calculated based on the event sample with highest concentration of microorganism in tile drains. This was generally at 0.03–0.04% for all microorganisms except the RV-A and somatic coliphages, these had a recovery of 0.13 and 0.34%, respectively, which was also reflected by their lower removal rate. When calculating the recovery of the microorganisms by summing all the event samples, they were comparable to the recovered concentration based on the maximum concentration sample; however, this was not true for RV-A (Table 4). The recoveries calculated on the two microorganisms with high concentration, RV-A and somatic coliphages, were far greater than the microorganisms with low concentration in manure.

Discussion

Limitations of the study

Comparing different microorganisms can be challenging due to the difference in available detection methods to assay each microorganism. rt-PCR was applied for detecting the genome of the porcine viruses, whereas phages and bacteria were detected by plaque assay and colony assay, respectively. These assays are not directly comparable as the plaque and colony assay accounts for viable phages and bacteria, versus the rt-PCR assays that target the genomes of viruses and not only infectious particles. The study of enteric viruses by the use of rt-PCR have previously been performed in water samples, where infectivity correlated very well with rt-PCR detection (Borchardt et al. 2012) and a correlation between the survival of somatic coliphages and viral genome quantification has also been reported (Skraber et al. 2004).

Similar, detection of bacteria is faced by the challenge due to the differentiation of dead and live bacteria, and the differentiation of these from culturable and viable but non-culturable (VBNC) bacteria depending on the method employed. The limitation in the use of culturing and plaque assay is that not all pathogens of the same family are equally

Table 3 Correlation between the six studied microorganisms and selected chemical constituents in weekly drainage water samples. Significant values are highlighted in *italic*

	pH	DOC	Particle-associated phosphorus	Total dissolved phosphorus
HEV	0.44	*0.96*	*0.99*	0.74
PCV2	0.55	*0.96*	*0.99*	0.74
RV-A	0.46	0.69	0.79	*0.94*
E. coli	0.40	*0.95*	*0.99*	0.77
Enterococcus spp.	0.31	*0.96*	*0.99*	0.74
Somatic coliphages	0.39	*0.96*	*0.99*	0.74

Table 4 Recovery (%) of the six microorganisms in drainage water

	HEV	PCV2	RV-A	Somatic coliphages	*E. coli*	*Enterococcus* spp.
BTC peak concentration (C_{max})	0.04	0.03	0.13	0.34	0.03	0.04
All event samples aggregated	0.04	0.05	0.28	0.40	0.03	0.04
All weekly samples aggregated	0.11	0.07	0.20	0.35	0.06	0.04

BTC breakthrough curve

suitable for culturing (Vaz-Moreira et al. 2013). Bacteria entering the VBNC- stage are unable to grow on traditional culture media (Colwell 2000), but the persistence of bacteria in soil does not seem to be promoted by entering the VBNC-stage (Mascher et al. 2000). Discrepancy in the amount of viable and culturable bacteria compared to quantification of cell numbers based on DNA, which include culturable and non-culturable or dead cells, have been reported in environmental samples (Amin et al. 2013; Artz et al. 2006; Bech et al. 2010). In the current study, only viable *E. coli*, enterococci and somatic coliphages naturally occurring in the slurry and adapted to this environment were employed; furthermore, using a natural setting does not allow for perfectly timed samplings. This resulted in very low resolution of samples in this study and the scarce drainage runoff made it impossible to do additional analysis on larger volumes of water for viral analysis and also made duplicate measurements impossible without diluting samples.

Leaching of microorganisms

Colloids in the subsurface are characterized as mobile abiotic or biotic particles with a diameter less than 10 μm and that possess an electric charge on their surface (McCarthy and Zachara. 1989). Colloids include layer silicates, sesquioxides, organic macromolecules and microorganisms, e.g. bacteria and viruses (Kretzschmar et al. 1999). Breakthrough was observed for all six microorganisms into drainage water 1 day after pig slurry was applied on the field. The leaching appeared to be influenced by preferential transport as evidenced by soil hydraulic properties and fast solute transport. Measured hydraulic characteristics in A and B horizons showed a marked increase in conductivity when approaching full saturation, also indicating a high degree of preferential flow through macropores when the soil is fully saturated (data not shown, see Lindhardt et al. 2001). Similar, manure applied microorganisms were detected in the drainage system only 1 day after application. The observed transport was thus considerably faster than that occurring through the soil matrix. Piston transport through the low-permeability soil matrix (Kjær et al. 2007) would involve a travel time to the drainage system of 69 days. This finding is consistent with previous transport studies conducted with other chemical compounds (Kjær et al. 2007, Norgaard et al. 2012, Naveed et al. 2016) as well as microorganisms (Bech et al. 2014) which showed similarly high degree of preferential flow at the Silstrup field. The leaching patterns of the microorganisms were interesting in that they appeared to have different leaching profiles. Somatic coliphages, HEV, PCV2 and *Enterococcus* spp. appeared immediately in drainage water in maximum concentrations followed by a rapid decrease, while leaching of *E. coli* and RV-A was observed for an extended period. The leaching of microorganism through soil depends on the extent of their retention in the soil profile as well as their ability to survive in the soil environment (Ginn et al. 2002; Yates and Yates 1990). In structured soils, most studies of microbial transport generally show a rapid movement of bacteria and viruses due to preferential flow through macropores, e.g. naturally occurring cracks, fractures, earthworm holes, and channels formed by plant roots resulting in an early breakthrough, thereby bypassing much of the soil matrix (Abu-Ashour et al. 1994; Pang et al. 2008). Similar leaching patterns have been reported for phages and *E. coli* in field studies (Schijven et al. 1999; Vinten et al. 2002). Differences in leaching between *E. coli* and enterococci have been ascribed to a stronger attachment of *E. coli* to soil particles (Evans and Owens 1972). In the present study, the removal rate was similar for *E. coli* and enterococci; however, *E. coli* was detected for a prolonged period in the weekly samples which could indicate slower migration due to increased attachment to soil particles which was also observed for RV-A. When comparing the abundant RV-A and somatic coliphages, a clearly distinct leaching profile was seen in the weekly samples, and interestingly, an opposite leaching profile was observed in the first four event samples. RV-A concentration increased in drainage water during the first 2 weeks, whereas the concentration of somatic coliphages decreased. These very distinct leaching profiles of the two microorganisms, indicates that RV-A differs considerably with respect to the initial redistribution of the liquid phase of slurry in soil. Leaching of RV-A correlated with dissolved phosphorus indicating less interaction with the slurry and soil particles within the soil matrix. Somatic coliphages correlated with particle-associated phosphorus indicating a rapid transport through the soil in the preferential flow path which could be due to size exclusion. Studies on particle size-fractionated separated pig slurry have shown that phosphorous was present in larger proportions in particles less than 25 μm (Peters et al. 2011). Similarly, Amin et al. (2013) found evidence for an effect of

microbial cell size on the redistribution and leaching potential of slurry-borne pathogens in soil. This indicates that somatic coliphages, due to difference in size and physiochemical properties, are inadequate indicators for RV-A and probably other viruses that share this difference. These observations may only hold true under the conditions reported here, e.g. soil type, temperature and hydrological characteristics.

The high concentration of RV-A detected in the shallow screen of the vertical monitoring well and the detection of RV-A in the deeper horizontal well was unique for RV-A. None of the other microorganisms were detected in the horizontal well (3.5 m bgs). The difference in the detected levels of RV-A and somatic coliphages in the vertical monitoring well could be caused by the faster transport of somatic coliphages, i.e. the coliphages had passed the vertical monitoring well at time of sampling, whereas RV-A peaked at time of sampling. The sudden increase in removal rate for somatic coliphages in the vertical well compared to drains, as well as the decrease in removal rate for RV-A in the horizontal well compared with the drains and vertical well, could also indicate this. The lower removal rate of RV-A is in line with Pang (2009) reporting lower removal rates of enteroviruses compared to bacteriophages and other human viruses. This detection of RV-A in this deeper well is of major concern, since RV-A is a known cause of enteric disease in humans and animals, and zoonotic transmissions have been described at several occasions (Martella et al. 2010; Midgley et al. 2012). In addition, *E. coli* and *Enterococcus* spp. were also detected in the monitoring well. Shallow wells have previously been reported to be particularly susceptible to contamination of fecal bacteria from agricultural activities in clay rich sediments (Conboy and Goss 2000). The occurrence of fecal coliforms in tile drainage water in field experiments has been reported previously (Patni et al. 1984; Scott et al. 1998; Vinten et al. 2002). In the study reported here, a recovery of 0.03–0.06% was found which is in accordance with results of similar field studies (Evans and Owens 1972; Vinten et al. 2002). This is further corroborated by the *E. coli* recovery obtained in a leaching experiment performed on intact soil cores using soil of similar composition (Forslund et al. 2011b). The recoveries of HEV and PCV2 were comparable to that of *E. coli* and *Enterococcus* spp., whereas the recovery of RV-A was similar to that of somatic coliphages.

Several factors have been ascribed to enhance migration of microorganisms through soil, e.g. straining, pH, precipitation and manure constituents (Abu-Ashour et al. 1994; Bradford et al. 2002). The viruses HEV, PCV2, and RV-A have been reported to have a diameter of approximately 32, 17 and 70 nm, respectively, whereas the somatic coliphages ranges from 24 to 200 nm (Burbano-Rosero et al. 2011). Viruses with smaller size can easily move into the soil matrix by diffusion and aggregate during the initial redistribution, which protects them from subsequent preferential flow events (Yates and Yates 1990). During subsequent heavy rain events the viruses can be released and leach through the soil profile; however previous reports have indicated that straining has a larger effect on bacteria (0.5–2 μm) than on viruses, as viruses generally are smaller than the pore size of the soil; hence, straining on viruses due to size should be negligible (Lance and Gerba 1984).

The isoelectric point, the pH where the net charge of the total particle surface is zero, is another important factor that may impact leaching of microorganisms (Dowd et al. 1998; Jamieson et al. 2002; Schijven and Hassanizadeh 2000). The isoelectric point of two different strains of RV-A have been reported with different results. An isoelectric point of 8.0 was measured on the simian rotavirus SA-11 (Butler et al. 1985) and more recently, a porcine rotavirus with an isoelectric point of 4.5 was reported (Gutierrez et al. 2009). The exceptional high isoelectric point of RV-A reported by Butler et al. (1985) could explain the difference in leaching pattern of RV-A compared to other microorganisms observed in this study, as the net positive charge of RV-A at neutral pH would allow for adhesion on the negatively charged soil particles. The low isoelectric point of 4.5 is in the range of many enteric viruses and phages (Michen and Graule 2010) which at neutral pH gives a negative net charge of the particle and would result in repulsion from the negatively charged soil particles. Even though the overall charge of the particle is negative, there can still be local areas of positive charge, thus a larger particle will allow for more positively charged areas, capable of interactions with soil particles (Dowd et al. 1998). In the work by Dowd et al. (1998) it was also shown that phages less than 60 nm in diameter were dependent on isoelectric point for adhesion, whereas size became the determining factor for adhesion properties of particles larger than 60 nm in diameter. This corresponds very well with data on viruses acquired during this study, where a retention of the larger RV-A particle (70 nm) compared to the much smaller HEV (32 nm) and PCV2 (17 nm) particles is observed.

Precipitation events occurring immediately after manure application facilitated a rapid transport of microorganisms via preferential pathways. In addition, heavy rainfall occurring 3 months after application promoted the leaching of *Enterococcus* spp. and RV-A to the tile drain and monitoring well. This is corroborated by a study showing that pathogen transport through soil to drinking water wells is promoted by heavy rainfall (Lamka et al. 1980).

Manure constituents such as DOC have been shown to enhance leaching of microorganisms by competing with favorable adhesion sites on soil particles (Bradford et al. 2006; Gerba 1984; Guber et al. 2007). Royer et al. (2007) showed that surface application of manure to a field resulted in immediate leaching of DOC in tile drains indicating preferential flow. Similar increased levels of DOC after application of pig slurry were also observed in the present study. Leaching

of all microorganisms except RV-A correlated well to the leaching of DOC and particle-associated phosphorus, i.e. phosphorous that is bound to particles and also phosphorous bound in microorganisms. Phosphorus is generally considered to be well adsorbed by the soil matrix, but it has been shown that particle-associated phosphorus adsorbed to a lesser extent than dissolved phosphorus (Glæsner et al. 2011). The transport of particle-associated phosphorus would, due to the particle mediated transport, be enhanced and be restricted to larger pores and fractures in the soil profile, thus indicating preferential flow . The leaching of RV-A on the other hand had a high correlation with the dissolved phosphorus which could indicate a lower adsorption to the slurry and soil particles resulting in slower transport through soil compared to particle mediated transport.

Environmental and water safety hazards

The survival of a microorganism is an important factor for determining water contamination and the potential hazard this entails. It was possible to detect *E. coli* and somatic coliphages for at least two months, while *Enterococcus* spp. was detected through the entire study period. Cools et al. (2001) reported the survival of enterococci to be higher than that of *E. coli* in multiple soil compositions at temperatures below 15 °C and at different moisture contents, which is in contrast to Pourcher et al. (2007) that reported no difference in survival of these two fecal indicators; however, in both studies enterococci and *E. coli* survived for more than 2 months. The survival of somatic coliphages has been reported to be high with detection of infectious somatic coliphages in soil for 143 days post manure application (Gessel et al. 2004). The survival of somatic coliphages has been shown to correlate to viral genome quantification, but not to fecal indicator organisms which survived for a shorter period (Skraber et al. 2004); however, it was found that leaching of *Enterococcus* spp. exceeded that of *E. coli* and somatic coliphages which again exceeded that of HEV and PCV2. The presence of HEV in the water environment of the western world has recently been recognized (Christou and Kosmidou 2013). There have previously been reports of HEV in urban sewage samples from Europe and USA (Clemente-Casares et al. 2003) and that it can survive for at least 1 month in sewage (Pina et al. 1998). These studies and the one reported here are all based on PCR detection and does not confirm the presence of viable viruses; furthermore, HEV viruses have been shown to survive for 9 weeks at fluctuating temperature in soil and for 10 weeks at 37 °C (Parashar et al. 2011). Surveys have found HEV in shellfish, and a study in the United Kingdom showed a prevalence of 50–96% HEV RNA in shellfish from coastal areas (Crossan et al. 2012). An epidemiological study of autochthonously acquired cases of HEV was linked to consuming water from a private drinking-water well in France (Renou et al. 2008). The majority of privately owned wells reside in rural areas, and therefore in close proximity of farmland, where wells are at risk of microbial contamination from land application of fecal originated wastes (Bradbury et al. 2013; Lamka et al. 1980). Since some viruses have been shown to be transported in water reservoirs for longer than 400 m horizontally and more than 60 m vertically (Keswick and Gerba 1980), the leaching of pathogens into the groundwater, after spread of manure, could expose humans to these pathogens by migrating into wells. Combined with the high stability of some of these viruses, there is a real risk for these viruses causing clinical illness. The survival of PCV2 have proved to be high with PCV2 being one of the most stable porcine viruses even withstanding disinfectants (Kim et al. 2009) and therefore difficult to eradicate in swineherds. The possibility that PCV2 circulates in the water environment could also contribute to the risk of introducing or reintroducing PCV2 into susceptible swine herds. RV-A have previously been detected in drinking water (Gerba et al. 1996). RV-A potentially poses a large problem, primarily due to the very large amount of virus excreted from animals and humans, high stability in water and zoonotic potential (Gerba et al. 1996; Martella et al. 2010). The survival of human rotavirus in river water and tap water has been shown to be at least 60 days at 4–20 °C (Raphael et al. 1985). Similarly, the time to reduce 90% of infectious calf rotavirus in distilled water and sewage was shown to be 73 and 84 days, respectively (McDaniels et al. 1983). In this field study it was found that RV-A was detectable in groundwater and persisted there for at least 3 months following application of pig slurry and the soil temperature measured during the study period would also favor an extended persistence of the microorganisms.

Conclusion

This field study investigated the simultaneous transport of RV-A, HEV, PCV2 and somatic coliphages under natural condition along with the leaching of *E. coli* and *Enterococcus spp.* through soil. The results showed immediate leaching of all microorganisms to the tile drain. *E. coli* and RV-A had similar leaching pattern but different from somatic coliphages, PCV2, HEV and *Enterococcus* spp. The data generated in the present study suggest that somatic coliphages can be used to model transport of some viruses in soil, e.g. HEV and PCV2 but does not necessarily cover all viruses. This paper reports different leaching patterns of RV-A. The exact reason for this is not clear based on the data, but size exclusion or difference in physiochemical properties are likely to play a key role here. Furthermore, only RV-A was detected in the horizontal well corresponding to leaching into the shallow groundwater, making RV-A a possible risk due to its high mobility, stable nature, high copy number and high prevalence in pig and cattle herds,

suggesting that this virus should be monitored more closely considering its zoonotic potential. As drainage water is led into surface water reservoirs this could pose a health risk to humans and animals exposed to these water resources. The authors therefore recommend future work on assessment of human health risk related to contamination of surface water reservoirs from manureborne microorganisms leaching to tile-drains.

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