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Abstract Wild birds have been shown to be significant sources of numerous types of pathogens that are relevant to humans and agriculture. The presence of large numbers of migratory birds in such a sensitive and important ecosystem as the Platte River in central Nebraska, USA, could potentially serve a significant source of bird-derived pathogens in the water/sediment and riverine environment. In 2009 and 2010, a study was completed to investigate the potential water-quality impacts of Sandhill Cranes and Snow Geese on the microbial water quality of the central Platte River during their spring migration period. Fecal material, river-bottom sediment, and water samples were collected from January through May of each year during the spring migration season of Sandhill Cranes in the Central Flyway of North America. Results indicate that several types of fecal indicator bacteria and from a range of viral, protozoan, and bacterial pathogens, *Campylobacter jejuni* were present in Sandhill Crane excreta, and at significantly higher frequency and densities in water and sediments when the Sandhill Cranes were present, particularly during evening roosts within the Platte River environment. Therefore, further investigation of the health significance of avian pathogens is warranted for the Platte River in Central Nebraska during migration of Sandhill Cranes and other waterfowl.

Keywords Sandhill Cranes · Microbial water quality · Nebraska

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1 Introduction

Migratory birds moving across national and intercontinental borders may act as long-range vectors of microbial pathogens (Waldenström et al. 2002; Meinersmann et al. 2013). These migratory birds may spread pathogens via

coprophagy or through airborne particles and water (Broman et al. 2000). For example, *Escherichia coli* O157:H7 and other waterborne enteric pathogens including *Cryptosporidium*, *Giardia*, and *Campylobacter* spp. have been detected in the excreta of various migratory gull species, Canada geese, and Whistling swans (Gould and Fletcher 1978; Demaré et al. 1979; Waldenström et al. 2002; Wetzel and Lejeune 2007; Graczyk et al. 2008; Lu et al. 2011). So, while as few as 30 ring-billed gulls (*Larus delawarensis*) may impact on the microbial water quality of a waterbody as measured by fecal indicator bacteria (FIB; Lévesque et al. 2000), the real concern is their potential co-introduction of potentially pathogenic species to humans and other animals. Indeed, most studies to date have focused on the FIB contribution from roosting gulls and other waterfowl (ducks, geese, and swans; Benton et al. 1983; Valiela et al. 1991), rather than the seasonal introductions of pathogens related to changing bird species and population densities.

The Platte River provides numerous beneficial uses to people in central Nebraska, such as irrigation, fishing, hunting, and groundwater recharge used as source drinking water. The river is also critical to the survival of wildlife that inhabits the region. The central Platte River valley is also an important stopover for migratory waterfowl, such as Sandhill (*Grus canadensis*) and Whooping (*Grus americana*) Cranes, in their yearly traversal of the Central Flyway (Mexico to Alaska), and is a bottleneck portion of the central flyway utilized by Sandhill Cranes and many other types of waterfowl. From late-February to early-April, approximately 600,000 Sandhill Cranes and a small number of Whooping Cranes stage along the central Platte River, Nebraska, building up their fat reserves and absorbing nutrients vital to their survival through the nesting season. Moreover, seven to ten million waterfowl, shorebirds, and other species also use the Platte River and the adjacent Rainwater Basin wetlands each spring (Buckley 2011).

Sandhill Cranes spend their days in the corn fields and then move to Platte River sandbars or surrounding wetlands for protection from predators as they roost during the nights. Roosting densities of cranes and other migratory birds within the central Platte River has increased in recent years as a result of lower flows and habitat degradation caused by drought-driven agricultural management decisions over the last decades (Krapu et al. 1984; Faanes and LeValley 1993; Kinzel et al. 2005). In addition, loss of wet meadows, which at one time were another prime location for crane roosting, have mostly been drained and cultivated for agricultural production within the last 40–50 years. Hence, during the height of the migration season, hundreds of thousands of cranes and other waterfowl roost within the river along a 50-mile stretch approximately between Kearney and Grand Island, Nebraska, at a density of up to 12,000 birds per mile (Buckley 2011).

Surprisingly, the microbial impact to the central Platte River from migratory Sandhill Cranes and other waterfowl has not been documented, even though deterioration of the microbial water quality could have an economic impact on local recreation, tourism, and animal agriculture. For example, avian disease outbreaks linked to migratory birds could impact the tourism industry surrounding bird watching (which is focused around Sandhill Cranes in this area), while and waterfowl hunting would be affected, not to mention the natural ecosystem that depends on them. Animal husbandry could be affected too, particularly with bacterial pathogens such as *Campylobacter* spp., which are a major cause of septic abortion in cattle, goats, and sheep (Sauerwein et al. 1993). Moreover, up to 20 % of *Campylobacter* cases in humans are said to attributable to environmental contaminants (Mead et al. 1999), and migratory waterfowl are considered high risk species for environmental contamination (Pacha et al. 1988; Aydin et al. 2001). Of increasing concern is avian influenza, particularly to chicken and turkey animal health (Clark 2003). The most recently available data showed that in 2007, Nebraska had 4.8 million broiler chickens with a production value of $12,330,000 and produced 2.984 million eggs with a production value of $185,092,000 (USDA 2008). In addition to poultry production, waterborne avian influenza has also been shown to cause disease outbreaks in swine in Ontario (Karsin and Olsen 2000).

Limited research has shown that cranes can have a significant impact on public health. Recently developed quantitative microbial risk assessment (QMRA) approaches based on pathogen estimates may provide more insights into potential human risks of waterfowl (e.g., Roser et al. 2006; Schoen and Ashbolt 2010). However, in order for QMRA models to be adequate, it is important to identify pathogens as well as fecal indicator levels in both fecal sources and in the environment. Assays that

can corroborate the likely sources of pollution in water are also relevant to better understand the environmental factors that contribute to the survival and transport of avian pathogens in the environment. This is especially true for avian influenza, which has been detected at significant frequencies from the sediment and water column during migratory bird migration in Georgia and North Carolina (Dalton et al. 2009).

Therefore, the objectives of the study were to collect a baseline dataset of viral, bacterial, and protozoan pathogens and various fecal indicators (*E. coli*, enterococci, *Bacteroidetes*, and a putative crane-specific marker) from the central Platte River, and to compare microbial impacts from cranes from the upper to downstream regions during the migration and non-migration periods.

2 Methods

2.1 Sampling Sites and Samples

Water and sediment sample locations were selected based on available USGS (2011) or gaging station flow data during non-ice conditions, and located so as to be upstream (Overton), middle (Denman), and downstream (Grand Island) of the predominant roosting and migration ranges of Sandhill Cranes in the Platte River valley. Fecal samples were collected from Sandhill Cranes and Snow Geese (*Chen caerulescens*) at a location near the Platte River Whooping Crane Maintenance Trust near Alda, Nebraska (Fig. 1). Few cranes roosted upstream of the Overton site and were exposed to considerably less Sandhill Crane fecal material during that portion of their migration season.

Sandhill crane excreta (n=10), water (n=61), and sediment (n=24) samples were collected from February to April, 2009 (Fig. 1). In 2010, Sandhill Crane excreta (n=64), snow goose (*C. caerulescens*) excreta (n=22), water (n=42), and sediment (n=30) samples were also collected at the same locations from January to May, 2010. All samples were transported on ice or hand delivered to the analytical laboratory and processed within 24 h of collection, except for the 2009 water samples collected at night and shipped to out-of-state laboratories that were analyzed within 36 h of collection.

2.2 Fecal Sample Collection

Crane and goose excreta were collected just after defecation during the 2009 and/or 2010 water/sediment sampling periods. Samples were generally placed into sterile plastic bags and immediately stored in ice coolers until delivered to laboratories. However, samples for avian influenza testing were collected via a Dacron swab and immersed in viral transport media (Docherty and Slota 1988), kept cool in the field, and sent on wet ice to the USGS National Wildlife Health Center for testing within 24 h of collection.

2.3 Water Sample Collection

Between February and April 2009, water samples were collected on Mondays [one day time (10 a.m.) and one night time (1 a.m.)] during sampling weeks with an Isco® refrigerated autosampler from three river sites (Fig. 1). The intake line for the autosampler was placed at the midpoint depth at a location near the bank, but in a thalweg of the braided river. Samples were retrieved from the field on the same day they were collected by the autosamplers. In 2009, sample collection occurred each week from February 16 to April 27 (8 daytime and 8 nighttime samples from each of three sample sites), which spanned the periods before, during, and after the normal migration season. The field parameters of specific conductance, turbidity, and pH were measured from a subset of the samples collected by the autosampler. In 2010, water samples were collected on Mondays as described above with the exception that samples were collected by hand-grab sampling, and some samples were also collected on Thursdays during the period in March when Sandhill Cranes were present. Sample collection occurred between January 18 and May 24, 2010.

At least two replicate water samples and one blank water sample were collected from each site during the study. Replicate samples were used to quantify variability during the collection and analysis process. These replicate samples were collected simultaneously to incorporate variability from collection, processing, and sample shipping (Vogel et al. 2009). The coefficient of variation for all replicate sample sets was calculated using log-transformed data so as to satisfactory normality of the data set for ANOVA. A total of 36 field blank samples, contamination-free water,

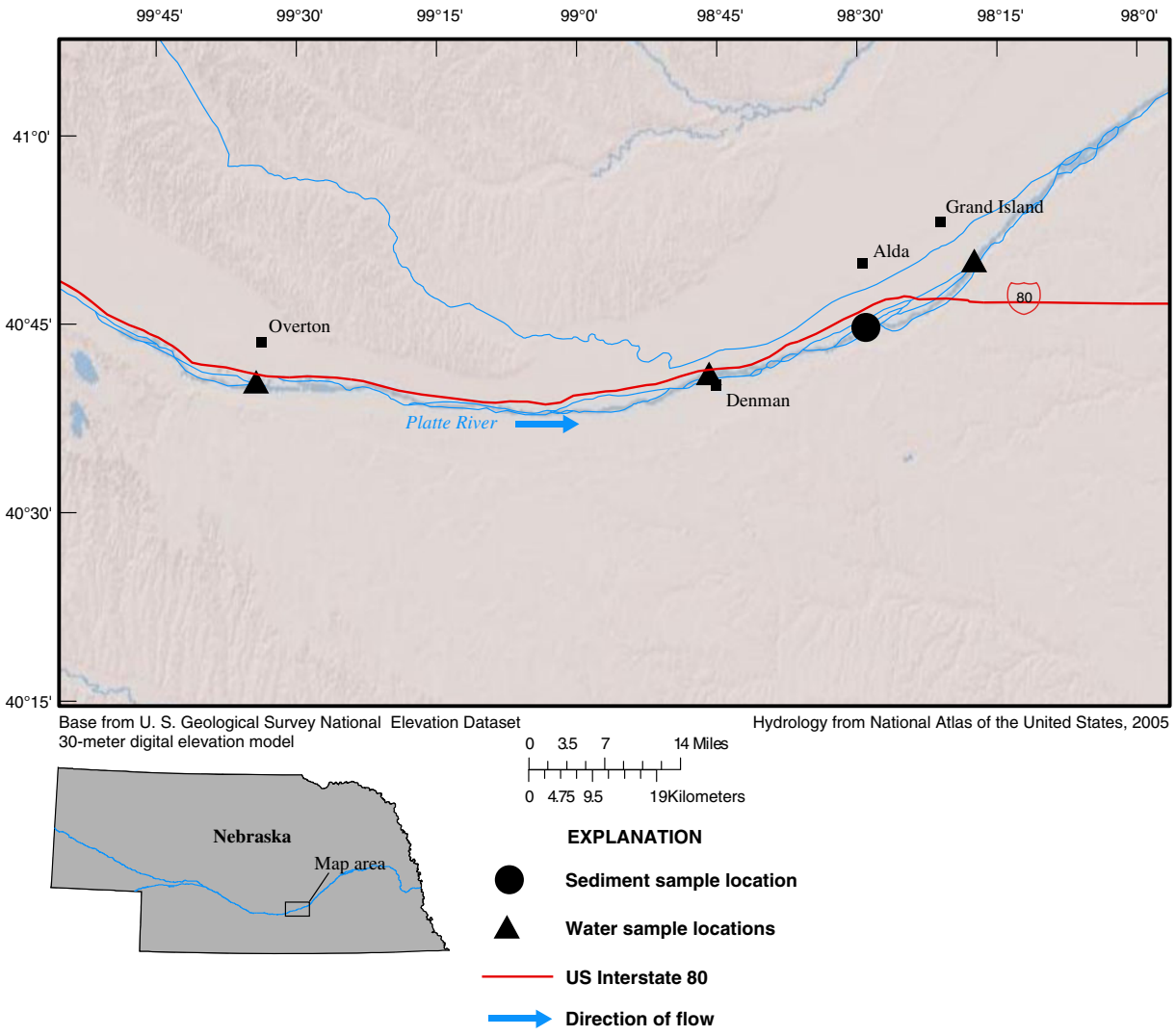

Fig. 1 Approximate sampling locations for the Platte River, Central Nebraska

were collected to test for contamination of samples over the course of the study and analyzed in the same manner as the test samples (Vogel et al. 2009).

2.4 Diurnal Study

Over a period of 72 h, water samples were collected by hand every 90 min on two separate occasions in March of 2010. The goal of this frequent sampling interval was to examine diurnal fluctuations of water quality parameters and to determine if the increased number of cranes roosting in the Platte River at night resulted in increased FIB (*E. coli* and enterococci) in the river. This increased sampling rate occurred during the crane roosting peak in 2010, specifically, March 15–18th and March 22–25th. Sampling was undertaken at the Platte River gaging station near Grand Island (Fig. 1), which was downstream of most of the roosting locations in the river. Samples were collected by hand as described previously for the 2010 samples.

2.5 Sediment Sample Collection

Sediment samples were collected in triplicate on the same days as water samples at approximately 11:00 a.m. from the sediment sampling site near Alda (Fig. 1) in sterile 250-mL bottles. Except for

analysis for avian influenza, the samples were collected as saturated sediment with a minimum amount of river water from above the sediment. The sediment samples for avian influenza analysis were diluted with surface water collected from the same location in a 1:10 (*v*/*v*) ratio.

2.6 Sample Analysis

Fecal indicator bacteria (E. coli and enterococci) Excreta, water, and sediment samples were analyzed within 24 h for *E. coli* in 2009 and for *E. coli* and enterococci for the 2010 samples at a local contract laboratory (Ward Laboratories, 4007 Cherry Avenue, Kearney, Nebraska), which was approved through the USGS external laboratory approval process (USGS 2002). Water samples were assayed at 1:1 and 1:10 dilutions and excreta/sediment over three ten-fold dilutions. Aliquots (1 g ww) of excreta and sediment were analyzed following vortexing in 10 mL of sterile phosphate buffer solution (PBS), then serial dilutions in PBS. One hundred milliliters of each sample was mixed with Colilert™ or Enterolert™ and sealed in a Quanti-Tray®/2000 and the most probable numbers (MPN) estimated as per the manufacturer's instructions (IDEXX Company, USA).

Fecal source tracking markers Cattle- and human-targeted *Bacteroidetes* qPCR assays (CF128 and HF183, respectively) were used to identify fecal source impacts to sediment and water samples collected in 2010, as described by Bernhard and Field (2000). These assays were used as cattle herds, and household septic systems associated with the numerous farms in the valley were likely sources of fecal pollution. A putative crane-specific marker targeting *Catellicoccus*-like spp. was used for the detection of Sandhill Crane excreta in water and sediment as described by Ryu et al. (2012a, b).

Campylobacter Excreta, sediment, and water samples collected in 2010 were analyzed for the presence of *Campylobacter* spp. at the US EPA National Exposure Research Laboratory in Cincinnati, Ohio. Sub-samples of crane excreta and river-bottom sediment (1–2 g) were vortexed in PBS for culture enrichment and total community DNA extraction. One milliliter of the PBS suspension was transferred to Bolton broth (CM0983; Oxoid, Basingstoke, UK) and incubated under microaerophilic conditions (5 % O_2, 10 % CO_2, and 85 % N_2) at 42 °C for 72 h. After 72 h, 1 mL of the pre-enriched cultures was centrifuged at 15,000×*g* for 10 min. DNA was extracted from the resulting pellet using MasterPure™ DNA Purification Kit following the manufacturer's instructions (Epicenter Biotechnologies, Madison, WI, USA). DNA extracts were further purified using the Genomic DNA Clean & Concentrator™ kit (Zymo Research Corp., Irvine, CA, USA).

Water samples (100 mL) were filtered through polycarbonate membranes (0.40-μm pore size, 47-mm diameter; GE Water and Process Technologies, Trevose, PA, USA). Membranes were aseptically cut into four pieces and placed into tubes containing lysing solution (Tissue & Cell Lysis Solution™, Epicenter Biotechnologies Madison, WI, USA), and processed using silica bead disruption and DNA extracted and further purification as described for excreta samples. Additionally, water samples (5 mL) were enriched for *Campylobacter* spp. and processed as described above.

Molecular methods used to determine the presence of *Campylobacter* included (1) cloning and sequencing of *Campylobacter* spp. PCR products (Linton et al. 1996), (2) PCR assays used for the speciation of pathogenic *Campylobacter* (Stucki et al. 1995; Gonzalez et al. 1997; Khan and Edge 2007), and (3) *Campylobacter* spp. qPCR assays (Lund et al. 2004). For *Campylobacter* qPCR assays, targeted cells in the extracts were quantified as spiked cell equivalents (CE). The detection limits and standard curves for *Campylobacter* spp. were determining using standard curves through spiking a *Campylobacter* strain with targeted sequences into the matrices originated from the same or similar sources to the samples assayed. Specifically, five crane excreta and water samples and four sediments, which previously tested negative for *C. jejuni*, were pooled, respectively, and used as *C. jejuni*-spiking matrices. Serial dilutions of *C. jejuni* from 10^8 to 10^1 were prepared in PBS and spiked into nine 1-mL triplicate samples of crane excreta–PBS buffer (eight dilutions and one negative control), sediment–PBS buffer, or river water. DNA was then extracted as described above as used in qPCR assays. The lowest number of gene copies that was detected consistently in triplicate assays was considered the qPCR limit detection. To test qPCR inhibition, each DNA extract was assayed using the original concentration, and after a ten-fold dilution in T&C lysis buffer (Epicenter Biotechnologies), and with the addition of the TaqMan Exogenous Internal Positive

Control Reagents (a VIC-labeled probe) manufactured by ABI™ (Applied Biosystems). The qPCR data from ten-fold dilutions were used, when any inhibition was identified. Each qPCR run included a DNA standard curve in the first row of the plate, and a no-template control for each row of each 96-well plate assayed. The qPCR efficiency was calculated using the following equation: Efficiency%=100×[10(−1/slope)−1] (Applied Biosystems).

Legionella, Cryptosporidium, and Giardia Samples were shipped on ice overnight on the day of sample collection and analyzed at the USGS Florida Integrated Science Center in Tallahassee, Florida. Aliquots of excreta (0.25 g) were transferred to a 15-mL tube containing 2 mL of sterile water. The samples were vortexed, and 1 mL aliquots of sample were transferred to separate 1.5-mL microcentrifuge tubes (one tube for *Legionella* spp. and one tube for *Cryptosporidium* and *Giardia* spp. analyses), and refrigerated until analyzed.

Sediment samples (5.0 g) were transferred to 15-mL tubes containing 10 mL of sterile water. Samples were then vortexed using a Vortex-Genie 2 (MO BIO Laboratories, Inc. Carlsbad, CA, USA) set at high speed for 30 s, followed by rotation in a tabletop rotator and then vortexed again for 30 s. The supernatant was then transferred to another 15-mL tube and 5 mL of sterile water added to the remaining sediment pellet and processed as described above. The resulting suspension was centrifuged at 3,000 rpm at room temperature, and the supernatant was discarded. The pellet was suspended to 2 mL in sterile water, vortexed, and split into separate 1.5-mL microcentrifuge tubes (one tube for *Legionella* spp. analyses and one tube for *Cryptosporidium* and *Giardia* spp. analyses). These two aliquots were stored at 4 °C or −20 °C (the latter for *Legionella* spp. PCR analyses) until assayed as described below. Most of the samples were stored for a period of days, but some of the 2009 samples were stored for a period of weeks.

For water samples, 1-L aliquots were centrifuged at 3,000 rpm for 15 min at room temperature using a swinging bucket rotor. The supernatant was discarded and the pellet suspended in 10 mL of original sample. The 10-mL sample was further concentrated to 2 mL by centrifuging at 4,400 rpm for 10 min and aspirating off the supernatant with a Pasteur pipette. The remaining sample was split into 1-mL aliquots and processed as described below.

In 2009, presumptive *Legionella* spp. were first culture from 210 μL sample aliquots, pre-treated at 50 °C for 30 min and then 100 μL spread-plated (in duplicates) using *Legionella* selective agar or *Legionella* selective agar DGVP (LSA and DGVP, Becton, Dickinson and Company, Sparks, MD, USA), incubated for 3 days at 35 °C, and colony forming units (CFU) were enumerated as per the manufacturer's instructions. Sixty-one out of 188 presumptive *Legionella* colonies were transferred to fresh plates of agar, and DNA extracted from single colonies was used as a template for the partial amplification of the 16S rRNA gene as described by Nadkarni et al. (2002). PCR products were sequenced using Sanger chemistry, and sequences were processed using Lasergene (DNASTAR, Inc., Madison, WI, USA) and Finch TV (Geospiza, Inc., Seattle, WA, USA). The BLAST tool (National Library of Medicine) was used for sequence identification.

In 2010, *Legionella* spp. PCR assays were performed by vortexing thawed aliquots, followed by centrifugation at 14,000 rpm for 5 min and removal of supernatant. The pellet was vortexed to resuspend the bacterial pellet, and DNA was extracted from the sample using the DNeasy Blood and Tissue Kit (Gram+protocol, QIAGEN, Inc., Valencia, CA, USA). Aqua Screen PCR kit (Minerva-Biolabs, Berlin) was utilized for detection of *Legionella* spp. using 5 μL of the sample as template and following the manufacturer's instructions for amplification conditions. Gel electrophoresis was used for visualization of amplicon (1.5 % agarose stained with ethidium bromide).

To analyze samples for *Cryptosporidium* and *Giardia* using PCR, the aliquots were briefly vortexed, and oocyst and cyst (oo/cysts) purification using Dynabeads GC-Combo kit as per the manufacturer's instruction (Immuno-magnetic separation, Invitrogen, Carlsbad, CA, USA). The final elution volume was 55 μL, subject to a freeze/thaw procedure to lyse the oo/cysts, specifically by using crushed dry ice floated in 200 proof ethanol and a boiling water bath. DNA was then extracted from the samples using the DNeasy Blood and Tissue kit (QIAGEN). Eluents (4 μL) were used as template for detection of each organism. *Cryptosporidium* spp. PCR primers targeted a 435-bp segment of the 18S rRNA gene (Johnson et al. 1995), and the *Giardia* spp. PCR primers targeted a 163-bp size segment of the heat shock protein 70 (HSP70; Abbaszadegan et al. 1997). *Cryptosporidium* and *Giardia* spp. amplicons were visualized by dot blots

as previously published (Johnson et al. 1995; Rochelle et al. 1997).

Avian influenza Avian influenza samples were shipped overnight and analyzed at the USGS Wildlife Health Research Laboratory in Madison, Wisconsin. Sediment samples analyzed for avian influenza were adjusted to 10 % (*w*/*v*), vigorously mixed with a Fisher Scientific Vortexer to disperse viruses into the liquid phase. The samples were then centrifuged at 1,000×*g* for 10 min, and the resultant supernatant was tested for influenza type A viruses. Potential viral RNA were recovered from the supernatant of sediment samples, from surface water samples, or fecal swab samples in viral transport media (Docherty and Slota 1988) by extraction of 50 μL aliquots using the Ambion MagMax™ AI/ND Viral RNA extraction kit according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Eight microliters of the extracted RNA were tested using the matrix gene RT-PCR reaction in a Stratagene MX3005P real-time thermal cycler (Agilent Technologies, Santa Clara, CA, USA). The matrix gene RT-PCR test is designed to detect all subtypes of influenza A viruses (Spackman et al. 2002). Any matrix RT-PCR-positive samples were further subtyped using H5- and H7-RT-PCR tests (Docherty and Slota 1988). Virus isolation was performed on all samples by inoculation into allantoic sac of 8-day-old embryonated specific pathogens-free chicken eggs (Docherty and Slota 1988). Allantoic fluids from each egg were collected after an incubation period of 3 days at 37 °C and tested for influenza type A viruses in a hemagglutination (HA) test using rooster-chicken and turkey red blood cells. HA-negative samples were retested by inoculation into a second round of embryonated eggs before the sample was considered negative. HA-positive samples were subtyped by nucleotide sequencing to determine the hemagglutinin and neuraminidase subtypes.

Data interpretation and quality assurance. Data analysis was undertaken in Excel, using a single-factor analysis of variance (ANOVA). If the probability (*p* value) of the test was less than 0.05, the values are considered to be significantly different. Quality assurance/quality control was undertaken on 36 water field blanks (6 and 30 in 2009 and 2010, respectively). None of the microbial analyses on the blank samples revealed detections. During the study, replicate water samples were tested for *E. coli* (2009 and 2010; $n=34$) and enterococci (in 2010; $n=10$). Mean coefficient of variation on the microbiological analyses was 28 % and 25 %, respectively, during the study. Comparison of relative standard deviation to sample concentration did not indicate a significant trend for any of these microbial analytes.

3 Results

3.1 Platte River Discharge

Two of the three sample sites had USGS discharge gages (Overton and Grand Island). In general, the flow in 2009 was much lower than in 2010 (Fig. 2). During the migration season (March 1–April 10) of 2009 and 2010, the mean discharge in the Platte River at the Overton site was 22 and 51 cfs, respectively, and 830 and 2,700 cfs, respectively, at the Grand Island site. In fact, there were no runoff events in 2009 during the migration period (March and early April). However, a large snowmelt in early March 2010 affected the discharge in 2010. Spikes or apparent increases in discharge in the baseflow periods were a result of releases from the upstream dam at Lake McConaughy or artifacts of ice in the river (during January, February, and the first half of March).

3.2 Local Region Sandhill Crane Population

The number of Sandhill Cranes present in the local region (comprised of the central Platte River Valley and surrounding area) was measured by the Platte River Whooping Crane Maintenance Trust (Fig. 3a, b). The peak measured population of Sandhill Cranes in the area occurred on March 17 in both 2009 and 2010 (Buckley 2011). Although Sandhill Cranes were distributed throughout the river reach studied, Buckley indicates that, in general, cranes tend to congregate towards the downstream portion of the reach during the first part of the migration period and congregate in the upstream portion during the later migration period.

Fecal indicators In general, geometric mean most probably numbers (MPN) of enterococci were 2–3 $\log_{10}$ higher in crane and Snow Goose excreta than geometric *E. coli* estimates (Table 1). In contrast, *E. coli* and enterococci estimates were of similar magnitude in sediments and water samples collected in 2009

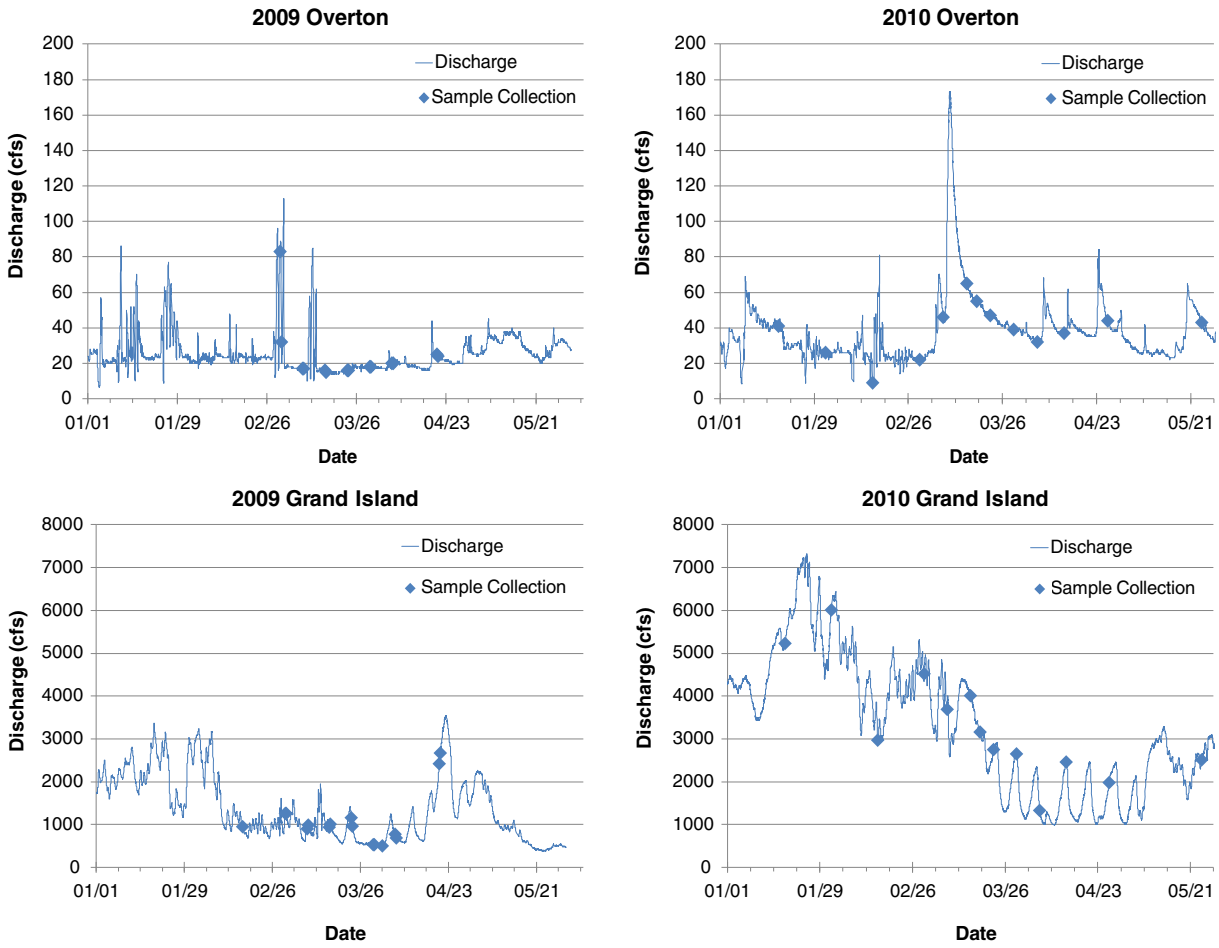

Fig. 2 Discharge at the Overton and Grand Island sampling sites (see Fig. 1) during the sampling period in 2009 and 2010. Raises in discharge during baseflow were a result of either upstream dam releases or an artifact of ice in the river (USGS 2011). *cfs* cubic feet per second

and 2010 (Table 1), but both peaked in sediments during the peak presence of cranes at the central roosting site (Fig. 3a–c). Further evidence of bird impact to the Platte River was evident from the fecal source markers examined (Table 1). In contrast to the absence of human- and cattle-targeted markers in bird excreta, the Crane1 marker was present in both crane and Snow Goose excreta and positive in stream sediment samples collected on March 8 (one of three samples), March 15 (two of three samples), and March 17, 2010 (two of three samples), which coincided with the peak of roosting cranes and the period when the enterococci concentrations were highest.

Diurnal studies of 2010 When water samples were collected every 90 min for a period of 72 h, overall *E. coli* densities generally peaked at around 0830 hours, while the enterococci densities generally peaked sometime between 2330 and 0830 hours (Figs. 4 and 5). These samples were collected at the most downstream site (Grand Island), so travel times from upstream roosting sites from pre-dawn hours before cranes left the stream are a potential reason for higher densities even later in the morning. The consistent peaks of *E. coli* in water samples could be a result of resuspension of overnight deposited bacteria from stream bottom sediment, as crane activity was highest right before they departed from the river.

Higher bacteria densities between the hours of 2200 and 1130 occurred at the Grand Island site, with *E. coli* p values <0.0001 (geometric mean=242 and 26 MPN/100 mL, respectively) and enterococci <0.001 (geometric mean=132 and 18 MPN/100 mL, respectively). Since both sampling times in 2009

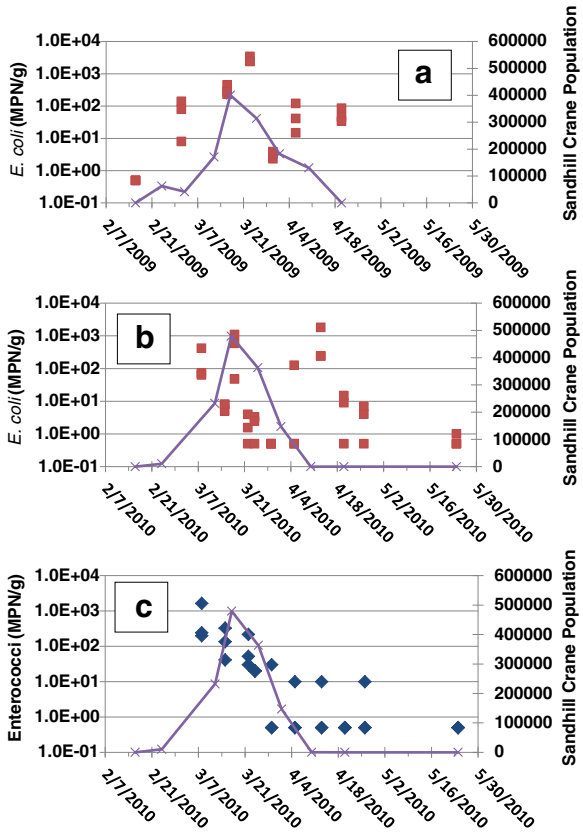

Fig. 3 Fecal indicator bacteria concentrations in sediment samples for (**a**) *Escherichia coli* in 2009, (**b**) *E. coli* in 2010, and (**c**) enterococci in 2010 at the Platte River Whooping Crane Maintenance Trust compared to 2010 Sandhill Crane population in the local region. Non-detects are shown as 0.5 MPN/g on the logarithmic scale. Sandhill Crane estimated population in the central Platte River Valley in Nebraska during their northern migration in 2009 and 2010 (adapted from Buckley 2011). *MPN* most probable number

spanned the period of crane activity (collected at 0200 and 1000 hours), this could help explain why day–night differences were not seen in the samples from 2009.

Avian impact on the Platte River samples Water samples for *E. coli* analysis were collected from the three tests sites between February 16 and April 20 in 2009 and between January 18 and May 24 in 2010. Statistically differences were noted for *E. coli* when cranes were present at Denman in 2010, Grand Island (combined 2009–2010 samples), and the combined Denman and Grand Island data from 2010 alone and 2009–2010 combined (Table 2). No significant differences were observed between night and day *E. coli* levels during March 2 and April 6, 2009 when cranes were migrating through the region (Table 2). There were no significant differences from one year to the next from all sites, from the subset including Denman and Grand Island, or from the subset from Denman and Grand Island when cranes are present. Furthermore, the geometric mean concentrations of enterococci for each sampling period and lack of significant difference from paired *t* tests for specific are shown in Table 2. The enterococci concentration for the combined Denman and Grand Island sites was significantly greater when cranes were present, but only at the $p<0.10$ level.

Further confirmation of avian and additional fecal source impacts was demonstrated from the source markers studied. Most water samples collected during March and the first week of April at the Denman and Grand Island sites (87.5 %, $n=16$) tested positive for the Crane1 marker. None of the remaining water samples collected in 2010 ($n=27$; all samples from Overton plus Denman and Grand Island during January, February, late April, and May) were positive for the Crane1 marker. Using a one-tailed Fisher's exact test (Preacher and Briggs 2001), the occurrence of the Crane1 marker at the Denman and Grand Island sites was significant ($p<0.001$). Of the 42 water samples screened for the presence of the *Bacteroidetes* cattle-targeted (CF128) and human-targeted (HF183) markers, a total of 13 samples tested positive for CF128 marker and 12 samples tested positive for HF183 marker. However, the spatial distribution of the positive tests differed for the Crane1 marker, with 38 % (10/26) and 31 % (8/26) testing positive for cattle-targeted and human-targeted markers, respectively, for all samples from Overton plus Denman and Grand Island during January, February, late April, and May. For the remaining samples (Denman and Grand Island during March and the first week of April), 21 % (3/16) and 29 % (4/16) of samples tested positive for cattle-targeted and human-targeted markers, respectively. Using a one-tailed Fisher's exact test (Preacher and Briggs 2001), the occurrence of the cattle-targeted and human-targeted markers at the Denman and Grand Island sites during March and the first week of April was not significantly different from the rest of the samples at the $p=0.05$ level ($p=0.16$ and $p=0.49$ for cattle-targeted and human-targeted, respectively). These results appear to reflect the local population of cattle (continuous), humans

Table 1 Results for microbial targets in bird excreta, sediments, and water for the central Platte River, Nebraska

Group	Excreta		Sediment	Water
	Crane	Snow goose		
Fecal indicator bacteria				
Enterococci [geometric mean in MPN/g (solids) or 100 mL (water), detections/number of samples]	1.6×10^{7} (31/31)	9.1×10^{5} (21/22)	57 (16/30)	45 (36/36)
E. coli [geometric mean in MPN/g (solids) or 100 mL (water), detections/number of samples]	2.8×10^{4} (27/30)	6.1×10^{3} (8/10)	44 (40/54)	74 (86/86)
Fecal source markers				
Crane (Crane1) ("+ vs. /n)	65/95	12/22	5/30	14[a]/43
Cattle (CF128) ("+ vs. /n)	0/95	0/22	0/30	13/42
Human (HF183) ("+ vs. /n)	0/95	0/22	0/30	12/42
Campylobacter spp. ("+ vs. /n)	34/48	11/22	0/30	15/42
Cryptosporidium	0/30	0/19	0/19	1/103
Giardia	6/30	0/19	0/19	12/87
Presumptive *Legionella* spp. ("+ vs. /n)	8[b]/20	0/19	1[b]/19	2[b]/42
Avian influenza virus ("+ vs. /n)	0/30	0/29	0/46	0/57

MPN most probably number, *n* number of samples

[a] Denman and Grand Island sites were the only ones positive for Crane1 marker

[b] Positive confirmation by PCR not completed

(continuous), and cranes (migratory) in the area. The high frequency of runoff during the 2010 season when these samples occurred likely helped facilitate the delivery of the cattle-derived *Bacteroidetes* to the River.

Pathogens Campylobacter were the most prevalent pathogen group recovered from excreta, sediments, and water samples (Table 1). Sandhill Crane excreta were positive in 71 % (34/48) and Snow Geese in 50 % (11/22) of samples collected in 2009 and 2010. This result is in the same range as the 81 % detection level for Sandhill Crane excreta reported in Pacha et al. (1988). Snow Geese fecal samples were not analyzed by Pacha et al. (1988), but are much higher

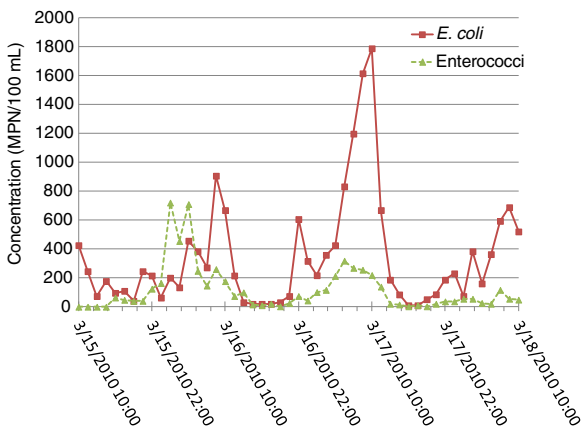

Fig. 4 *Escherichia coli* and enterococci densities (MPN/100 mL) March 15–18, 2010 at the Platte River gage near Grand Island. *MPN* most probable number

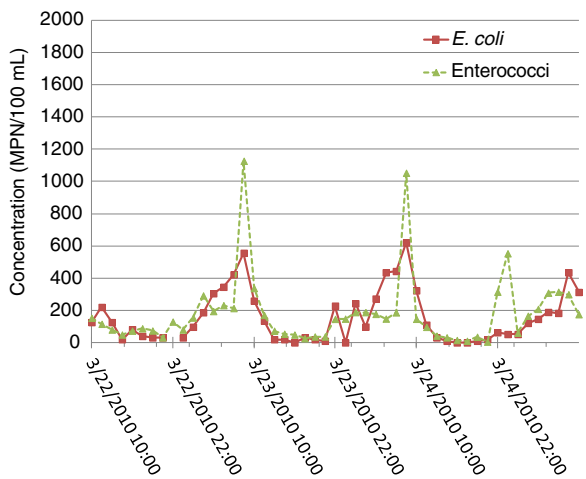

Fig. 5 *Escherichia coli* and enterococci densities (MPN/100 mL) during March 22–25, 2010 at Grand Island. *MPN* most probably number

Table 2 Selected statistics and analysis of variance (ANOVA) tests for *Escherichia coli* data collected in 2009 and 2010

Descriptor 1	Descriptor 2	Descriptor 3	Factor 1 and geometric mean (MPN/100 mL)	Factor 2 and geometric mean (MPN/100 mL)	*p* value
2009	Overton	–	Cranes, 19 (n=12)	No Cranes, 28 (n=3)	0.77
2010	Overton	–	Cranes, 50 (n=8)	No Cranes, 105 (n=5)	0.14
2009–2010	Overton	–	Cranes, 28 (n=20)	No Cranes, 64 (n=8)	**0.044**
2009	Denman	–	Cranes, 207 (n=12)	No Cranes, 28 (n=3)	0.25
2010	Denman	–	Cranes, 328 (n=8)	No Cranes, 80 (n=5)	**0.036**
2009–2010	Denman	–	Cranes, 249 (n=20)	No Cranes, 54 (n=8)	*0.061*
2009	Grand Island	–	Cranes, 104 (n=14)	No Cranes, 36 (n=3)	0.27
2010	Grand Island	–	Cranes, 119 (n=8)	No Cranes, 16 (n=5)	*0.054*
2009–2010	Grand Island	–	Cranes, 109 (n=22)	No Cranes, 22 (n=8)	**0.041**
2009	Denman and Grand Island	–	Crane, 143 (n=26)	No Cranes, 32 (n=6)	0.17
2010	Denman and Grand Island	–	Cranes, 198 (n=16)	No Cranes, 36 (n=10)	**0.007**
2009–2010	Denman and Grand Island	–	Cranes, 162 (n=42)	No Cranes, 34 (n=16)	**0.021**
2009	Denman and Grand Island	Cranes	Day, 153 (n=13)	Night, 133 (n=13)	0.83
All data	–	–	2009, 64 (n=47)	2010, 89 (n=39)	0.53
Denman and Grand Island	–	–	2009, 108 (n=32)	2010, 102 (n=26)	0.36
Denman and Grand Island	Cranes	–	2009, 143 (n=26)	2010, 198 (n=16)	0.61

ANOVA probability values (p values) less that 0.05 are considered significant and designated in *bold*. p values significant at the p=0.10 level are designated in *italics*

MPN most probably number, *p value* probability returned by the analysis of variance, *Cranes* samples collected between March 1 and April 15, *No Cranes* samples collected before March 1 and after April 15, *n* number of samples

than their 5 % detection frequency for Canadian geese. Whereas *Campylobacter* spp. did not persist in river sediments, the regular detections in water samples are reported in Table 3. Using a Fisher's exact test (Preacher and Briggs 2001), no significant differences were seen on the detection frequencies (p =0.104 when comparing the downstream sites during migration to all other sites and dates). Of note, however, is that the three *Campylobacter* detections during the early period (one from each site) were on the January 18 sampling date. The source of these *Campylobacter* was from a source other than Sandhill Cranes or Snow Geese, which were generally not yet present in the area.

Cryptosporidium and *Giardia* oo/cysts were generally not detected in any sample, with the exception of two Snow Goose (2/4) and one Sandhill Crane (1/4) excreta samples (Table 1) and a limited number of *Giardia* detections in water samples (Table 4). All *Giardia* detections shown in April in Table 4 were from samples collected on April 6 before Sandhill Cranes left the area.

While presumptive *Legionella* were detected in crane excreta (8/40) in 2010, confirmation by sequence analysis was not performed. Furthermore, no *Legionella* were confirmed from sediments or water samples. From the water samples collected in 2009, a total of 188 presumptive *Legionella* colonies were identified from the water samples, but a subsample of 41 were not confirmed as *Legionella* when identified via 16S rRNA sequencing, and this culture-based work was not continued in 2010. Presumptive legionellae isolates were identified as *Acidovorax* sp. (n=2), *Acidovorax* or *Pseudomonas* sp. (n=4), *Alphaproteobacteria* (n=4), *Ancylobacter*

Table 3 *Campylobacter* detections per water samples collected for each collection period in 2010

	Overton	Denman	Grand Island	Total
January–February	1/3	1/3	1/3	3/9
March–mid April	2/9	6/9	4/9	12/27
April–May	0/2	0/2	0/2	0/6
Total	3/14	7/14	5/14	15/42

Table 4 *Giardia* detection at each site in water samples during the months of February, March, and April in 2009

	Overton	Denman	Grand Island	Total
February	0/1	0/1	0/1	0/3
March	2/10	4/10	2/10	8/30
April	1/4	1/4	0/4	2/12
Total	4/15	5/15	1/15	10/45

Number of detects/samples tested

polymorphus (n=2), *Bosea* sp. (n=1), *Brevibacillus laterosporus* (n=7), *Brevundimonas* sp. (n=3), *Brevundimonas* or *Mycoplana* sp. (n=11), *Paenibacillus* sp. (n=5), and *Xanthobacter polyaromaticivorans* (n=1). In water, all of the bacteria identified as species of *Brevibacillus* and *Paenibacillus* were isolated from samples collected on or after April 6, 2009.

In 2010, a total of 42 water samples were screened for presence of *Legionella* using PCR. Only two samples, collected at Denman on March 8 and at Grand Island on March 15, were positive, but confirmation by sequence analysis was not completed. No avian influenza virus PCRs were positive for any sample.

4 Discussion

Numerically, migrating Sandhill Cranes contribute a large mass of excreta each spring, with over 500,000 birds stopping by the central Platte River. The data from this study indicated that fecal indicator bacteria (FIB) and, in some cases, potential pathogens relevant to humans and agriculture were present in 2009 and 2010 (Table 1). However, the significance of the contribution by migrating bird excreta is complicated by multiple potential sources for microbial contamination to the central Platte River. Therefore, we used multiple lines of evidence to identify fecal contributions by cranes and co-migrating Snow Geese.

4.1 Key Markers of Crane Pollution

Enterococci in crane and goose excreta outnumbered *E. coli* by some two orders of magnitude (Table 1). These FIB findings are consistent with data generally reported for avian excreta (Geldreich 1978; Byappanahalli et al. 2012), but there are known exceptions [e.g., gulls (Gould and Fletcher 1978; Fogarty et al. 2003)]. However, *E. coli* and enterococci provide no clear source attribution data. Despite early attempts to utilize the higher ratio of fecal coliforms (*E. coli*) to enterococci to discriminate human from animal (bird) fecal sources (Geldreich and Kenner 1969; Geldreich 1976), due to varying differential die-off (and even growth) of either *E. coli* or enterococci in different aquatic environments (Davies et al. 1995; Genthner et al. 2005), interpretation of such ratios is generally confounded. Hence, alternative approaches have been explored, and despite continued efforts, no robust *E. coli* nor enterococci markers have been reported (Field et al. 2003; Jiang et al. 2007; Whitman et al. 2007; Mohapatra et al. 2008; Lanthier et al. 2010). There are promising bird markers, however, which come from *Catellicoccus* 16S rRNA gene markers (Lu et al. 2011; Green et al. 2012; Ryu et al. 2012a, b), and one demonstrated to identify Sandhill Crane excreta (Ryu et al. 2012a, b) was used and supported in the current study (Table 1). A further line of evidence comes from the limited detection of *Bacteroidetes* in Sandhill Crane and Snow Goose excreta compared to other animal/human fecal sources, which has also been reported for other avian species (Lu et al. 2007, 2008, 2009; Gourmelon et al. 2007; Dubinsky et al. 2012). When including the high occurrence of likely human pathogenic *C. jejuni* from Sandhill Crane excreta, but lack of other pathogens identified during the current study (Table 1; Lu et al. 2013), we now focus on the lines of evidence of this fecal pollution within Platte river water and sediments.

4.2 Pre- and Post-Crane Migration Data

Within river-bottom sediment at the crane roosting site (Fig. 2), enterococci and the crane-specific marker were detected during the migration season but were absent during the non-migration season. *E. coli* ($p<0.05$) and enterococci ($p<0.10$) densities in water were higher at the downstream sites during the migration season than at the upstream site and all sites during the non-migration season. Diurnal studies at the most downstream site (Grand Island) in 2010 indicated highest water sample FIB densities between 2230 and 1100 hours, which is reflective of the normal crane roosting period plus travel time in the stream. Furthermore, the crane-specific marker was only amplified from water samples collected from the two downstream sites during the migration season, not from upstream nor any site during the non-migration season. *Giardia*, which was detected in Sandhill Crane feces but not Snow Goose feces, was

only detected in water during the migration period during 2009 when there were no significant runoff or snowmelt events. The higher flows in 2010, as a result of significant snowmelt and precipitation events, may have diluted cysts below the detection limit.

Nonetheless, the FIB detected in all sediment samples (2009 and 2010) could be the result of multiple fecal sources in the watershed and, for *E. coli*, may be a result of slower die-off in the sediment compared to enterococci (Davies et al. 1995). Indeed, the presence of *Bacteroidetes* and occasional human- and bovine-targeted markers in the river water (Table 1) indicated background contamination from at least these two additional fecal sources. Overall, however, pathogen risks appear to be dominated by *Campylobacter* from crane excreta during the migration period.

Several reports have indicated the possible presence of legionellae in bird excreta when using culture-based approaches. Similarly, in the current study, yet in the current work, confirmation based on partial 16S rRNA gene sequence data indicated that none were in fact *Legionella*. Hence, care is needed when interpreting CFU *Legionella* data, and verification of presumptive colonies is strongly recommended. Nonetheless, shorebirds have been confirmed to carry legionella (e.g., Santos et al. 2012), and bird exposure appeared to be associated with legionellosis cases in Iowa (Helms et al. 1981). In general, however, avian diseases such as psittacosis are considered more likely than legionellosis following exposures to birds (Gacouin et al. 2012). In addition, emerging avian zoonoses such as West Nile virus appear largely associated with corvids, the American robin, and the house sparrows, not waterfowl (Dridi et al. 2013; Crowder et al. 2013; Reisen et al. 2013; Valiakos et al. 2012). Lastly, despite the concern over avian influenza virus zoonosis, in the current study, the lack of avian influenza virus detection could have resulted from insufficient sample volumes being examined and that this virus does not persist in the Platte River environment.

5 Conclusions

Numerous types of FIB and pathogens relevant to humans and agriculture including *E. coli*, enterococci, *Bacteroidetes*, *Cryptosporidium*, *Giardia*, and *Campylobacter* were present in the Platte River during the spring migration periods in 2009 and 2010. The Platte River is a complex system with multiple potential sources for microbial contamination. Nonetheless, results of this study indicate that Sandhill Cranes are likely increasing the concentration of FIB and certain pathogens in the Platte River including the following:

- Within river-bottom sediment at a crane roosting site, enterococci and the crane-specific *Bacteroidetes* marker were detected during the migration season but not during the non-migration season;
- *E. coli* (at the $p<0.05$ level) and enterococci (at the $p<0.10$ level) densities in water were higher at the downstream sites during the migration season than at the upstream site and all sites during the non-migration season;
- Diurnal studies at the most downstream site in 2010 indicated that both *E. coli* and enterococci were greater in water samples collected between 2230 and 1100 hours, which is reflective of the normal crane roosting period plus travel time in the stream;
- The crane-specific *Bacteroidetes* marker in water samples was amplified only for samples collected from the two downstream sites during the migration season, but not for samples from the upstream site or any site during the non-migration season; and,
- *Campylobacter jejuni* appears to be a prevalent pathogen excreted by Sandhill Cranes, and along with *Giardia* (detected in Sandhill Crane feces but not Snow Goose feces), which was only detected in water during the migration period during 2009 when there were no significant runoff or snowmelt events.

E. coli in river-bottom sediment was detected throughout the sampling periods in 2009 and 2010, likely as a result of multiple sources in watershed and slow die-off in the sediment compared to enterococci, and may not be a good indicator of crane impact to the stream. Of the pathogens analyzed during this study, pathogens risks appear to be dominated by *Campylobacter* from crane excreta during the migration period. However, further research is needed to determine if there may be increased avian or human health risk as a result of the presence of any or all of the pathogens in the Platte River during this period.

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