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Detection of hepatitis E virus and other livestock-related pathogens in Iowa streams



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HIGHLIGHTS

- Assessment of manure application as a source of pathogens to the environment.
- Overland runoff and tile drainage facilitates pathogen transport to surface waters.
- Detected hepatitis E virus in surface water following manure application.
- Increased pathogen gene detections and indicator bacteria concentrations post-manure.
- Manure application can potentially impair water quality and environmental health.

GRAPHICAL ABSTRACT



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ABSTRACT

Manure application is a source of pathogens to the environment. Through overland runoff and tile drainage, zoonotic pathogens can contaminate surface water and streambed sediment and could affect both wildlife and human health. This study examined the environmental occurrence of gene markers for livestock-related bacterial, protozoan, and viral pathogens and antibiotic resistance in surface waters within the South Fork Iowa River basin before and after periods of swine manure application on agricultural land. Increased concentrations of indicator bacteria after manure application exceeding Iowa's state bacteria water quality standards suggest that swine manure contributes to diminished water quality and may pose a risk to human health. Additionally, the occurrence of HEV and numerous bacterial pathogen genes for *Escherichia coli*, *Enterococcus* spp., *Salmonella* sp., and *Staphylococcus aureus* in both manure samples and in corresponding surface water following periods of manure application suggests a potential role for swine in the spreading of zoonotic pathogens to the surrounding environment. During this study, several zoonotic pathogens were detected including Shiga-toxin producing *E. coli*, *Campylobacter jejuni*, pathogenic enterococci, and *S. aureus*; all of which can pose mild to serious health risks to swine, humans, and other wildlife. This research provides the foundational understanding required for future assessment of the risk to environmental health from livestock-related zoonotic pathogen exposures in this

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region. This information could also be important for maintaining swine herd biosecurity and protecting the health of wildlife near swine facilities.

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1. Introduction

Runoff from land application of animal manure is one possible pathway of transport of bacterial, fungal, protozoan, and viral pathogens to surface waters (Khaleel et al., 1980; Vanotti et al., 2007). Animal manure harbors not only animal-specific pathogens (e.g. bovine viral diarrhoea virus), but also zoonotic pathogens (e.g. hepatitis E virus, *Campylobacter jejuni*) capable of infecting humans (Gordoncillo et al., 2013; Haack et al., 2015; United States Environmental Protection Agency, 2013; Ziemer et al., 2010). Many of these pathogens are able to survive, persist, and move through the agricultural landscape. Agricultural runoff whether via overland flow (Davies et al., 2004) or through the outflow of tile-drain systems (Jamieson et al., 2002; Joy et al., 1998; Wilkes et al., 2014) facilitates pathogen transport and can consequently contaminate and significantly impair the water quality of adjacent surface waters (Crane et al., 1983; Meinhardt et al., 1996). Pathogens may not only be transported via stream water, but also subsequently may be adsorbed into streambed sediment and later resuspended when the sediment becomes disturbed with animal movement or increased flow via storm events (Goss and Richards, 2008).

Surface waters and bed sediment can be reservoirs of pathogens that affect both wildlife and human health. In an animal agriculture-dominated region in south Alberta, Canada, researchers found a higher prevalence of zoonotic pathogens in surface waters used for drinking, irrigation, and recreational purposes and also a higher incidence of gastrointestinal illness linked to *Escherichia coli* O157:H7 and *Salmonella* than in neighboring non-agricultural regions, suggesting a link between animal agriculture, contaminated surface waters, and human health (Jokinen et al., 2012). Current swine manure management practices utilize injection or tillage to incorporate manure to potentially reduce risks of nutrients or pathogens being directly transported to surface water in runoff after rainfall (Dell et al., 2011; Sterk et al., 2013). Still, this case study along with similar research suggests that both animal and human health are potentially at risk from ongoing manure management practices and subsequent surface water contamination (Gordoncillo et al., 2013; Haack et al., 2016; Haack et al., 2015; United States Environmental Protection Agency, 2013; Ziemer et al., 2010). For example, genes indicative of potentially zoonotic Shiga toxin-producing *E. coli*, enterohemorrhagic *E. coli*, and the *Enterococcus* esp. gene, which is more frequently expressed in isolates from human patients with bacteremia and urinary tract infections (Shankar et al., 1999), were found in surface waters and sediment up to 25 days after a swine manure spill (Haack et al., 2015).

Of additional concern when examining the role of manure in regard to surface water quality and spread of zoonotic pathogens is understanding the potential risk associated with understudied and emerging zoonotic pathogens, such as hepatitis E virus (HEV). Human HEV infections can be serious with a 1% mortality rate and increased mortality rate of 27% among pregnant women (Chau et al., 2006; Kumar et al., 2004). Several HEV outbreaks in developing countries have been linked to waste-contaminated drinking water (Krawczynski, 1993; Satou and Nishiura, 2007) or contaminated food sources (Satou and Nishiura, 2007). Such infections, however, are rare in developed countries (Christou and Kosmidou, 2013; Kase et al., 2009). Among the four HEV genotypes, only genotype 3 has been reported in the United States and has been found in swine and other domestic animals and also in wild rodents, deer, and wild boar (Christou and Kosmidou, 2013; Satou and Nishiura, 2007; Takahashi et al., 2004; Tei et al., 2003; Yugo and Meng, 2013).

Swine are clinically unaffected carriers of HEV and this virus appears to be prevalent among U.S. swine farms and has been detected in swine and in swine manure and waste lagoons (Huang et al., 2002; Kase et al., 2009; Kasorndorkbua et al., 2005). A previous swine study from Iowa documented HEV genotype 3 in approximately 68% of storage pits and 38% of waste lagoons (Kasorndorkbua et al., 2005). This particular genotype is linked to human HEV infections in developed countries (Christou and Kosmidou, 2013; Meng, 2003) and is of particular concern because researchers have found almost genetically indistinguishable swine and human strains of HEV (Kase et al., 2009; Meng et al., 1997). A study examining the prevalence of HEV among midwestern United States swine herds noted that there was sequence homology between swine HEV and clinical isolates from human HEV cases (Meng et al., 1997) suggesting a potential role for swine in human transmission of this virus. In addition, cross-species infection (e.g. swine to human, deer to human, swine to monkey) indicates that animal strains of HEV not only pose a zoonotic risk to humans but also a health risk to other wildlife (Christou and Kosmidou, 2013; Meng et al., 1998; Meng et al., 1997; Yugo and Meng, 2013).

While HEV has been detected in both swine feces and in stored liquid waste that is often applied to agricultural lands (Gentry-Shields et al., 2015; Kase et al., 2009), few studies have been conducted to date to ascertain the environmental occurrence of swine HEV (Gentry-Shields et al., 2015; Kasorndorkbua et al., 2005). Kasorndorkbua et al. (2005) did not detect HEV in 28 surface water samples taken near swine farms during late summer and autumn. The objective of this study was to determine the presence of HEV and other livestock-related bacterial, protozoan, and viral pathogens (Supplemental Tables 1 and 2) in relation to periods of swine manure application in a basin (South Fork Iowa River) with extensive swine production (around 840,000 hogs). This research will provide the foundational understanding required for any future assessment of the risk to environmental health from HEV and other animal agricultural-related pathogen exposures in this region. Such risk could include spread from swine to human, spread from swine to other wildlife (e.g. deer and rodents), or spread among swine farms. This information could be important for maintaining swine herd biosecurity and protecting the health of wildlife near swine facilities.

2. Materials and methods

2.1. Sample sites and sample collection

To determine the environmental prevalence of HEV and other microbiological contaminants, 22 water samples from six stream sites in central Iowa were collected (Fig. 1, Table 1). The sampling network included five stream sites located in Beaver Creek, Tipton Creek, and the South Fork Iowa River within the South Fork Iowa River basin and one out-of-basin control site in the Walnut Creek basin where no swine were raised. The South Fork Iowa River basin was selected because: (1) it is an area of intense swine production (roughly 840,000 hogs in the 78,000 ha watershed), (2) about 85% of the basin is in row-crop agriculture and 30 to 60% of the basin receives 93 to 186 m³ ha⁻¹ of swine manure annually, (3) the basin has an extensive network of subsurface drainage (tiles) to artificially enhance water drainage providing a rapid transport mechanism for contaminants from the land surface to corresponding streams, and (4) a network of streamflow gages were available throughout the basin to provide important ancillary data on streamflow (Tomer et al., 2008). The sampling network in the South

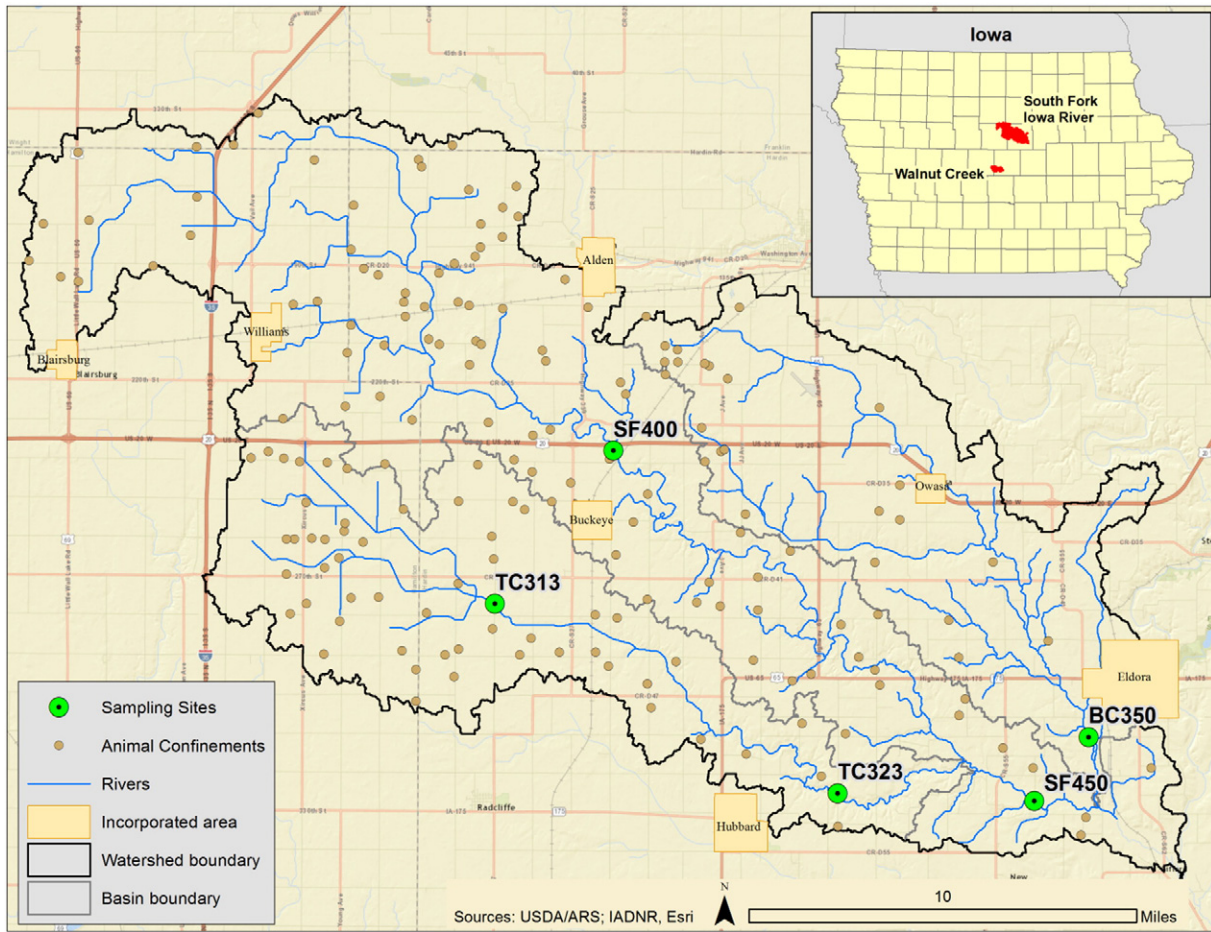


Fig. 1. Diagram of South Fork Iowa River basin sites. IASF400 (SF400)-South Fork River near Buckeye, Iowa; (TC313)- Tipton Creek near D Avenue in Buckeye, Iowa; (TC323) -Tipton Creek East near Hubbard, Iowa; (SF450)-South Fork Iowa River NE near New Providence, Iowa; (BC350)- Beaver Creek near 250th Street in Eldora, Iowa. The subset shows the location of the South Fork Iowa River basin (top) in reference to the Walnut Creek control (bottom) basin where no swine were raised. Swine confinement operations as of February 2014.

Table 1
Station IDs, station locations, and environmental parameters at the time of sampling.

| Sampling date | USGS station ID | Station name | Water temperature (°C) | Discharge m ³ s ⁻¹ | Dissolved oxygen (mg L ⁻¹) | pH water | Specific conductance (µS cm ⁻¹) |
|---------------|-----------------|--------------|------------------------|--|--|----------|---|
| 8/09/2011 | 5451110 | SF400 | 27 | 0.096 | 8.8 | 7.9 | 607 |
| | 5451140 | TC313 | 26.6 | 0.057 | 13.4 | 8 | 677 |
| | 5451148 | TC323 | 22.4 | 0.184 | 8.5 | 7.9 | 563 |
| | 5451210 | SF450 | 22.4 | 0.623 | 7.9 | 8 | 539 |
| | 5451260 | BC350 | 23.8 | 0.224 | 11.2 | 7.8 | 546 |
| 8/10/2011 | 5471009 | WC | 15.8 | 0.003 | 8.4 | 7.9 | 801 |
| 11/03/2011 | 5451110 | SF400 | 6.3 | 0.113 | 7.6 | 7.4 | 580 |
| | 5451140 | TC313 | 7.9 | 0.014 | 15.1 | 7.8 | 661 |
| | 5451148 | TC323 | 6.2 | 0.108 | 13.1 | 7.8 | 580 |
| | 5451210 | SF450 | 5.6 | 0.190 | 10.6 | 7.3 | 547 |
| | 5451260 | BC350 | 5 | 0.099 | 10.4 | 6.7 | 510 |
| 3/07/2012 | 5451110 | SF400 | 2.6 | 0.663 | 13 | 7.7 | 582 |
| | 5451140 | TC313 | 10.2 | 0.283 | 14 | 7.8 | 615 |
| | 5451148 | TC323 | 7.6 | 0.496 | 13.2 | 7.9 | 648 |
| | 5451210 | SF450 | 6.6 | 1.133 | 11.5 | 7.6 | 581 |
| | 5451260 | BC350 | 7.9 | 0.357 | 12.8 | 7.8 | 587 |
| 3/08/2012 | 5471009 | WC | 5.3 | 0.011 | 14.8 | 8 | 730 |
| 4/15/2012 | 5451110 | SF400 | 17.2 | 0.906 | 9.9 | 8 | 719 |
| | 5451140 | TC313 | 16.6 | 0.736 | 12.6 | 8 | 683 |
| | 5451148 | TC323 | 15.3 | 1.218 | 10.6 | 8 | 609 |
| | 5451210 | SF450 | 13.7 | 2.633 | 8.7 | 7.6 | 532 |
| | 5451260 | BC350 | 13.5 | 1.586 | 9.2 | 7.7 | 604 |

SF400-South Fork River near Buckeye, Iowa; TC313- Tipton Creek near D Avenue in Buckeye, Iowa; TC323-Tipton Creek East in Hubbard, Iowa; SF450-South Fork Iowa River NE near New Providence, Iowa; BC350- Beaver Creek near 250th Street in Eldora, Iowa; and WC- Walnut Creek near 510th Avenue in Ames, Iowa. Water samples were not collected at Walnut Creek for the November 2011 sampling because of a dry streambed or for the April 2012 sampling due to sampling logistics.

Fork Iowa River basin consisted of three tributary and two main-stem sampling sites (Fig. 1). Walnut Creek near Ames, Iowa, was selected as a nearby (within 80 km) out-of-basin control as this basin has similar soils and land use (e.g. tile-drained row crops) but no livestock production and no known swine manure application (Tomer et al., 2003).

Water-quality field measurements were made by use of standard USGS methods (Gibs et al., 2007) (Table 1). Daily mean streamflow was calculated at the sampling sites over the course of the study using methodology described by Tomer et al. (2008). Hydrographs were created for the sampling sites for the date of sampling and seven preceding days (Supplemental Fig. 1).

Stream water samples were collected in the South Fork Iowa River basin at four time points: before fall manure application (August 9, 2011), after fall manure application (November 3, 2011), before spring manure application (March 7, 2012), and after spring manure application (April 15, 2012). Sampling dates were chosen to capture the first precipitation event causing a rise in the hydrograph after a majority of the manure had been applied to the basin. Water samples were collected from the control site at Walnut Creek on August 10, 2011, and March 8, 2012. Samples were not collected at Walnut Creek for the November 2011 sampling because of a dry streambed or for the April 2012 sampling due to sampling logistics. Water grab samples ($n = 22$) for enrichment PCR analyses for 19 bacterial antibiotic resistance and pathogen gene markers (bacterial gene markers, BGMs) were collected from the centroid of flow. Water samples ($n = 22$) for qPCR analyses for HEV and other bacterial, protozoan, and viral targets were collected using a portable pumping system connected to a glass wool filtration with a pre-filter for concentration of bacteria and viruses (Lambertini et al., 2008; Millen et al., 2012). The mean sample volume filtered was 337 L and ranged from 201 to 837 L. A negative control of the glass wool filtration equipment consisted of sterile phosphate buffered saline ($1 \times$ PBS) and was performed in the field during every sampling period (four total) and all were negative for all qPCR targets. Bed sediment samples were collected at the same location as the water samples during each sampling period and consisted of the top 2–3 cm of bed material composted from multiple points in the depositional zones (i.e. areas where the bed consisted of finer grained materials). Bed sediment samples were analyzed for indicator bacteria and presence of bacterial pathogen genes. Additionally, hog slurry (manure) samples ($n = 2$; 1-L in sterile amber glass) were collected from two swine producers in South Fork basin during active 2011 fall manure application. While all sites were visited, fecal samples from wild deer ($n = 3$) were only found near three sampling sites in March 2012. Deer feces were collected in the riparian zone of the stream adjacent to water sampling locations. Fecal droppings were composited by sampling site in a 1-L sterile amber glass jar and homogenized, and then sub-sampled into three sterile specimen cups. All samples were placed on ice immediately and shipped overnight to Michigan Bacteriological Research Laboratory (MI-BaRL; USGS Michigan Water Science Center, Lansing, MI) for enumeration and enrichment PCR analysis and to the Agricultural Research Service (Marshfield, WI) for qPCR analysis.

2.2. Enumeration of indicator bacteria

Water (50 mL, 10 mL, 3 mL, and 1 mL), sediment and manure (1 and 3 mL of a 1:10, 1:100, and 1:1000 dilution) samples were plated on mFC (fecal coliforms), modified mTEC (*E. coli*), mEI (enterococci), and Baird Parker (*Staphylococcus* spp.) for enumeration. Water samples (50 mL) were filtered in the laboratory using sterilized equipment and standard methods for membrane filtration (American Public Health Association, 1998) and subsequent enrichment. Laboratory blanks consisting of sterile PBS ($1 \times$) were processed with all environmental samples and were absent of contamination.

2.3. Enrichment PCR to detect bacterial gene markers

To determine if selected bacterial gene markers (BGMs) were in each sample, multiple types of growth enrichment were conducted. After indicator bacteria counts were recorded from membrane filtration enumeration, all growth (target and non-target) from the 50-mL filter (or maximum volume) of each enumeration was subsequently used to detect BGMs from target genera which grew upon that media (Supplemental Table 3). Additionally, enrichments were prepared for *Campylobacter* and *Salmonella* with a filter through which 50 mL of sample water had been passed, or directly with 1 g sediment or solid waste, or 1 mL liquid waste.

Filters containing colony growth (target and non-target) from mFC (*Shigella* enrichment), modified mTEC (*E. coli* enrichment), mEI (enterococci enrichment), and Baird Parker (*Staphylococcus* enrichment) media were aseptically transferred into sterile PBS, agitated for 15 min, and then centrifuged at 3400 rpm for 20 min at 4 °C to form a pellet. For Bolton broth (*Campylobacter* enrichment) and Rappaport Vassiliadis R10 broth (*Salmonella* enrichment), membrane filters were aseptically removed from broth cultures and the remaining enrichment was centrifuged as above to form a pellet. For all samples, the supernatant was decanted and the pellet was resuspended in 1 mL of 20% glycerol/0.5 \times PBS. The resulting glycerol stocks of all enrichments were stored at -70 °C for further analysis.

To determine if BGMs were detected in target and non-target growth from each medium's enrichment (including enumeration media), glycerol stocks were thawed and homogenized and 100 μ L (Gram negative cultures) or 400 μ L (Gram positive cultures) was used for DNA extraction (Qiagen DNeasy DNA extraction kit; Qiagen, Valencia, CA). Extracted DNA was stored at -20 °C until PCR analysis was performed.

PCR was used to detect the presence of 19 BGMs from target populations from enrichment media (Supplemental Table 1) using methods referenced in Supplemental Table 1 and elsewhere (Haack et al., 2013). Standard quality control checks of PCR reactions included running a positive and negative PCR control with every 20 reactions. Blanks of DNA extraction methods were also performed for every group of extractions processed. Throughout the study at least one sample that was previously determined to produce a negative PCR result for each virulence gene was spiked with positive-control DNA and repeated to confirm a positive result, and the absence of PCR inhibition (36, 37). All laboratory QC samples produced expected results throughout the study (data not shown).

2.4. Microbiological analyses – qPCR

Using the methods described in Millen et al. (2012), glass wool filters and pre-filters were eluted and the eluent further concentrated by polyethylene glycol. The two final concentrated sample volumes (FCSV) for each pair of glass wool filter and pre-filter composing a sample were combined (mean = 5.9 mL, range 2.4–11.0 mL, $n = 22$) and stored at -80 °C until analysis.

An FCSV aliquot of 280 μ L was extracted for nucleic acids by first using the freeze-thaw extraction procedure for *Cryptosporidium* oocyst DNA (Di Giovanni and LeChevallier, 2005) followed by extraction with QIAamp DNA blood mini kit and buffer AVL (Qiagen, Valencia, CA) (Borchardt et al., 2012). The extraction final volume was 150 μ L. Every sample was checked for qPCR inhibition and, if necessary, inhibition was mitigated following the methods described by Gibson et al. (2012).

qPCR was conducted for genes specific for the following pathogens: HEV, Influenza A virus, six human viruses (adenovirus, enterovirus, GI norovirus, GII norovirus, hepatitis A virus (HAV), rotavirus), eight bovine viruses (bovine viral diarrhea virus Types 1 and 2 (BVDV1 and BVDV2), group A rotavirus, group C rotavirus, enterovirus, coronavirus, adenovirus, polyomavirus), four bacteria (*C. jejuni*, *Salmonella* spp., enterohemorrhagic *E. coli*, *Mycobacterium avium*

subsp. *paratuberculosis*, and two protozoa (*Giardia lamblia* and *Cryptosporidium parvum*). qPCR standard curve parameters and references for the primers and hydrolysis probes are reported in Supplemental Table 2. qPCR and reverse-transcription (RT) procedures were the same as those described in Borchardt et al. (2012) with one update; the reverse transcriptase was SuperScript® III (Life Technologies, Grand Island, NY). All qPCR assays included no-template controls for extraction, RT, and PCR steps and throughout the study these controls were negative (i.e., no fluorescence). Sources of the positive controls and their enumeration for creating standard curves are described in the Supplemental material.

HEV amplicons from qPCR-positive samples were sequenced to confirm identity. Amplified DNA was visualized by gel electrophoresis and the band size corresponding to HEV was excised from the agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified DNA was cloned with pGEM-T Easy Vector System (Promega, Madison, WI) and sequencing reactions of the cloned plasmids were conducted in both directions with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Products were submitted for sequencing to the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility (Madison, WI). Consensus sequences were constructed and aligned with Lasergene (DNASTAR, Madison, WI). Sequence identifications were confirmed using BLAST (National Center for Biotechnology Information, Bethesda, MD).

2.5. Statistical analyses

All statistical analyses were conducted in R statistical software using the vegan package (Oksanen et al., 2015; R Core Team, 2015). Mann Whitney U tests were used to determine if there was a statistically significant difference between indicator bacteria (fecal coliforms, *E. coli*, enterococci, and *Staphylococcus* spp.) between the Walnut Creek (control site) and the South Fork Iowa River sites. Mann Whitney U tests were also performed to determine the statistical significance between HEV concentration pre- and post- manure application and between main-stem and tributary sites. Paired Wilcoxon signed-rank tests were performed to assess whether there was a significant difference between fecal coliforms, *E. coli*, enterococci, and *Staphylococcus* spp. concentrations between pre-manure samplings; between post-manure samplings; and between pre- and post-manure applications. Fisher's exact test was performed to determine whether there was a significant difference in the number (presence/absence) of pathogen gene of BGMs detected pre- and post- manure application. Spearman's rank correlation coefficient (ρ) was used to measure the statistical correlation between environmental variables and indicator bacteria concentration, number of BGMs detected, and HEV, *C. jejuni*, and bovine polyomavirus concentrations. Relations and correlations were considered significant when $p < 0.05$. Box plots were constructed in R using the reshape package (Wickham, 2007) and the ggplot2 (Wickham, 2009) and cowplot (Wilke, 2015) graphics packages.

3. Results

3.1. Detection of bacterial gene markers in fecal sources

Ten of the 19 BGMs (53%) were detected in at least one of the two swine manure samples that were collected from farms within the South Fork Iowa River basin (Supplemental Table 4). In addition to the *eaeA* (*E. coli* intimin marker), *ddl faecium* (*E. faecium* marker), and *ddl faecalis* (*E. faecalis* marker) genes, the *Enterococcus* esp. (human marker) and *hirae* (bovine marker) and *E. coli* STII (swine marker) were detected in both (100%) swine manure samples. All BGMs detected in swine manure samples were subsequently detected in either water and/or sediment samples from subsequent samplings within the South Fork Iowa River basin. Both swine manure samples were positive for HEV RNA with high concentrations of 3.5×10^7 and 2.8×10^6 genomic copies L^{-1}

(Supplemental Table 5). One of the manure samples was also positive for Influenza A at a concentration of 3.4×10^3 genomic copies L^{-1} .

Few BGMs ($n = 5$) were detected in the three fecal deer pellet samples except for *Salmonella invA* which was detected in all (100%) of the samples (Supplemental Table 4). All of the BGMs present in deer fecal samples were also detected in at least one of the water and/or sediment samples collected within the South Fork Iowa River basin. The deer fecal samples were also positive for HEV RNA at concentrations of 7.0×10^4 genomic copies g^{-1} (Supplemental Table 5). Concentrations of HEV within manure and deer fecal samples were on average 4–5 logs greater than concentrations found in surface waters of the South Fork Iowa River basin.

3.2. Temporal variation in indicator bacteria concentrations

Fecal coliforms, *E. coli*, enterococci, and *Staphylococcus* spp. were enumerated in water and sediment samples at all sites pre-and post-manure application (Fig. 2, Supplemental Table 6). There was no statistically significant difference between the concentrations of these indicator bacteria in water or sediment between both the pre-manure samplings (August 2011 and March 2012) or between both the post-manure samplings (November 2011 and April 2012). Conversely, mean concentrations of fecal coliforms, *E. coli*, enterococci, and *Staphylococcus* spp. in all water samples from the South Fork Iowa River basin sites were significantly different between pre- and post- manure application ($p = 0.013$, $p = 0.013$, $p = 0.009$, and $p = 0.006$ respectively). In water, the largest concentrations of fecal coliforms, *E. coli*, and *Staphylococcus* were found in April post-manure application. In sediment, indicator bacteria concentrations were not significantly different between the two pre-manure samplings, between the two post-manure samplings, or between the pre-and post-manure application.

Considering the State of Iowa's water quality standard for *E. coli* of 2.35×10^2 CFU $100 mL^{-1}$ for Class A1 (primary contact) waters and 2.88×10^3 CFU $100 mL^{-1}$ for Class A2 (secondary contact) waters (Iowa Department of Natural Resources, 2015a), 80% of South Fork Iowa River basin samples ($n = 8$) from the August 2011 (pre) and November 2011 (post) and 100% of samples ($n = 5$) from the April 2012 (post) sampling exceeded the *E. coli* standard level for Class A1 waters (Fig. 2). Additionally, 80% of the April 2012 (post) water samples ($n = 4$) exceeded the bacteria standard level for Class A2 waters. Samples ($n = 2$) collected at the control site at Walnut Creek exceeded the Class A1 *E. coli* standard level for both the August 2011 and March 2012 (pre-manure application) water samples (Supplemental Table 6). Beaver Creek, Tipton Creek, Walnut Creek, and the South Fork Iowa River all include regions that are classified as Class A1 and Class A2 waters (Iowa Department of Natural Resources, 2015b).

Concentrations of indicator bacteria were similar ($\mu = 0.54$, $\sigma = 0.49$ log difference) between the Walnut Creek (control) and South Fork Iowa River basin sites (Supplemental Table 6). The only significant difference between these two basins was observed for enterococci concentrations with the control site having statistically higher enterococci levels ($p = 0.04$). Indicator bacteria concentrations were only measured at Walnut Creek pre-manure application (August 2011 and March 2012) because of the lack of flow during November 2011 and sampling logistics during April 2012.

3.3. Temporal and spatial variation in pathogen detection

The human viruses enterovirus, GII norovirus, HAV, and rotavirus; the bovine viruses BVDV1, BVDV2, group A rotavirus, group C rotavirus, enterovirus, and coronavirus; *M. avium*; the protozoan *G. lamblia*, and the BGM for the enterotoxigenic *E. coli* ST (human marker) were not detected in any water or sediment samples. Additionally the *vanB* gene (antibiotic resistance marker) and GI norovirus were both detected at the control site, but not within the South Fork Iowa River basin (Supplemental Tables 4 and 5).

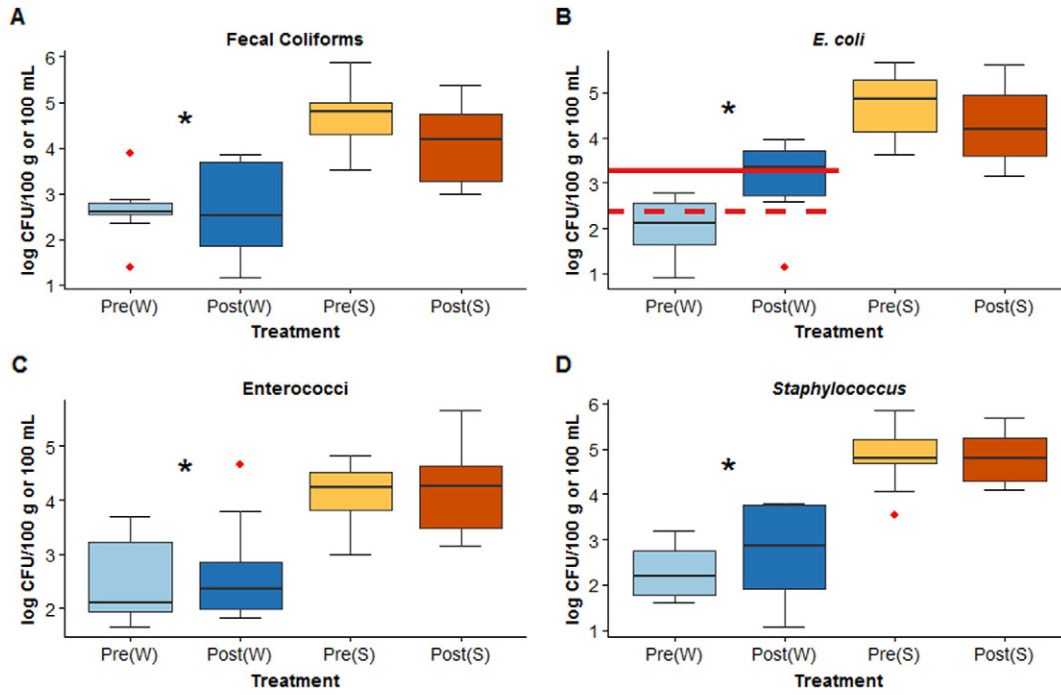


Fig. 2. Box plot of fecal coliform (A), *E. coli* (B), enterococci (C), and *Staphylococcus* (D) concentration in water (log CFU 100 mL⁻¹) and sediment (log CFU 100 g⁻¹) from the five sites in the South Fork Iowa River basin. Blue boxes denote pre-manure (August 2011 and March 2012; n = 10) and post manure (November 2011 and April 2012; n = 10) concentrations in water. Brown boxes denote pre-manure (August 2011 and March 2012; n = 10) and post manure (November 2011 and April 2012; n = 10) concentrations in sediment. For *E. coli* (B), the dashed red line indicates the State of Iowa *E. coli* water quality standard of 2.35×10^2 CFU 100 mL⁻¹ in Class A1 (primary contact) waters. The solid red line indicates the *E. coli* water quality standard of 2.88×10^3 CFU 100 mL⁻¹ for Class A2 (secondary contact) waters. The lower and upper borders of the box correspond to first and third quartiles (or 25th and 75th percentiles). The whiskers extend to 1.5× inter-quartile range. Data outside of the whiskers are plotted as outliers and plotted as red points for each box plot. Asterisks indicate statistical significance (p < 0.05; paired Wilcoxon rank-sum test) between the two paired groups (pre-and post-manure).

All other BGMs were detected in the main-stem or tributaries of the South Fork Iowa River (Supplemental Table 4). The most frequently detected BGMs in water samples (n = 20) were *E. coli eaeA* (90%), *E. faecium ddl* (90%), *E. faecalis ddl* (65%), and *E. coli stx1* (55%) (Table 2). It is important to note that the *E. coli eaeA* BGM (attachment marker) was detected in both (pre) samples and the *E. faecium ddl* and *E. faecalis ddl* BGM (species markers) were detected during the March (pre) sampling from the control basin indicating a likely background population of these markers (Supplemental Table 4). The *E. coli* STII BGM (swine marker) was not detected in any of the water samples, but was found in a single sediment sample from one of the tributaries during post-manure fall (November 2011) application. Additionally, *C. jejuni/coli* 16SrRNA, *E. hirae bov*, *E. coli* STII, *E. coli* stx2e, and *Staphylococcus aureus mecA* BGMs were only detected in water and sediment samples from the South Fork Iowa River basin post manure application (Supplemental Table 4). There was an overall significant (p = 0.005) difference in the

number of BGMs detected between the pre-manure application (52 BGMs) compared to the post-manure application (90 BGMs) samples. This difference was most notable between the March 2012 pre application and the April 2012 post application samplings (p = 0.01) where a 12% increase (additional 22 BGMs) in the number of BGMs detected was observed. Additionally, there was a significantly (p = 0.03) higher proportion of BGMs detected when water samples exceeded the Class A2 bacteria standard level of 2.88×10^3 *E. coli* CFU 100 mL⁻¹, when compared to samples that were below this standard.

By qPCR, HEV (45%, n = 20), *C. jejuni* (35%, n = 20), and bovine polyomavirus (25%, n = 20) were the most frequently detected pathogens in the South Fork Iowa River basin (Table 3, Supplemental Table 5). While *C. jejuni* and bovine polyomavirus were detected more frequently in the post-manure application samples compared to the pre-manure application samples, this trend was not statistically significant.

Table 2

Occurrence frequencies (%) of bacterial pathogen genes detected in surface waters by P/A PCR in the South Fork River Basin. Three pathogen genes (*vanB*, STII, STH) were not detected in any water samples at any of South Fork River Basin sites. The pathogen gene *vanB* was detected in both water and sediment samples at the WC site during August 2011. The STII gene was detected once in sediment at the TC313 site during November 2011 after fall manure application. Dashes indicate non-detect.

| Sampling date | <i>C. jejuni</i> or <i>coli</i> | | <i>Enterococcus</i> | | | | <i>E. coli</i> | | | | | <i>Salmonella</i> | | <i>S. aureus</i> | | | | |
|-----------------------------------|---------------------------------|----|---------------------|-------------|-------------------------------|------------------------------|---------------------|-----|---------------------|--------------------|-------------|-------------------|--------------|------------------|-------------|-------------|-------------|-------------|
| | 16S | | <i>esp</i> human | <i>vanA</i> | <i>ddl</i> <i>faecalis</i> | <i>ddl</i> <i>faecium</i> | <i>hirae</i> bov | 16S | <i>LT11a</i> bov | <i>rfb</i> O157 | <i>stx1</i> | <i>stx2</i> | <i>stx2e</i> | <i>eaeA</i> | <i>invA</i> | <i>spvC</i> | <i>femA</i> | <i>mecA</i> |
| 8/09/2011 (pre manure) n = 5 | - | - | - | - | 20 | 80 | - | 100 | 20 | 20 | 40 | 20 | - | 100 | 20 | 20 | 40 | - |
| 11/03/2011 (post manure) n = 5 | 40 | 20 | 20 | - | 60 | 100 | 60 | 100 | 20 | - | - | 20 | 80 | 20 | - | - | - | 20 |
| 3/07/2012 (pre manure) n = 5 | - | - | - | 20 | 100 | 100 | - | 100 | - | 20 | 80 | - | 80 | 40 | - | - | - | - |
| 4/15/2012 (post manure) n = 5 | 40 | - | - | 20 | 80 | 80 | 80 | 100 | - | 40 | 100 | 60 | 100 | - | - | - | 60 | 60 |
| All dates (n = 20) | 20 | 5 | 5 | 10 | 65 | 90 | 35 | 100 | 10 | 20 | 55 | 20 | 5 | 90 | 20 | 5 | 25 | 20 |

Table 5

Spearman rank correlation coefficient (ρ) between indicator bacteria (fecal coliforms, *E. coli*, enterococci, and *Staphylococcus* spp.), array of bacterial gene markers (BGMs), hepatitis E virus (HEV), and *Campylobacter jejuni*, bovine polyomavirus with measured environmental parameters for South Fork Iowa River basin sites.

Bold values indicate a statistically significant relation where $p < 0.05$.

| Target | n | Daily mean flow | Water temperature | Discharge | Dissolved oxygen | pH | Specific conductance |
|-----------------------|----|-----------------|-------------------|-------------|------------------|--------------|----------------------|
| Fecal coliforms | 20 | 0.14 | 0.11 | 0.09 | −0.39 | −0.18 | −0.23 |
| <i>E. coli</i> | 20 | 0.16 | 0.18 | 0.14 | −0.47 | −0.10 | −0.16 |
| Enterococci | 20 | 0.06 | −0.55 | 0.14 | 0.02 | −0.50 | −0.16 |
| <i>Staphylococcus</i> | 20 | 0.27 | −0.21 | 0.37 | −0.19 | −0.13 | 0.07 |
| Array of BGMs | 20 | 0.31 | −0.21 | 0.35 | 0.23 | −0.08 | 0.21 |
| HEV | 20 | 0.16 | −0.36 | 0.09 | 0.38 | −0.19 | 0.11 |
| <i>C. jejuni</i> | 20 | −0.12 | −0.29 | −0.19 | 0.21 | −0.59 | −0.52 |
| Bovine polyomavirus | 20 | 0.65 | 0.25 | 0.71 | −0.20 | 0.26 | 0.37 |

However, there were significant correlations between the detected concentrations of bovine polyomavirus and daily mean streamflow ($\rho = 0.65$) and discharge ($\rho = 0.71$).

4. Discussion

The occurrence of numerous BGMs and significantly-increased frequency and concentration of HEV in stream water after swine-manure application and runoff generating rain indicates a link between swine manure management and zoonotic pathogens to the environment. In two previous studies, HEV in surface waters adjacent to animal-based agriculture was detected once at a single site (Gentry-Shields et al., 2015; Kasorndorkbua et al., 2005). However, HEV was detected more frequently in the current study suggesting a higher incidence of the virus in swine farms in this region. There are several other factors that may have contributed to the increased detection of HEV in the South Fork Iowa River basin including 1) differences in manure management, 2) the intensity of hog production with 30 to 60% of the basin receiving manure, and 3) that the basin is extensively tile drained facilitating rapid transport of these pathogens to streams. HEV was not detected in the Walnut Creek control basin also suggesting that swine are a source of this virus to the South Fork Iowa River watershed. Additionally, deer fecal samples were positive for HEV RNA suggesting that deer may also be a potential source of HEV to the environment.

The *E. coli* STII (swine marker) BGM and HEV were detected in swine farm manure, but were not always detected in adjacent surface waters. Although Haack et al. (2015) found that the STII BGM increased in concentration and persisted in surface water following a swine manure spill, this BGM was also found upstream of the manure spill suggesting that STII may be endemic in this stream dominated by a multitude of swine sources. The current study supports this finding by detecting the STII BGM once in the sediment post-manure application, but not in a similar broad distribution that was found after a swine manure spill (Haack et al., 2015).

The *E. hirae* (bovine) BGM was detected in both swine manure samples and frequently in sediment and water samples post manure applications. Cattle are present in this watershed particularly at TC313 and TC323 where in some cases the cattle have direct access to the stream. However, this particular marker has been detected previously in stream water following a swine manure spill suggesting that this marker is not solely specific to cattle manure (Haack et al., 2015; Oksanen et al., 2015). Furthermore, the *E. coli* LTIIa (bovine) BGM which is another bovine indicator gene was not detected in the swine manure samples. Likewise, the esp. (human-infection associated *Enterococcus*) BGM which was detected in a single post-manure water sample and in both swine manure samples has also been previously detected in swine and other manure (Haack et al., 2015; Willems et al., 2001).

Elevated concentrations of indicator bacteria after manure application exceeding the Iowa's state bacteria water quality standard indicate that swine manure contributes to diminished water quality and that risk to human health may be increased from waters exceeding this standard. These *E. coli* and enterococci concentrations are consistent with

previous reports on this watershed (Tomer et al., 2008) where investigators found the highest concentrations of *E. coli* and enterococci in summer and lower concentrations in winter. Other researchers have documented that manure application can result in dramatic increases in water quality impairments (Dean and Foran, 1992). However, concentrations of culturable *E. coli* and enterococci in the same range in the Walnut Creek control site (where no animal agriculture existed) and the study sites and detections of *Enterococcus*-specific BGMs in Walnut Creek suggest additional sources are present (i.e. humans and wildlife).

Indicator bacteria and selected BGMs were detected in the control Walnut Creek basin where there was no known swine or livestock manure applications taking place. However, there are wildlife in this basin that may be a vector of indicator bacteria and BGMs. Previous studies found that background levels of fecal coliforms could be attributed to wildlife and changing environmental conditions (Farnleitner et al., 2010; Gessel et al., 2004; Somarelli et al., 2007). Additionally, there was a single detection of GI norovirus within this basin that potentially suggests septic influence. Septic systems may also have influenced concentrations of indicator bacteria (Sterk et al., 2013).

Although there were no significant correlations between daily streamflow or discharge and HEV and BGMs, increased detection and concentration of HEV and the number of BGMs detected post-manure application suggests that runoff and tile input are important transport mechanisms of both HEV and BGMs to corresponding surface waters. This finding is consistent with the hydrology of the South Fork Iowa River which is dominated by subsurface drainage that artificially enhances the transport of water to corresponding streams. Approximately 90% of baseflow in this basin is from subsurface drainage (Green et al., 2006). Overland flow after storm events is also a potential transport pathway and both surface and subsurface flow contribute to the transport of *E. coli* and *Enterococcus* (Tomer et al., 2010). Thus, it is likely that both subsurface drainage and overland flow contributed to the transport of HEV and other manure-related constituents to adjacent surface water.

Increased storm event flows coupled with increased flow from seasonal snowmelt prior to the March sampling (pre manure), may have resulted in the resuspension of bacteria containing BGMs in the sediment and increased detection of BGMs during this pre-manure sampling. Several BGMs including *E. faecalis* *ddl*, *E. faecium* *ddl*, *E. hirae* *bov*, *E. coli* *stx1*, *Salmonella* *invA*, and *S. aureus* *femA* were routinely detected in the sediment pre- and post-manure application suggesting that the sediment may be a sink and reservoir for BGMs as has been documented previously (Haack et al., 2015). High stream flows and storm events after the April 2012 manure application flushed freshly applied manure-derived constituents into the environment resulting in this study's highest pathogen concentration and number of BGMs detected.

Indicator bacteria concentrations for fecal coliforms, *E. coli*, and *Staphylococcus* and the number of BGMs detected were significantly correlated suggesting that these constituents have similar transport pathways and transport rates in the environment. Although bovine polyomavirus concentration was related to increased indicator bacteria

concentrations and BGM detections, HEV concentration was not correlated to the detection or concentrations of these constituents. There is little documentation of virus transport in tile drainage, but Wilkes et al. (2014) reported the detection of coliphage in water from tile-drained watersheds. Previous studies have shown that various viruses, including hepatitis A, can be transported through soil to groundwater (Borchardt et al., 2012; De Serres et al., 1999) and from surface to groundwater (Bradford et al., 2013).

5. Conclusion

This study detected several antibiotic resistance and pathogen gene markers from bacteria (Table 2, Supplemental Table 4) that can pose mild to serious health risks to swine, humans, and wildlife were detected including Shiga-toxin producing *E. coli*, *C. jejuni*, enterococci, and *S. aureus* (based on detection of *mecA* and *femA* genes). The spread of HEV and other zoonotic pathogens in the environment is of concern to both animal and human health. Manure management is linked to pathogen load and indirectly influences pathogen survival and transport through the environment (Goss and Richards, 2008). Application of fresh manure containing bacterial and viral pathogens to fertilize agricultural land can potentially impair groundwater, surface water quality and consequently influence environmental and human health. Further research is needed to: 1) better understand the persistence and survival of manure-related zoonotic pathogens in water and sediment, 2) assess the risk to animals and humans from manure-related zoonotic pathogens, and 3) ascertain manure management techniques that can reduce zoonotic pathogen load to the environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2016.05.123>.

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