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Source tracking swine fecal waste in surface water proximal to swine concentrated animal feeding operations

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HIGHLIGHTS

- We studied the sanitary quality of surface water proximal to swine CAFOs.
- Fecal indicator bacteria levels suggest poor water quality proximal to swine CAFOs.
- Swine-specific Bacteroidales were more prevalent proximal down- vs proximal upstream.
- Swine-specific Bacteroidales can help track fecal waste proximal to swine CAFOs.

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ABSTRACT

Swine farming has gone through many changes in the last few decades, resulting in operations with a high animal density known as confined animal feeding operations (CAFOs). These operations produce a large quantity of fecal waste whose environmental impacts are not well understood. The purpose of this study was to investigate microbial water quality in surface waters proximal to swine CAFOs including microbial source tracking of fecal microbes specific to swine. For one year, surface water samples at up- and downstream sites proximal to swine CAFO lagoon waste land application sites were tested for fecal indicator bacteria (fecal coliforms, *Escherichia coli* and *Enterococcus*) and candidate swine-specific microbial source-tracking (MST) markers (*Bacteroidales* Pig-1-Bac, Pig-2-Bac, and Pig-Bac-2, and methanogen P23-2). Testing of 187 samples showed high fecal indicator bacteria concentrations at both up- and downstream sites. Overall, 40%, 23%, and 61% of samples exceeded state and federal recreational water quality guidelines for fecal coliforms, *E. coli*, and *Enterococcus*, respectively. Pig-1-Bac and Pig-2-Bac showed the highest specificity to swine fecal wastes and were 2.47 (95% confidence interval [CI] = 1.03, 5.94) and 2.30 times (95% CI = 0.90, 5.88) as prevalent proximal down- than proximal upstream of swine CAFOs, respectively. Pig-1-Bac and Pig-2-Bac were also 2.87 (95% CI = 1.21, 6.80) and 3.36 (95% CI = 1.34, 8.41) times as prevalent when 48 hour antecedent rainfall was greater than versus less than the mean, respectively. Results suggest diffuse and overall poor sanitary quality of surface waters where swine CAFO density is high. Pig-1-Bac and Pig-2-Bac are useful for tracking off-site conveyance of swine fecal wastes into surface waters proximal to and downstream of swine CAFOs and during rain events.

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1. Introduction

Hog production in the United States (US) has shifted from numerous small family farms to fewer large vertically integrated concentrated animal feeding operations (CAFOs) (MacDonald and McBride, 2009; Reimer, 2006). In North Carolina (NC) between 1991 and 1998, the number of swine increased from 3.7 million to over 10 million, placing NC as the second leading state in US pork production (Edwards and Ladd, 2000). Since 1998, NC has remained the second leading US pork producer with recent total hog and pig inventory estimates ranging mostly between 8 to 9 million (NCDACS, 2012; USDA, 2007, 2012, 2013, 2014). Swine CAFOs are disproportionately located in the eastern coastal plain region of NC (Wing et al., 2000) and house large numbers of animals whose waste is collected and stored in open-pits called lagoons before the liquid waste is sprayed onto agricultural fields.

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According to 2012 county-level estimates of the North Carolina Department of Agriculture and Consumer Services, the top five NC hog-producing counties (Duplin, Sampson, Bladen, Wayne, and Jones) are contiguous and have a population of over 5.6 million swine (NCDACS, 2012). Government officials, agricultural experts, and neighbors of swine CAFOs have expressed concern that this scale of swine production and the associated quantity of manure produced in a small area of land could lead to over-application to agricultural fields and off-site conveyance of fecal pollution and contamination of surface waters (USGAO, 2008).

The NC Department of Environment and Natural Resources (NCDENR) permits swine CAFOs as non-discharge facilities. Swine CAFO permits and regulations include nutrient management plans for the application of liquid waste according to agronomic rates of nutrient uptake of crops grown on the permitted land application spray fields (Edwards and Ladd, 2000; NCGA, 1995). However, questions remain about whether fecal pollution from swine CAFOs in NC can be conveyed off-site of permitted spray fields and whether there are impacts on the sanitary quality of surface waters proximal to swine CAFOs (Jongbloed and Lenis, 1998; Krapac et al., 2002; Thurston-Enriquez et al., 2005).

In 2012, Duplin County, NC had an estimated swine population of 2,040,000 and an estimated poultry population (broiler and other meat-type chickens as well as turkeys) of 88,500,000 (NCDACS, 2012). Because sources of fecal contamination of surface water can be diverse – with numerous potential animal and human inputs – better tools and technologies are needed to track species-specific sources of fecal wastes. Microbial source tracking (MST) methods are designed to improve the identification of sources of fecal contamination (Boehm et al., 2013; Dancer et al., 2014; EPA, 2005). Several candidate swine-specific fecal MST markers have been proposed (Mieszkin et al., 2009; Okabe et al., 2007; Ufnar et al., 2007) with variable specificity and unresolved questions about the generalizability of the markers in different geographic locations (Santo Domingo et al., 2007; Stewart et al., 2013). Application of the proposed microbial source tracking markers to help evaluate management practices in agricultural watersheds has also been limited, although studies in Ontario have used *Bacteroidales* markers to assess livestock exclusion practices (Wilkes et al., 2013) and to compare tile drainage management techniques (Wilkes et al., 2014). Determining whether candidate swine-specific fecal MST markers can be detected in environmental waters in NC, an area with high swine density, is important to assess whether these markers could be useful tools to evaluate and implement best management practices (BMPs).

In this study we aimed to evaluate the impact of swine CAFO liquid waste land application on the sanitary quality of proximal surface waters in NC. The study's specific objectives were to estimate concentrations of fecal indicator bacteria (fecal coliforms, *Escherichia coli*, and *Enterococcus*) in surface waters proximal to swine CAFO liquid waste land application spray fields and to field test candidate MST markers of swine fecal wastes in surface water samples proximal to swine CAFO liquid waste land application sites.

2. Methods

2.1. Study location

Sampling was conducted in the coastal plain region of eastern NC where there is a high density of swine, chicken, and turkey CAFOs as well as beef cattle on pasture. Swine CAFOs typically use liquid waste management systems (lagoons and spray fields), whereas most poultry CAFOs in the area use dry litter waste management systems in which waste-laden litter is applied to fields. Many rural homes in the area use septic systems for sewage disposal. Sampling locations were selected proximal upstream and proximal downstream of three swine CAFO liquid waste land application fields (Sites 1–3), where streams could be sampled from a public right-of-way. We use the letters A and B to denote proximal upstream and proximal downstream locations, respectively, at each swine CAFO surface water sampling site; however, "A"

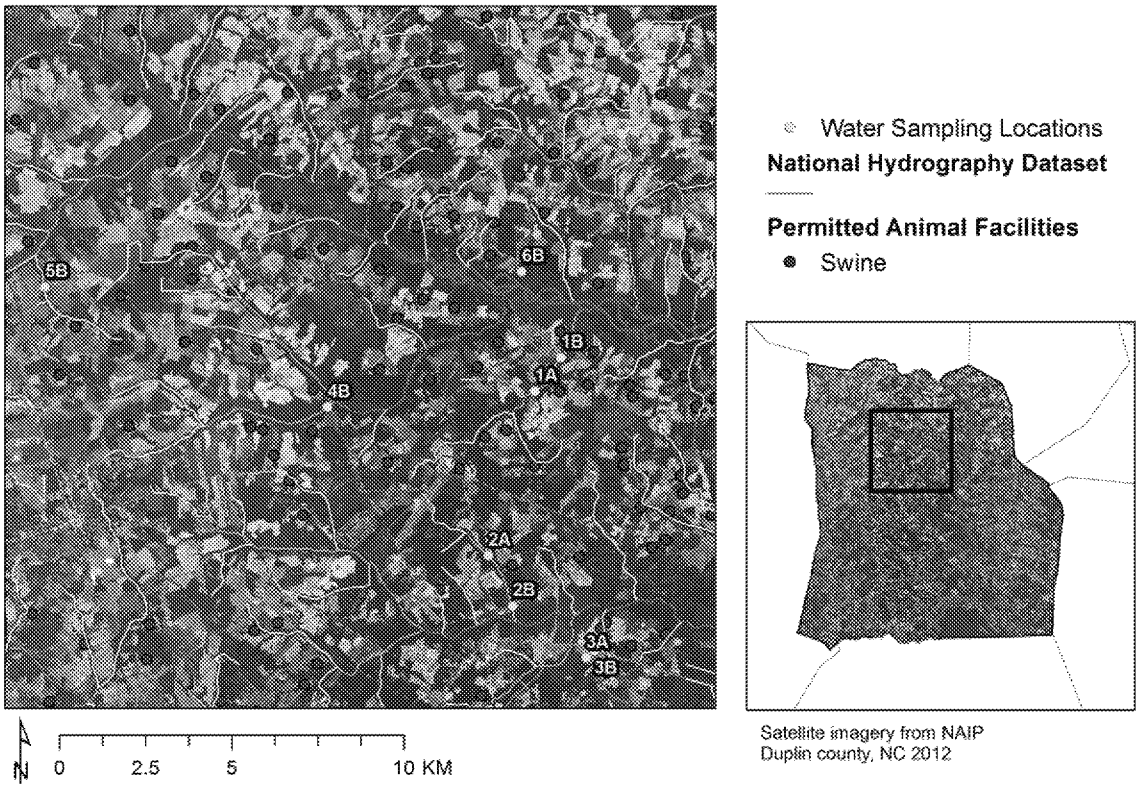

Fig. 1. Map of surface water sampling sites proximal to swine concentrated animal feeding operation spray fields, North Carolina.

sampling locations were proximal and downstream of numerous other swine CAFOs. We could not identify accessible sampling locations in the study watersheds where there were no upstream swine CAFOs.

2.2. Sample collection

A total of 187 surface water samples were collected via weekly sampling for six months (from mid-February to mid-August 2010) and monthly sampling (from mid-September 2010 to mid-January 2011) to capture seasonal trends. Surface water samples were collected from public access waters proximal to swine CAFO liquid waste land application sites (Fig. 1). Seventy six samples were collected at Site A (proximal upstream) locations and 109 at Site B (proximal downstream) locations (2 samples were missing site A/B designations). Sterile 4-liter Nalgene bottles were used for collection after they were washed and autoclaved for 15 minutes at 121 °C. Sample bottles were coded so that sample processors were blinded during laboratory analysis. After collection, samples were transported on ice. All samples were analyzed for fecal coliform bacteria within 24 hours of sample collection. Known-source fecal waste samples (swine lagoon, swine wallow-water, swine feces, and other animal feces) were collected in sterile containers and transported to the laboratory in coolers on ice for analyses. Rainfall data were obtained from a State Climate Office of North Carolina weather station within 27–47 km of the sampling locations. Hourly increments of rainfall (inches) were combined to tabulate the cumulative amount of rain (inches) that fell during the 24 and 48 hours before sampling.

2.3. Fecal indicator bacteria estimates

Fecal indicator bacteria were quantified using standard membrane filtration techniques (APHA, 2006). Fecal coliforms were quantified by membrane filtration using modified fecal coliform (mFC) agar. *Enterococcus* were quantified by EPA method 1600 using modified mE medium (mEI) containing the chromogenic substrate indoxyl-beta-D-glucoside (EPA, 2009a). *E. coli* were quantified by EPA method 1603 using modified m-TEC media (EPA, 2009b). Negative controls were included in each membrane filtration analysis. Samples were filtered in dilutions to obtain counts in the 30–300 colony forming units (CFU)/100 mL range. To test reproducibility of fecal indicator bacteria methods within the laboratory, samples were filtered in duplicate 20% of the time, or every fifth set of samples. All duplicates were within an order of magnitude of each other.

2.4. Swine fecal microbial source-tracking (MST) markers

To examine DNA in each surface water sample, 500 mL of water was filtered using a 0.22 μm Durapore® (Millipore, Billerica, MA) membrane. Excess filter paper, i.e. paper that was not exposed to the sample, was cut aseptically and discarded before placing the filter in a PowerBead tube to extract DNA using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. Similarly, this kit was used to extract DNA from 0.5 g of each known-source fecal sample with use of provided PowerBead tubes, as recommended by the manufacturer. Swine lagoon and wallow water samples were collected in sterile centrifuge bottles and 250 mL of liquid were centrifuged at 3000 ×g for 20 minutes. The supernatant was removed to allow access to the pellet, and 0.5 g of the pellet was placed into a PowerBead tube. Instead of utilizing the MO BIO Vortex Adapter tube holder to vortex the PowerBead tubes for 10 minutes as recommended by the manufacturer, the PowerBead tubes were vortexed using the high energy Mini-Beadbeater (BioSpec Products, Bartlesville, OK) for one minute. DNA extractions were stored at −80 °C and were used for multiple PCR assays.

A series of PCR assays were performed for swine-specific markers. PCR assays for Pig-1-Bac and Pig-2-Bac were performed using a Qiagen QuantiTect Probe PCR kit and the Pig-Bac-2 and P23-2 assays were performed using 5 PRIME MasterMix with the appropriate amount of deionized water and primers according to manufacturer's instructions (Supplemental Table S1). Reactions for Pig-1-Bac and Pig-2-Bac assays were conducted in duplicate using primers and probes described by Mieszkin et al. (2009) using a Cepheid Smart Cycler model SC1000-1. Although Pig-1-Bac and Pig-2-Bac assays were run on a real-time machine quantitative results are not reported because: (1) a standard curve was not consistently run so we are not confident reporting quantitative results; and (2) we wanted to be consistent in our reporting across the assays. Reactions for Pig-Bac-2 and P23-2 assays were performed in duplicate as described by Okabe et al. (2007) and Ufnar et al. (2007), respectively. Reactions were carried out using an Eppendorf MasterCycler gradient thermal cycler; then products were visualized on a 1.5% agarose gel. All assays were performed with negative controls. An internal amplification control (IAC) for the P23-2 assay was used as described by Ufnar et al. (2007). This IAC was also tested to determine the lower limit of detection (10^{-5} μM). For the *Bacteroidales* PCR assays, extracts from a positive lagoon sample and two pig fecal samples were used as positive controls. The same samples were consistently used as positive controls, although multiple extracts were utilized from the samples over the course of the study.

A separate PCR assay using salmon sperm DNA was performed to test for inhibition in each DNA extract (Haugland et al., 2005). A known amount of salmon sperm DNA was injected into each DNA extract as well as a positive control. Duplicate PCRs were performed using a Qiagen QuantiTect Probe PCR kit in a Cepheid Smart Cycler model SC1000-1. The sample was considered inhibited if the difference of cycle threshold (C_T) between extract and control was greater than 3.3. If inhibited, the DNA extract was diluted tenfold and tested for inhibition again. Once an extract was considered to not be inhibited, it was retested for the four swine assays: Pig-1-Bac, Pig-2-Bac, Pig-Bac-2, and P23-2.

To examine the sensitivity and specificity of the four candidate swine-specific fecal microbial source-tracking markers, we tested pig fecal (n = 6), pig wallow water (n = 2), pig waste lagoon (n = 7) as well as chicken (n = 6), turkey (n = 3), goat (n = 2), cow (n = 4), horse (n = 1) and human (n = 3) fecal samples collected from sites in NC. Sensitivity of each of the four candidate swine-specific fecal microbial source-tracking markers was calculated as the proportion of known-source swine fecal samples that tested positive for each marker. Specificity was calculated as the proportion of known-source non-

Table 1
Fecal coliform, *E. coli*, and *Enterococcus* concentrations (CFU/100 mL) in surface waters at A and B sites proximal to swine concentrated animal feeding operation spray fields in North Carolina.

	Fecal coliforms (CFU/100 mL)				*E. coli* (CFU/100 mL)				*Enterococcus* (CFU/100 mL)			
	N	Range	Geo. mean	*p*-Value[a]	N	Range	Geo. mean	*p*-Value[a]	N	Range	Geo. mean	*p*-Value[a]
All A sites 1–3	76	0.5, 9091	111		76	0.4, 2090	78		75	1, 8517	89	
All B sites 1–3	76	0.5, 140,000	187	0.09	76	1, 5400	106	0.22	75	1, 10,400	103	0.64
All B sites 4–6	33	10, 117,273	331	–	33	10, 3167	121	–	33	10, 4267	220	–

Note. Site A = proximal upstream sampling location. Site B = proximal downstream sampling location. CFU = colony forming unit.
[a] T-test statistic from fixed-effects generalized linear regression model to account for repeated measures at each site.

swine fecal samples (i.e., chicken, turkey, goat, cow, horse, human) that tested negative for each marker.

2.5. Statistical analysis

Descriptive statistics were calculated for each of the fecal indicator bacteria estimates in surface water. T-test statistics were estimated using conditional fixed-effects linear regression models to account for repeated sampling at each site (Allison, 2005). Estimates of the concentration of each fecal indicator bacteria were compared to recommendations set by the North Carolina Department of Environment and Natural Resources (DENR) Division of Water Quality (DWQ) "Redbook" (NCDENR, 2007) and the United States Environmental Protection Agency (EPA) recreational water quality guideline values (EPA, 2012). We calculated the proportion of samples that exceeded state (NCDENR, 2007) and federal (EPA, 2012) recreational fresh water quality guideline values by tabulating the number of samples greater than 200 CFU/100 mL, 235 CFU/100 mL, and 70 CFU/100 mL for fecal coliforms, *E. coli*, and *Enterococci*, respectively. Exact chi-square tests were calculated to compare the frequency of exceedance of each water quality criterion by CAFO sampling site and by B versus A site. Odds ratios (OR) and 95% confidence intervals (CI) were estimated using conditional fixed-effects logistic regression models to account for repeated sampling at each site (Allison, 2005).

To quantitatively compare concentrations of fecal indicator bacteria at A and B locations within Sites 1–3, the mean and 95% confidence interval were calculated for each fecal indicator's pair-wise difference of Site B minus Site A concentrations by site. A positive mean value indicates that the concentration of fecal indicator bacteria was higher at the Site B compared to Site A location. A negative mean value indicates the concentration of a fecal indicator was lower at the B site compared to the A site at each water sampling location.

The frequency of detection of candidate MST markers was tabulated across all sites and by site. Exact chi-square tests were calculated to compare the frequency of detection of candidate MST markers by site. Fixed effects linear and logistic regression models were used to estimate associations between fecal indicator bacteria, presence of swine markers, and rainfall (Allison, 2005). Cumulative rainfall during the 24 and 48 hours before sample collection was considered in analyses with fecal indicator bacteria and MST markers as a continuous (inches) and a binary (>versus ≤ the mean of cumulative inches of rainfall) variable.

Because this is not a randomized study, statistical significance cannot be interpreted as the probability that an observed difference would occur by chance if there is truly no difference between groups being compared. However, *p*-values are presented so that results can be easily compared with other studies. Fecal indicator bacteria concentrations were $\log_{10}$-transformed prior to analysis. All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Fecal indicator bacteria concentrations in surface waters proximal to swine CAFOs

The highest maximum concentrations of fecal coliforms, *E. coli*, and *Enterococci* observed were 140,000, 5400 and 10,400 CFU/100 mL, respectively, and were measured at Site B locations (Table 1). In general, the Site B samples had higher geometric mean and maximum fecal indicator bacteria values compared to Site A samples (Table 1). The highest concentrations of fecal indicator bacteria were detected in the spring and summer months (Fig. 2a-c).

3.2. Exceedance of recreational water quality guideline values proximal to swine CAFOs

For fecal coliforms, *E. coli*, and *Enterococcus*, 74/187 (40%), 43/187 (23%), and 112/185 (61%) of all surface water samples exceeded the respective recreational water quality guideline values of 200 CFU/100 mL, 235 CFU/100 mL, and 70 CFU/100 mL (Table 2). Across Sites 1–3, recreational water quality guideline value exceedance was 1.86 (95% confidence interval (CI) = 0.96, 3.62), 1.73 (95%

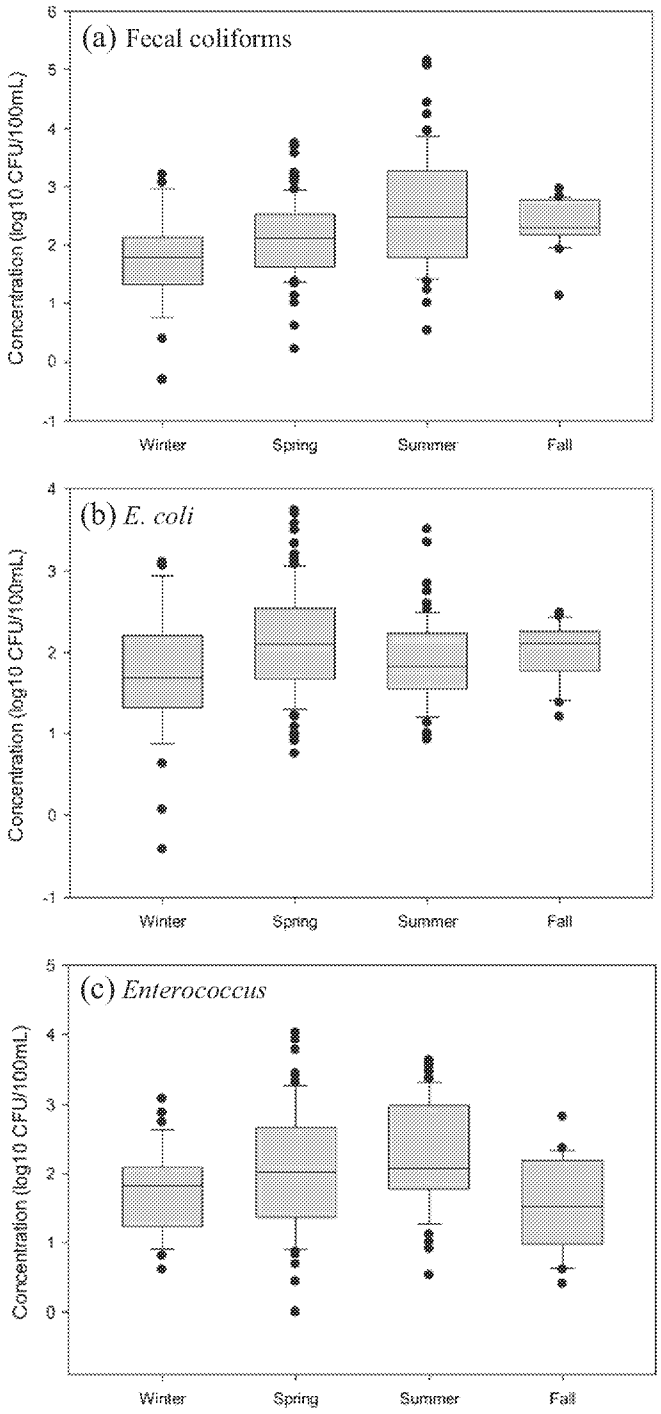

Fig. 2. a–c. Boxplot comparison of concentrations ($\log_{10}$ CFU/100 mL) of: (a) fecal coliforms (b) *E. coli* and (c) *Enterococcus* by season for all surface water samples at sites proximal to swine concentrated animal feeding operation spray fields in North Carolina. Median line and interquartile range depicted by boxes; range depicted by whiskers; outliers depicted by circular dots.

CI = 0.79, 3.78), and 1.49 (95% CI = 0.77, 2.88) times as prevalent at Site B compared to Site A locations (Table 2). For each of the fecal indicator bacteria, the greatest frequency of exceedance of recreational water quality guideline values was observed in the summer, followed by the spring (data not shown).

3.3. Mean pair-wise differences in fecal indicator concentrations

Across Sites 1–3, the means of the pair-wise differences (Site B value minus Site A value) for all three fecal indicator bacteria were positive (greater than the null value of mean equal to zero) (Table 3). The site-specific pair-wise differences were all positive except for *E. coli* at Site 3 and *Enterococcus* at Site 2 (Table 3). These two negative values were the smallest absolute differences in means observed.

3.4. Swine-specific fecal microbial source tracking markers in surface water proximal to swine CAFOs

The sensitivity of the three *Bacteroidales* markers Pig-1-Bac, Pig-2-Bac and Pig-Bac-2 was 80%, 87%, and 93%, respectively. The methanogen candidate swine-specific marker P23-2 was not detected in any of the known-source samples (while its internal amplification control was observed in every reaction). The specificities of Pig-1-Bac, Pig-2-Bac, and Pig-Bac-2 were 100%, 100%, and 37%, respectively.

The two *Bacteroidales* markers with 100% specificity for swine fecal pollution, Pig-1-Bac and Pig-2-Bac, were detected in 17% and 14% of surface water samples, respectively (Table 4). Pig-1-Bac was present each time Pig-2-Bac was detected and was also detected in six more samples than Pig-2-Bac. At sites where both A and B samples were collected (Sites 1–3), the difference in detection frequency at B compared to A sites was pronounced (Table 4). The odds of detecting the swine-specific fecal *Bacteroidales* marker Pig-1-Bac at Site B locations was 2.47 (95% CI = 1.03, 5.94) times the odds at Site A locations (Table 4). Site 1 demonstrated the most prominent difference in detection frequency between Site B and Site A (Pig-1-Bac OR = 6.76; 95% CI = 1.12, 40.8). The only instance in which the frequency of detection was higher at Site A than Site B was at Site 2 for *Bacteroidales* Pig-Bac-2. But Pig-Bac-2 was not a specific microbial source tracking marker for swine fecal waste. At Site 2, the two swine specific fecal *Bacteroidales* microbial source-tracking markers (Pig-1-Bac and Pig-2-Bac) were never detected at the Site A location. The swine-specific *Bacteroidales* markers Pig-1-Bac and Pig-2-Bac were most prominent during the winter (n = 32) months, with a detection frequency of 59% and 53%, respectively (data not shown). Pig-1-Bac and Pig-2-Bac were detected less frequently (15% and 10%, respectively) during the spring (n = 73) and were not detected during the summer (n = 62) and fall (n = 17) (data not shown).

3.5. Relation of rainfall with fecal indicator bacteria and swine-specific fecal microbial source tracking markers

In the 48 hours preceding sampling, the maximum cumulative inches of rainfall was 2.94 inches (Table S2). Mean fecal coliform, *E. coli* and *Enterococcus* levels increased as antecedent cumulative rainfall increased (Fig. 3; Table S3). Fecal coliforms, *E. coli*, and *Enterococcus* concentrations ($\log_{10}$ CFU/100 mL) increased 0.29 (95% confidence interval [CI] = 0.09, 0.49), 0.45 (95% CI = 0.27, 0.59), and 0.50 (95% CI = 0.31, 0.69), respectively, for every one-inch increase in cumulative rainfall in the 48 hours before sample collection, adjusting for season (Table S3).

Across all sites, the swine-specific fecal microbial source tracking markers Pig-1-Bac and Pig-2-Bac were detected more frequently when 48 hour antecedent cumulative rainfall (inches) was greater than versus less than or equal to the mean (Table 5). The odds of detecting Pig-1-Bac during time periods when 48 hour antecedent cumulative rainfall was greater than the mean were 2.87 times (95% CI = 1.21, 6.80) the odds during time periods when 48 hour antecedent cumulative rainfall was less than or equal to the mean (Table 5). Fecal indicator bacteria concentrations were not observed to be associated with swine-specific fecal microbial source tracking markers Pig-1-Bac and Pig-2-Bac (data not shown).

4. Discussion

The results of our study suggest an overall diffuse and poor microbial quality of surface waters proximal to swine CAFO liquid waste land application sites in NC, the second largest hog-producing state in the US. Fecal indicator bacteria were detected at concentrations that exceeded federal and state recreational water quality guideline values, with the highest concentrations observed immediately downstream of swine CAFO spray fields and in the spring and summer seasons. While some mean differences in fecal indicator bacteria were detected at Site A (proximal upstream) and Site B (proximal downstream) surface water sampling locations (e.g., higher Site B maximum values; positive mean pair-wise difference values; higher frequency of exceedance of fecal indicator guideline values at Site B compared to Site A locations), fecal indicator bacterial contamination was observed at both A and B locations.

While the study design allowed a comparison of Site A (upstream) and Site B (downstream) locations proximal to swine CAFO liquid waste land application sites, it is important to note that the Site A locations did not represent pristine non-impacted sites. Because the study sites in eastern NC were located among one of the top hog-dense counties in the US (Feedstuffs, 2013a,b; USDA, 2007), the Site A (proximal upstream) locations in our study were potentially influenced by numerous upstream swine CAFO liquid waste land application sites as well as poultry CAFO dry litter land application sites. Because fecal indicator bacteria (fecal coliforms, *E. coli*, *Enterococcus*) are non-specific indicators

Table 2
Frequency of exceedance of recreational water quality guideline values for fecal coliforms, *E. coli*, and *Enterococcus* at A and B sites proximal to swine concentrated animal feeding operation spray fields in North Carolina.

	Fecal coliforms		*E. coli*		*Enterococcus*	
	(200 CFU/100 mL)[a]		(235 CFU/100 mL)[b]		(70 CFU/100 mL)[b]	
	N exceed/total (%)	OR (95% CI)[c]	N exceed/total (%)	OR (95% CI)[c]	N exceed/total (%)	OR (95% CI)[c]
All sites	74/187 (40)	–	43/187 (23)	–	112/185 (61)	–
All A sites 1–3	24/76 (32)	Ref	13/76 (17)	Ref	40/75 (53)	Ref
All B sites 1–3	35/76 (46)	1.86 (0.96, 3.62)	20/76 (26)	1.73 (0.79, 3.78)	47/75 (63)	1.49 (0.77, 2.88)
All B sites 4–6	15/33 (46)	–	10/33 (30)	–	25/33 (76)	–

Note. Site A = proximal upstream sampling location. Site B = proximal downstream sampling location. OR = odds ratio. CI = confidence interval. CFU = colony forming unit. Ref = referent category.
[a] Based on North Carolina Department of Environment and Natural Resources surface water standards (NCDENR, 2007).
[b] Based on 2012 USEPA recreational water quality criteria beach action values (BAV) (EPA, 2012).
[c] Odds ratio and 95% confidence interval derived from fixed-effects logistic regression model to account for repeated measures at each site.

Table 3
Mean of pair-wise differences of fecal indicator bacteria concentrations (CFU/100 mL) in surface waters at B sites minus A sites proximal to swine concentrated animal feeding operation spray fields in North Carolina.

	Fecal coliforms			*E. coli*			*Enterococcus*		
	CFU/100 mL			CFU/100 mL			CFU/100 mL		
	N[a]	Mean[b]	95% CI	N[a]	Mean[b]	95% CI	N[a]	Mean[b]	95% CI
All sites 1–3	75	2266	−1180, 5712	75	129	−49, 307	74	89	−103, 281
Site 1	13	384	−357, 1125	13	504	−347, 1355	13	341	−145, 827
Site 2	31	4387	−3886, 12,660	31	117	−83, 317	30	−32	−350, 286
Site 3	31	934	−228, 2096	31	−19	−156, 118	31	99	−177, 375

Note. Site A = proximal upstream sampling location. Site B = proximal downstream sampling location. CI = confidence interval.
[a] Number of pair-wise samples.
[b] Mean of the pair-wise differences of concentrations of each fecal indicator bacteria (B sites minus A sites).

of fecal pollution – reflecting inputs from diverse fecal waste inputs, including hog and poultry CAFOs as well as other diffuse sources – this could account for the elevated levels of fecal indicator bacteria at Site A (proximal upstream) compared to Site B (proximal downstream) locations.

Bacteriodales markers Pig-1-Bac and Pig-2-Bac, which were developed and validated in other regions of the world, were tested against known-source swine and other animal fecal samples from NC and both showed a specificity of 100% to known-source swine fecal wastes. This supports the findings of Mieszkin et al. (2009) who also observed specificities of 100% for both markers in France. The lower sensitivity of Pig-1-Bac (80%) and Pig-2-Bac (87%) than observed in France (98–100%) may be explained by our inclusion of swine wallow water as a potential source of swine waste, which was not investigated in the French study (Mieszkin et al., 2009). Exclusion of these swine wallow water samples (which tested negative) would have resulted in a higher sensitivity for Pig-1-Bac (92%) and Pig-2-Bac (100%).

This is the first study to examine whether Pig-1-Bac and Pig-2-Bac would be appropriate as indicators of swine-specific fecal waste runoff under field conditions at ambient surface water locations proximal to swine CAFO liquid waste land application sites in NC. The presence of swine-specific Pig-1-Bac and Pig-2-Bac fecal MST markers off-site in these surface waters indicates that swine CAFO liquid waste land application practices in NC can lead to off-site migration of swine fecal wastes. Our observation that Pig-1-Bac was 2.47 times as prevalent at proximal downstream compared to proximal upstream sampling locations also suggests that fecal wastes from swine CAFO liquid waste land application sites can negatively influence proximal downstream surface water quality.

During our study period, the maximum cumulative rainfall 48 hours antecedent to sampling was 2.94 inches (Table S2), which is not suggestive of heavy rainfall conditions. The low amount of rainfall during our study is relevant to the NC regulatory framework because it requires that animal waste management systems "not cause pollution in the waters of the State, except as may result because of rainfall from a storm event more severe than the 25-year, 24-hour storm" (NCGA, 1995). Neighbors and community groups in NC have observed swine CAFO operators spraying before forecasted rainfall and also during rain events to avoid an overflow or breach of waste lagoons.

Rainfall was strongly associated with fecal indicator bacteria concentrations in our study – particularly *E. coli* and *Enterococcus* – which is consistent with a loading mechanism of increasing fecal indicator bacteria levels in surface waters during rainfall-induced run-off. Future studies should employ a sampling strategy to capture the effects of rainfall through targeted sampling at multiple time points during storm events to characterize the temporal dynamics of fecal pollution loading during run-off conditions. Future studies should also target specific swine liquid waste spraying events — i.e., sampling at times during and after swine liquid lagoon wastes are sprayed onto fields.

Rainfall was strongly associated with the frequency of detection of Pig-1-Bac and Pig-2-Bac MST markers. Pig-1-Bac and Pig-2-Bac were detected roughly three times as frequently during periods when cumulative antecedent 48 hour rainfall was greater than versus less than or equal to mean rainfall. This association between rainfall and swine-specific MST markers Pig-1-Bac and Pig-2-Bac provides evidence of a rainfall-induced loading mechanism of swine fecal wastes in surface waters proximal to and off-site of swine CAFO liquid waste land application sites. However, the sample size was too small to draw conclusions about rainfall-swine MST marker associations at Site B (proximal downstream) compared to Site A (proximal upstream) locations.

Concentrations of fecal indicator bacteria and exceedances of recreational water quality guideline values were not associated with the presence of swine MST markers (data not shown). Because fecal indicator bacteria reflect both point and non-point sources of fecal pollution from warm-blooded animals as well as other non-fecal sources (e.g., bacterial re-growth in the environment (Byappanahalli et al., 2006)), it is not surprising that these measures were observed to be poor predictors of MST markers specific to swine fecal wastes.

Mieszkin et al. (2009) reported that Pig-2-Bac was a more suitable marker than Pig-1-Bac because it was detected more frequently in water samples. Our field assessment in NC slightly contradicts these findings because we detected Pig-1-Bac in six samples in which Pig-2-Bac was not detected, while Pig-2-Bac was never detected in the absence of Pig-1-Bac. Our results suggest that it may be advisable to utilize both markers together, as protocols involving two PCR assays from the same DNA extract do not involve much additional cost or effort compared to protocols involving one PCR assay.

Table 4
Occurrence of two swine-specific fecal *Bacteroidales* microbial source tracking markers in surface water samples at A and B sites proximal to swine concentrated animal feeding operation spray fields in North Carolina.

	Pig-1-Bac		Pig-2-Bac	
	N pos./total (%)	OR (95% CI)[a]	N pos./total (%)	OR (95% CI)[a]
All sites	31/182 (17)	–	25/182 (14)	–
All A sites 1–3	10/74 (14)	Ref	8/74 (11)	Ref
All B sites 1–3	20/75 (27)	2.47 (1.03, 5.94)	16/75 (21)	2.30 (0.90, 5.88)
All B sites 4–6	1/33 (3)	–	1/33 (1)	–

Note. Site A = proximal upstream sampling location. Site B = proximal downstream sampling location. OR = odds ratio. CI = confidence interval.
[a] Odds ratio and 95% confidence interval derived from fixed-effects logistic regression model to account for repeated measures at each site.

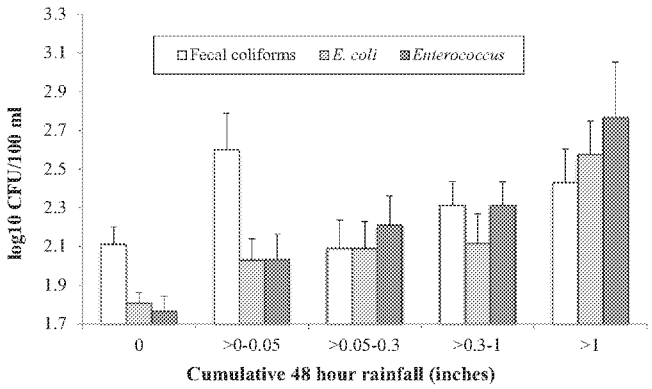

Fig. 3. Mean fecal indicator bacteria concentrations (log10 CFU/100 mL) by cumulative amount of rainfall (inches) during the 48 hours prior to sampling at sites proximal to swine concentrated animal feeding operation spray fields in North Carolina. Error bars represent the standard error of mean fecal indicator bacteria concentrations.

It is possible that swine fecal wastes were present in surface water samples when Pig-1-Bac and Pig-2-Bac were not detected. Sensitivity below 100% indicates that the MST marker was not detected in all known-source swine fecal waste samples. Furthermore, the persistence of these *Bacteriodales* MST markers (which are based upon anaerobic bacteria) is not well understood under ambient surface water conditions. A study of the effect of oxygen and temperature on the persistence of Pig-1-Bac and Pig-2-Bac reported a one-log reduction of the markers after eight to ten days in microcosms at 20 °C under aerobic conditions (Marti et al., 2011).

The seasonal variability of Pig-1-Bac and Pig-2-Bac in this study was somewhat surprising considering Mieszkin et al. (2009) reported temporal stability of Pig-1-Bac and Pig-2-Bac over a 48-month period. However, Mieszkin et al. (2009) likely meant that the markers were stable from year to year, as they did include enough samples to test seasonal differences. Recent research has established that lower temperatures result in slower *Bacteroidales* 16S rRNA gene decay (Bell et al., 2009; Schulz and Childers, 2011). Because Pig-1-Bac and Pig-2-Bac may persist in colder environments and decay more rapidly in warmer environments, it is possible that they were either absent in the environmental samples collected in NC during the warmer months, or were present at levels below the assay detection threshold. The warmer temperatures in NC could explain why these markers were not detected throughout the year.

This seasonal pattern, where the swine-specific MST markers were detected more frequently in winter, is in direct contrast to the typical seasonal pattern observed for fecal indicator bacteria. In this study and elsewhere (Cha et al., 2010; Tiefenthaler et al., 2009; Wilson et al., 2007), measures of fecal indicator bacteria in water are typically higher in warmer (summer) than in colder (winter) months. This marked difference in seasonal patterns is most likely attributable to the fact that traditional measures of fecal indicator bacteria are culture-based and target vegetative bacterial cells accustomed to growing in the warm environment of mammalian guts. Microbial source tracking markers, on the other hand, typically rely on detection of DNA specific to the cells of anaerobic bacteria. Both the cells and the DNA degrade more quickly in warm weather, likely causing lower frequencies of their detection in summer months (Schulz and Childers, 2011). Rainfall, which was higher during the spring and summer months of our study, may also contribute to the observed seasonal pattern of Pig-1-Bac and Pig-2-Bac presence.

The low specificity of Pig-Bac-2 (37%) demonstrates that this marker was not useful to distinguish swine from other animal sources of fecal waste. This marker had a low specificity because it was detected in chicken, cow, goat, horse, human, and turkey fecal samples. To our knowledge no other study has investigated the sensitivity and specificity of Pig-Bac-2 since publication of the assay, which included test samples from humans, cows and swine (Okabe et al., 2007). Lamendella et al. (2009) also observed a poor specificity of Pig-Bac-1, the other swine *Bacteroidales* marker proposed by Okabe et al. (2007), because it was detected in cattle, human, chicken, raccoon, and horse fecal samples. Since we did not detect Methanogen P23-2 in any known source sample (swine or other animal) or in any surface water samples, it appears to have limited utility for detecting swine waste in surface water samples in NC.

Several study limitations should be considered. We did not sample known-source swine fecal wastes from the lagoons of the swine CAFOs proximal to our selected surface water sampling sites. Future studies could improve understanding of off-site transport through on-site sampling of swine CAFOs spray-field run-off and of lagoon waste in addition to the proximal surface waters. We did not generate quantitative PCR results for Pig-1-Bac and Pig-2-Bac. Although assays were run on a real-time PCR machine, materials for a standard curve were not available and cycle threshold values were not recorded, which restricted analysis of these markers to their presence versus absence. Due to the high density of swine and other animal CAFOs in the study area we were unable to sample at un-impacted or pristine upstream sites. Future studies should attempt to include such un-impacted sites and also consider use of additional microbial source tracking markers to evaluate the relative contribution of swine versus other animal sources (e.g., chicken, turkey, human) of fecal pollution.

5. Conclusions

Evidence of high concentrations of fecal indicator bacteria and the presence of swine-specific fecal MST markers in surface waters proximal to swine CAFO liquid waste land application sites is relevant to evaluating the effectiveness of current technologies and policies for protecting the sanitary quality of surface waters proximal to swine CAFOs. These results could inform management decisions about liquid waste disposal practices, particularly landscapes where swine density is high and that are susceptible to over-land run-off from rainfall and flooding (e.g., NC coastal plain) (Wing et al., 2002). Use of swine-specific fecal MST markers Pig-1-Bac and Pig-2-Bac could help identify surface waters for targeted restoration, and help inform rules governing permitting, waste management (including storage,

Table 5
Relation between occurrence of swine-specific fecal *Bacteroidales* microbial source tracking markers in surface water samples and cumulative rainfall in the 48 hours before sample collection at sites proximal to swine concentrated animal feeding operation spray fields in North Carolina.

	Pig-1-Bac		Pig-2-Bac	
	N pos./total (%)	OR (95% CI)[a]	N pos./total (%)	OR (95% CI)[a]
All sites				
Cum. rainfall ≤ mean[b]	16/131 (12)	Ref	12/131 (9)	Ref
Cum. rainfall > mean[b]	15/53 (28)	2.87 (1.21, 6.80)	13/53 (25)	3.36 (1.34, 8.41)

Note. OR = odds ratio. CI = confidence interval.
[a] Odds ratio and 95% confidence interval derived from fixed-effects logistic regression model to account for repeated measures at each site.
[b] Stratified by time periods > vs ≤ the mean cumulative inches (0.248) of rainfall in the 48 hours before sample collection.

treatment, and disposal), and swine stocking density. Future studies should utilize swine-specific *Bacteroidales* fecal MST markers as they appear to represent important tools to advance understanding of impacts on water quality in areas with intensive swine production.

Abbreviations

CAFO	concentrated animal feeding operation
CFU	colony forming unit
PCR	polymerase chain reaction

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

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Appendix A. Supplementary data

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