Detection of Antimicrobially Resistant *E. coli* and Nitrates in Groundwater on or Near Swine Farms in Eastern North Carolina

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ABSTRACT

Antibiotic use for growth promotion and disease treatment in commercial swine production has caused high proportions of multiply antibiotic-resistant enteric bacteria to be fecally shed by animals and raised concerns about waterborne spread of these bacteria via land application and lagoon leakage. Nitrate is another environmental impact on groundwater quality from irrigating with fertilized water or fecal wastes. A study was conducted to quantify and compare the presence of antibiotic-resistant *E. coli* and nitrates in groundwater on swine and crop farms. Four study sites (2 swine farms, 2 reference sites) with known groundwater flow paths were screened for *E. coli* and nitrates four times over 1.5 years. A total of 100 biochemically-confirmed *E. coli* were isolated from groundwater. There were statistically significantly higher *E. coli* concentrations at the two swine farms than at the reference sites by Kruskall-Wallis χ^2 test. Bacterial isolates were tested for antibiotic resistance using a panel of 17 drugs that are typical of human and veterinary use. There were 19 and 71 *E. coli* isolates from swine farms #1 and #2 that were resistant to 0-2 and 0-6 antimicrobials, respectively. Of the 10 *E. coli* isolates from reference sites only one from each site showed antimicrobial resistance traits. Nitrate was found on all four sites, but at especially elevated levels in the shallow wells at swine farm #1. There were higher groundwater nitrate levels at swine farm #1 than at swine farm #2, but there were fewer *E. coli* in groundwater at swine farm #1 than at swine farm #2. These findings demonstrate that elevated levels of antibiotic-resistant *E. coli* and nitrates are present in groundwater of swine farms having lagoon and land application system for waste management. However, nutrient concentrations are not predictive of bacteria densities in groundwater and vice-versa.

INTRODUCTION

The commercial swine industry uses antibiotics for growth promotion and disease treatment which has led to high proportions of multiply antibiotic-resistant enteric bacteria being shed by these animals. There are rising concerns about the spread of these enteric bacteria into the environment. North Carolina is second in swine production in the United States, with nearly 10 million animals raised on more than 3000 farms, predominantly in the Eastern, coastal plain regions of the State. The porous soils and seasonally high water tables of this coastal plain region can cause underlying groundwater to be vulnerable to surface contamination from a variety of waste sources, including human and animal wastes. Previous studies have documented groundwater contamination by nitrates in the vicinity of swine farms, especially near the

anaerobic lagoons in which swine waste is stored prior to periodic land application of the lagoon liquid. However, little is known about the extent to which swine farm wastes pose risks of enteric microbial contamination of groundwater.

Escherichia coli bacteria are universally present in feces of all mammals at concentrations of over one billion per gram of feces, and are the most fecal-specific member of the coliform group (Edberg et al., 1997). *E. coli* is the only member of the coliform group that is unquestionably an inhabitant of the intestinal tract; hence it has come to be the definitive organism for demonstrating fecal pollution in water (Anonymous, 1987). Because the presence of *E. coli* is considered evidence of a direct public health risk from enteric pathogens, there is zero tolerance for these bacteria in drinking water. Therefore, *E. coli* has been deemed by some authorities to be the most efficacious indicator for public health protection (Edberg et al., 1997). The half-life of *E. coli* is conservatively estimated to be at least eight days under groundwater conditions, and therefore, the recovery of *E. coli* from a groundwater contamination event should be possible for weeks, although the large dilution factor of aquifers must be taken into consideration (Edberg et al., 1997). Due to the carriage of *E. coli* by many other warm-blooded mammals besides humans, its mere presence is not a specific indicator of the source of contamination (Parveen et al., 1999), and the absence of *E. coli* is not conclusive evidence of absence of fecal contamination and enteric pathogens.

Bacterial multi-drug- to pan- resistance is an important emerging public health issue. Antimicrobial resistant bacteria are starting to spread outside of hospitals, which have been the traditional United States reservoir (CDC, 2002). About half of the antibiotics produced globally flow into the agriculture industry to treat infections and promote growth. Many studies have documented high percentages of antimicrobial resistant bacteria in livestock waste, and human exposure to animal fecal bacteria has been documented. The pathways of exposure to these resistant bacteria must be established. Waterborne exposure is a possibility through drinking water that is contaminated with farm-origin antimicrobial resistant bacteria. One study (Marshall et al., 1990) demonstrated the stability of resistant strains of bacteria in the environment. Pigs were inoculated with an antimicrobial resistant strain of swine *E. coli*, and they found the resistant strain in water, bedding materials, mice, flies, and a human caretaker during the four-month follow up period. Another study (Mathew et al., 1999) determined that resistance patterns differed between farm types and pigs of differing ages, indicating that pig age and degree of

antibiotic use affect the resistance of fecal *E. coli*. Therefore, as antibiotic use changes, so do bacterial patterns of antibiotic resistance.

Testing for nitrate is considered the best indicator for nutrient levels in groundwater due to the rapid conversion of ammonia and nitrite to nitrate under most conditions, the high solubility and mobility of nitrate and the typical lack of phosphorus present in groundwater (Brady and Weil, 1999). Nitrate is also considered an environmental indicator for contamination from activities such as irrigating with fertilized water. Nitrate is very mobile in groundwater and will move with it without transformation and little or no retardation (Freeze and Cherry, 1979). The quantity of nitrate lost in drainage water will depend on the rate of water leaching through the soil, the concentrations of nitrates in the drainage water, precipitation and irrigation rates, and the soil texture and structure. When irrigating with fertilized water, heavy nitrogen fertilization can exceed what the plants are able to utilize, and thus be a major source of nitrogen leaching. Therefore, ineffective management of manure from concentrated livestock production facilities is a potential cause of nitrate contamination of groundwater.

Shallow groundwater under some farm conditions has been shown to contain nitrate in excess of the legal limit for drinking water in the United States (levels at 45 mg/L) (Brady and Weil, 1999). The maximum contaminant level (MCL) for nitrate in drinking water in the United States is 10 mg/L as elemental nitrogen (USEPA, 2002). The accumulation of high levels of nitrates in groundwater is associated with methemoglobinemia, a potentially lethal blood disorder that affects infants under six months of age (Savard, 2000). Therefore, the timing of nitrogen inputs is critical so as to minimize the potential for nitrate leaching, and this potential is greatest where inputs of water (irrigation, rainfall) and nitrates are high, and the removal of water and nitrates from the soil (evaporation and plant uptake) are low. These conditions typically occur in late fall, winter, and early spring in humid temperate climates.

The purpose of this study is to quantify the extent of release of antibiotic-resistant *E. coli* and nitrates from commercial swine farms into groundwater so as to determine the relationship between current swine waste management practices in Eastern North Carolina and the effects on microbiological and chemical groundwater quality.

MATERIALS AND METHODS

Four study sites that are located in the Neuse River Basin in Eastern North Carolina, have United States Geological Survey (USGS) monitoring wells and with known groundwater flow paths (Spruill, 2000) were screened for *E. coli* and nitrate nitrogen in groundwater. The first site (swine farm #1) has swine from the feeder to finish production state and has a design capacity for 5000 animals. The swine waste is flushed from the barns with running water and is sent to one lagoon at the end of the barns via pipes for storage and partial treatment prior to land application. The lagoon liquid is applied to fields surrounding the lagoons via pumps that take the material directly from the upper level of the lagoon and apply it with a sprayer. There are a total of 25 groundwater wells at this site, and they are located both up- and down-gradient of the lagoon and in the middle of the sprayfields. The second site (swine farm #2) is a farrow to finish swine production operation with a design capacity for 1500 animals. There is an alternative technology in place at this facility in which the waste solids are removed from the barn flush and are de-watered via compaction. The solids are applied to the field, and the liquid is directed to the lagoon. There are 12 groundwater wells at this site, and they are all located down gradient of the swine barns and lagoon with regards to groundwater flow path. The third site (reference #1) has crops such as corn, soybeans, and wheat that are grown on the field that forms the recharge area for the wells at this site. Commercial fertilizer of unknown quantity was applied on two dates prior to the onset of sampling, and a small herd of cattle of unknown type were also allowed to graze about 80 feet up gradient from the location of the wells. There are a total of 7 groundwater wells at this site. The fourth site (reference #2) is an agricultural site with crops, but does not apply swine waste to the land nor are there any agricultural animals present. The study site consists of an upland field where corn, soybeans, wheat and tobacco are grown, and there are 6 groundwater wells at this site.

Groundwater samples were collected four times over 1.5 years for microbial indicator and nutrient analyses. They were collected aseptically as grab samples in sterile 4-L bottles using standard procedures and placed in coolers with freezer packs. The tubing in the sampling pump was cleansed with 70% ethanol between samples to ensure no cross-contamination of the water samples from well to well. The water samples were analyzed within 30 hours of collection for *E. coli* densities. Bacteriological analyses for *E. coli* were performed by filtering water samples through 47 mm, 0.45-μm pore-sized cellulose ester filters in standard sterile membrane filter

apparatus. After filtration, each membrane filter was placed on an appropriate culture medium and incubated according to standard procedures (APHA, 1998). Selected isolates of *E. coli* colonies from samples were picked and streaked onto tryptic soy agar for purification and subsequent confirmation and biochemical characterization by Enterotube II (Becton Dickenson, Sparks, MD) with an ATCC strain of *E. coli* (# 25922) used as a positive control for the confirmation tests. The confirmed *E. coli* isolates were subjected to antibiotic susceptibility testing using customized commercially prepared plates (Sensititre 18-24 Hour Susceptibility System, TREK Diagnostics Inc., Westlake, OH). Minimum inhibitory concentrations (MIC) were generated for 17 antimicrobials that reflect those that are common for human therapy, and for use in the swine and cattle industries (Table 1). The *E. coli* isolates were first grown on tryptic soy agar, and 3-5 colonies were picked and resuspended in 4 mL of deionized water to achieve a turbidity of 0.5 MacFarland standard (~10^6 cfu/mL). A calibrated pipette was used to transfer 10 μL of this suspension into a tube of 50 mL of Mueller Hinton broth. This inoculated broth was then vortexed and 50 μL were transferred to each well of the custom plate using an eight-channel pipette. The plate was then sealed and incubated at 37 °C for 18-24 hours. The plates were then read after incubation, where the collection of bacteria at the bottom of a well was scored as positive. There were three positive control wells within the plate to confirm the quality of the test, with *Escherichia coli* ATCC 25922 strain as the positive control.

Nutrient analyses of selected wells for nitrate (NO_3-N) were done according to procedures described in Standard Methods (APHA, 1998) by Eric Fischer in Dr. Steven Whalen's laboratory at the University of North Carolina at Chapel Hill. The water samples for nitrate analyses were first filtered using a 0.45 μm Gelman a/e glass fiber filter. The filtered water was used to rinse the bottle three times, and then it was filled and capped. The two 125-mL bottles filled with sample water (unfiltered and filtered) were frozen without any preservatives. Nitrate levels were only measured during the second, third and fourth rounds of sampling at all four study sites.

RESULTS and DISCUSSION

E. coli Occurrence and Nitrate Concentrations in Groundwater

Swine Farm #1

A total of 134 presumptive *E. coli* were collected from the four study sites, and 100 were confirmed to be *E. coli* through biochemical testing. The swine farm #1 site yielded a total of 19

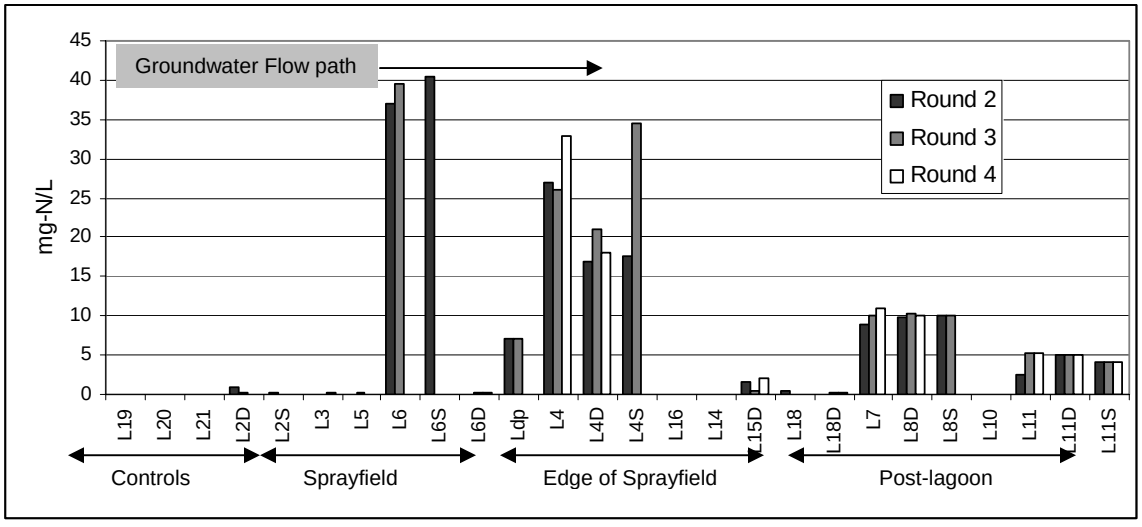
confirmed *E. coli* colonies in the groundwater. Only one *E. coli* colony was found in three wells each in the first round of sampling (March/May 2001): two wells were located in the middle of a land application sprayfield (L6 and L6S) and one well was located down gradient of the lagoon (L10). The second round of sampling (Feb./April 2002) had higher *E. coli* concentrations than the first with five positive wells and concentrations ranging from 1 to 6 cfu/100mL. The positive wells were two shallow wells located in the middle of sprayfields (L6, L6S), two wells located at the edge of a sprayfield (L14, L15S), and one well located down gradient from the lagoon (L11). The third round of sampling (May/June 2002) yielded only one *E. coli* colony that was located in one of the control wells up gradient of the swine farm (L21). The fourth round (Sept. 2002) yielded no *E. coli* colonies in the groundwater at all due to the continuing drought conditions, and several wells were unable to be sampled in the last round due to a lack of water in the wells. Therefore, *E. coli* were found more often and in higher concentrations in areas associated with the land application of swine waste (sprayfields) and in wells located down gradient from the lagoon than in the control wells at the swine farm #1 site.

There were relatively high levels of NO_3-N found at the swine farm #1 site, as shown in Figure 1. Two wells that exceeded the MCL for nitrate concentrations in drinking water (10 mg-N/L) by a factor of four (L6, L6S) are shallow wells located in the middle of the land application field (Figure 1). The other three wells that also showed levels in excess of the MCL are found at the edges of the sprayfields (L4, L4S and L4D). Lower concentrations of nitrate nitrogen that are below the MCL were found along the flow path gradient post the swine lagoon and sprayfields on this site. It is clear that land application practices are contributing nitrate to the groundwater in the shallow wells (3 to 12.6 feet) of this site. Similar concentrations are seen during both the second and third rounds. The only deviations seen in the fourth round are much higher concentrations in wells L4 and L4S, which are located at the edge of a sprayfield. Well L16 is part of this cluster, but at 50 feet compared to 8.6 to 12.6 feet deep for the L4 wells. Wells L14 and L15D (56.6 and 22.7 feet) are part of a cluster at the edge of a sprayfield that were not as impacted by nitrate contamination. Wells L18 and L18D (21.6 and 26.5 feet), which are located by an abandoned farm house down gradient from the lagoon, also had only small amounts of nitrate contamination. It should be noted that well L6S was not sampled during the third and fourth rounds due to a lack of water in the well probably caused by the continuing drought conditions experienced during the study period. Well L4S was not sampled during the third

round, and wells L6, L8S, and the drive point were also not sampled during the fourth round. Overall, the control wells show little to no nitrate concentrations in the groundwater, the concentrations spike up to high levels (up to 40 mg/L) greatly exceeding the drinking water MCL at the wells in the middle or on the edge of land application fields, and then the concentrations begin to decrease several-fold (to about 5 and 10 mg/l) at wells further down gradient from the land application fields and the lagoon.

Statistical comparisons of nitrate concentrations in the control wells located up gradient of the farm, the land application wells, and the wells that are located down gradient of the lagoon were made using the Wilcoxon Exact Scores two-sample test. The up gradient concentrations were statistically significantly different than those found in the land application wells ($p = 0.03$), and the up gradient concentrations were also significantly different from the down gradient groundwater concentrations ($p = 0.0001$). However, there were no statistically significant differences between the nitrate concentrations in the land application and down gradient locations. These findings confirm that there was little presence of nitrate in the groundwater up gradient of the land application fields and lagoon, and higher concentrations in the groundwater under the land application fields and also post-sprayfields and lagoon in the down gradient wells on the swine farm #1 site.

Figure 1. Nitrate Concentrations in Groundwater, Swine Farm #1 Site



At this site, the *E. coli* were predominantly found in wells L6 and L6S where there were high nitrate concentrations. However, *E. coli* were found in wells L10, L14, L15S, and L21

where no nitrate levels were found. Therefore, the presence of nitrate is not a good indicator for the presence of *E. coli* bacteria in groundwater at this site.

Swine farm #2

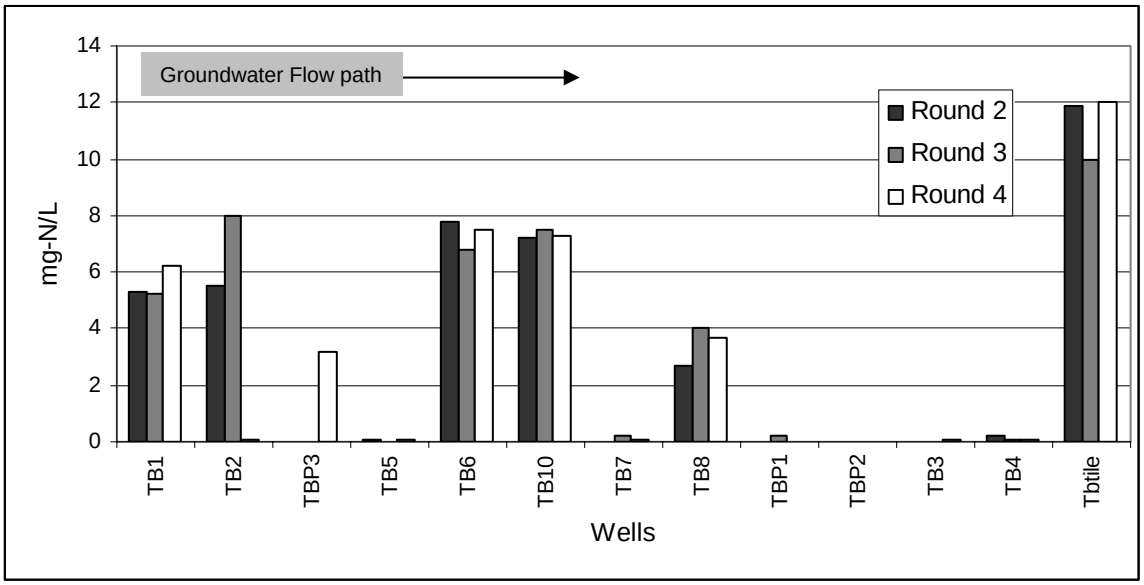
All of the wells are located down gradient of the lagoon, and are present on or at the edge of the land application field at the swine farm #2 site. There are no control wells that are not potentially impacted with swine waste within this site. The swine farm #2 study site yielded a total of 71 *E. coli* colonies from groundwater samples. The first round (May/June 2001) had one well and two piezometers positive for *E. coli* out of the 12 wells. The well (TB5) yielded only one *E. coli* colony (0.5 cfu/100mL) and one piezometer (TBP2) yielded 16.4 cfu/100mL of *E. coli.* The second piezometer (TBP1) had 1,045 cfu/100 mL *E. coli* and inspection of the piezometer proved that it might be a conduit for contamination in the aquifer. The second round (Dec. 2001) showed four wells (TB5, TB6, TB7, TB8) and one piezometer (TBP2) to be positive for *E. coli* (0.5 to 32.7 cfu/100mL). The piezometer is close to the sprayfield and also close to the stream, whereas two positive wells are in the sprayfield and the other two are located down gradient from the swine lagoon. The third round (April 2002) yielded only one piezometer (TBP1) positive for *E. coli* (1.8 cfu/100mL) and the final round of sampling (Sept. 2002) had three wells (TB2, TB5, TBP2) positive with *E. coli* also at low levels (1.0-1.8 cfu/100mL). Round one showed higher *E. coli* concentrations in groundwater than all other rounds. Round two also revealed positive *E. coli* colonies in the groundwater samples, but by rounds three and four the *E. coli* concentrations were at non-detectable levels in all wells due to summer and drought conditions, which was similar to the findings at the swine farm #1 site.

The overall nitrate concentrations at this farm were lower than at the swine farm #1, with a sample maximum of 8.0 mg/L in well TB2 (Figure 2). All of the nitrate concentrations were lower than the MCL of 10 mg/L, except for levels found in the tile drain during the second and fourth rounds of sampling that were close to 12 mg/L. The three rounds of sampling revealed generally consistent results, except for the dramatic decrease in nitrate in well TB2 in the fourth round of sampling. Of note, TBP3 was only sampled during the fourth round. Statistical comparisons of nitrate concentrations between the groundwater sampled from wells located at the upper end of the land application field, the middle of the field, and at the end of the study site post-land application field were done using the Wilcoxon Exact Scores two-sample test. There were no significant differences between the nitrate concentrations found in the upper end of the

land application field and those located in the middle of the field (p = 0.29). However, there were statistically significant differences between the wells at these two locations and the down gradient wells (upper wells vs. down gradient p = 0.002, and middle field wells vs. down gradient p = 0.009). This relationship is supported by the lack of nitrate found in wells TB3, TB4, TBP1 and TBP2, which are considered the down gradient wells.

The majority of the *E. coli* isolates were found in wells TBP1 and TBP2, which revealed little to no levels of nitrate in the same water samples. Well TB2 had *E. coli* present during the fourth round of sampling when there was very low nitrate levels found, and TB5 also had consistent positive samples for *E. coli*, but very low nitrate levels. Well TB7 also had the highest level of *E. coli* found water during round 2 when no nitrate was detected. Therefore, the same lack of a direct correlation between *E. coli* and nitrate concentrations in groundwater is found at the swine farm #2 site.

Figure 2. Nitrate Concentrations in Groundwater, Swine Farm #2 Site



Reference #1

Low *E. coli* concentrations (4.5 cfu/100mL) appeared in only one well (CF1) out of seven at the reference site #1 during the first round of sampling (June/Aug. 2001) and not in the subsequent three sampling rounds (Feb.2002, May 2002, and Aug. 2002). This well is located off to the East of the rest of the wells, and is not in line of the groundwater flow path that the rest of the wells represent. There was the potential for cattle manure to penetrate through the soil matrix due to a small herd of cattle (approximately 10 animals) that were allowed to graze about 80 feet

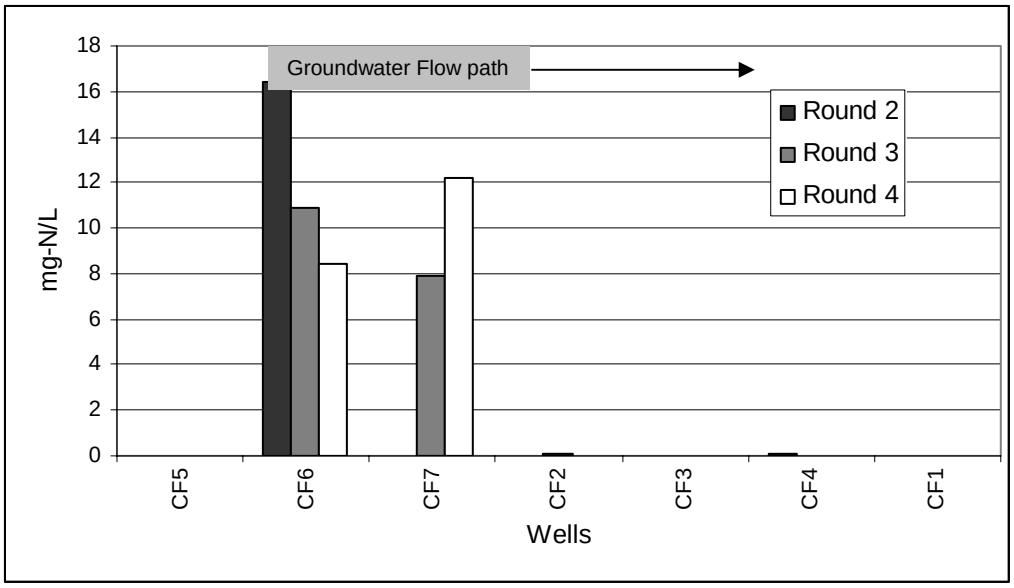
up gradient from the location of the wells. Therefore, the results from this site may indicate bacteria from cattle sources, but the absence of detectable bacteria in groundwater samples from down gradient wells indicates that their waste did not have a detectable impact during the study period. This site is considered to be an agricultural reference site that does not contain the presence of swine or their waste material. Most of the wells at the reference #1 site are deeper (10 to 40 feet) than at the reference #2 site (7 to 18 feet). The deeper wells on this site are located below an aquiclude (confining or dividing layer between aquifers) and are not representative of the shallow waters that are showing positive results at the other sites. However the shallower wells at 10 feet at this site are representative of the surficial aquifer and were not positive for *E. coli* bacteria.

The nutrient concentrations at the reference #1 site (Figure 3) were all near zero (non-detectable) with two exceptions. One well located in the middle of the site (CF6, 14 feet) that is in the surficial aquifer had a nitrate level of 16.6 mg-N/L during the second round, which is above the MCL of 10 mg-N/mL for nitrate in drinking water. The levels dropped off to 10.9 mg/L during the third round and dropped even lower to 8.5 mg/L in the fourth round of sampling, which was below the MCL. However, a value of 8.5 mg-N/L for groundwater is still considered very high. The well right next to this one (CF5) is 40 feet deep and in the deeper confined aquifer, and had only 0.01 mg/L of nitrate in the water. The only other well that had detectable nitrate concentrations was well CF7, which is located down gradient of CF6, in the middle of the field and is 15 feet deep. Interestingly, detectable nitrate concentrations appeared in this well starting in the third round of sampling at just below 8 mg-N/L, and increased to above the MCL of 10 mg-N/L during the last round of sampling. This may show that the nitrate was moving down the groundwater gradient since it was first present in the shallow upstream well (CF6) during the second round (May 2002) and not in CF7, but finally appeared in the third round (June 2002) and persisted until the fourth round of sampling (August 2002). The wells down gradient from this cluster of wells (10, 15 and 38 feet deep) also had negligible levels of nitrate in the water for all rounds of sampling. Although animal waste fertilizer was not applied during the study period, chemical fertilizer containing nitrogen was applied on the following dates: March 14, 2001; May 24, 2001; February 27, 2002; and September 25, 2002. The first round of sampling started in June and finished in August of 2001, just one month after nitrogen was applied to the fields. The second round of sampling was on February 11, 2001, which was

just before the next application of nitrogen. The third round of sampling was May 8, 2002, just over two months after the last application of nitrogen fertilizer during the study period. Therefore, no further nitrogen was applied by the fourth round of water sampling on August 6, 2002. The last record of application of nitrogen fertilizer to the fields was in September 2002 after the water collection had been completed. The direct application of nitrogen fertilizer to the fields as chemical fertilizer is an obvious potential source for this nutrient at this site and not from the application of animal waste material as fertilizer.

Statistical comparisons were made of the nitrate concentrations found in the groundwater at the upper end of the field, the middle of the field, and the wells located at the end of the field that are on the bank of the surface water for this study site using the Wilcoxon Exact Scores two-sample test. There were no statistically significant differences between the upper and middle field groundwater nitrate concentrations ($p = 0.29$), nor between the upper wells and the down gradient wells ($p = 0.07$). The only statistically significant differences were between the nitrate concentrations found in the groundwater in the wells located in the middle of the field and the down gradient wells ($p = 0.01$) due to the lower concentrations found in the down gradient wells. The only well to yield *E. coli* colonies in the groundwater was CF1 where no nitrate was detected in the groundwater.

Figure 3. Nitrate Concentrations in Groundwater, Reference #1 Site



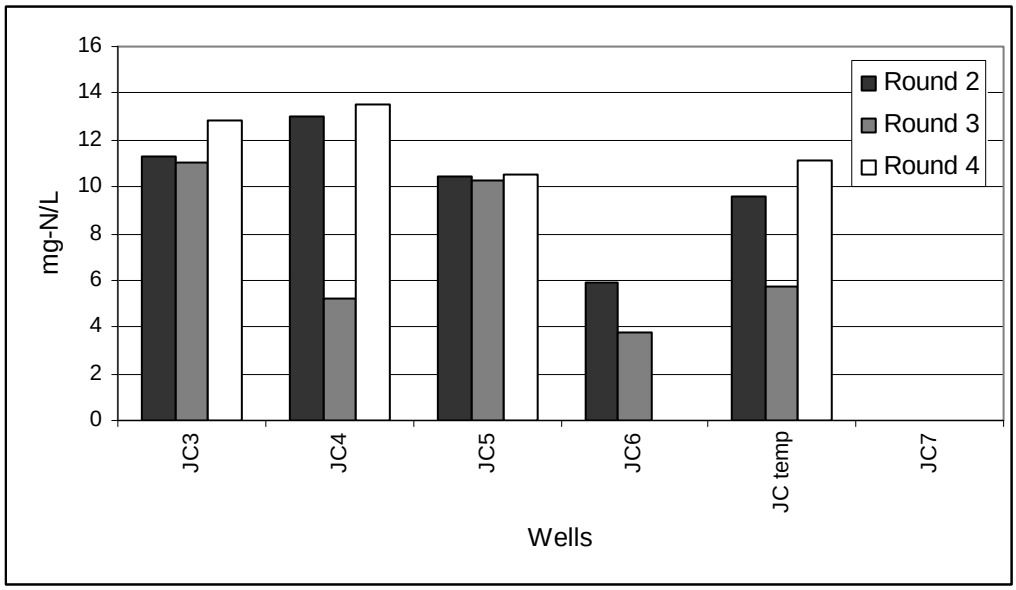
Reference #2

Only one *E. coli* isolate (0.5 cfu/100mL) appeared in one well (JC7) out of six at the reference #2 site during the first round of sampling (Sept./Oct. 2001) and not in the subsequent three rounds (April/May 2002, June 2002, and Aug.2002). The one positive well is 8.9 feet deep and is located only a few feet from the surface water on the site.

The levels of nitrate found in the groundwater at this reference site (Figure 4) are similar to those found at the swine farm #2 site. However, there are nitrate concentrations higher than 10 mg-N/L in the two wells that are located off to the North of the other line of wells (JC3 and JC4). These wells were initially installed to determine if the riparian buffer did indeed reduce nutrient levels. The nitrate concentration at JC4 was above 10 mg/L during the second and fourth rounds of sampling, but not the third, suggesting that there was a decline and then an input of nitrogenous nutrients between the sampling events. JC3 is an 18 feet deep well, and JC4 is a 10 feet deep well. A similar pattern to well JC4 was seen in the temporary well (JC8, 7 feet) where the nitrate concentrations where high, declined, and then went back up again in the last round of sampling. Only the last round had nitrate concentrations higher than the MCL for drinking water. The well JC5 (16 feet) also had consistent nitrate levels just above the 10 mg/L MCL during all rounds of sampling. The JC6 well, at 10 feet deep, had nitrate concentrations lower than 10 mg-N/L, and it was not sampled during the last round. Perhaps more recent events of application of chemical fertilizers may be the reason for the increase in nitrate concentrations found in oxygen rich wells at this site.

Statistical comparisons of nitrate concentrations in the groundwater from the upper, middle and down gradient wells were made using the Wilcoxon Exact Scores two-sample test. There were significant differences in nitrate concentrations between the upper and middle wells ($p = 0.02$), the upper and down gradient wells ($p = 0.01$), and the middle and down gradient wells ($p = 0.006$), which is reflective of the lack of nitrate in the down gradient well (JC7). Finally, the one well where one colony of *E. coli* bacteria were isolated (JC7) showed no detectable levels of nitrate during all rounds of sampling.

Figure 4. Nitrate Concentrations in Groundwater, Reference #2 Site



***E. coli* Statistical Analysis**

The Kruskall-Wallis χ^2 test was employed to determine if there were statistically significant differences in bacteria concentrations in all of the groundwater samples between the four study sites. These analyses reveal that there were statistically significant differences in the *E. coli* concentrations between the two swine farms ($p = 0.003$), which may be due to the larger *E. coli* concentrations present at swine farm #2 along with differences in numbers of animals, waste management practices and geo-hydrological or other environmental conditions. There also were statistically significant differences in *E. coli* concentrations between the swine farm #2 site and the reference sites (#1: $p = 0.003$; #2: $p = 0.005$). There were no statistically significant differences when comparing the swine farm #1 site to the references sites (#1: $p = 0.36$; #2: $p = 0.41$).

***E. coli* Antimicrobial Resistance**

Knowing the antimicrobial usage at the two swine farms would reveal if there is a direct positive association between antimicrobial use and finding antimicrobial resistant isolates in groundwater on or near these farms. However, this information was unavailable, and even deemed proprietary when it comes to what antibiotics are included in the swine feed. What is known is that: (1) the following antimicrobials in the Sensititre panel used for antimicrobial testing are approved for use in swine feed: streptomycin, tetracycline, chlortetracycline,

sulfamethoxazole, neomycin, tiamulin, and tylosin base, and (2) the following antimicrobials are approved for use as a water soluble or for injection to treat disease: erythromycin, gentamicin and ampicillin. The *E. coli* isolates were tested for antibiotic resistance using a panel of 17 drugs that are typical of human and veterinary use (Table 1).

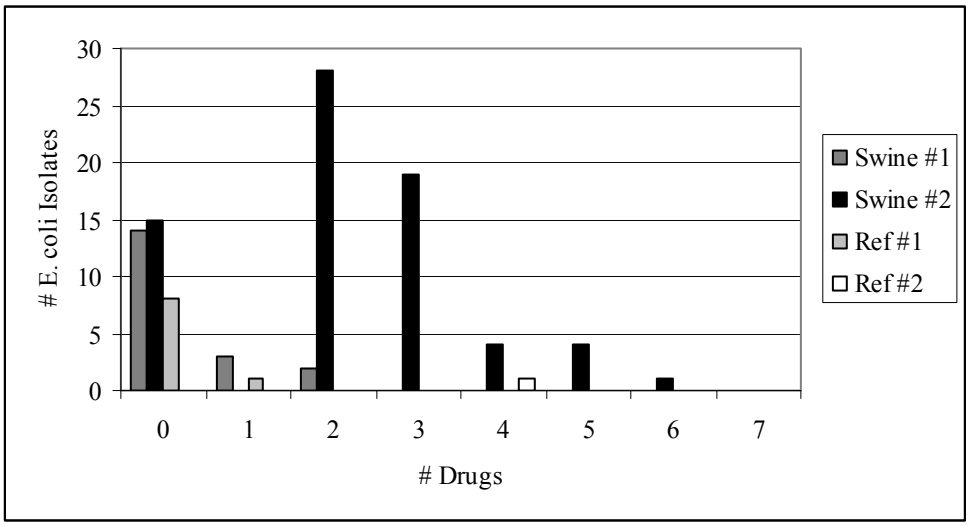
Table 1. Antimicrobials, Dilution Ranges, and MIC Breakpoints

Antimicrobial	Abbreviation	Dilution Range (µg/mL)	MIC Breakpoints
Streptomycin[f,h]	STR	32-1024	R ≥ 64; S ≥ 32
Vancomycin[h]	VAN	8-256	R ≥ 32
Chlortetracycline[f]	CTET	0.5-64	R ≥16; S ≥ 4
Tetracycline[f,h]	TET	0.5-64	R ≥16; S ≥ 4
Trimethoprim[h]	TMP	2-64	R ≥4; S ≥ 2
Sulfamethoxazole[f,h]	SMX	32-1024	R ≥512; S ≥ 256
Chloramphenicol[h]	CHL	4-128	R ≥32; S ≥ 8
Tiamulin[f]	TIA	8-64	R ≥ 32
Erythromycin[d,h]	ERY	4-32	R ≥ 8
Enrofloxacin[c]	ENRO	1-16	R ≥ 4
Ciprofloxacin[h]	CIP	1-16	R ≥ 4; S ≥ 1
Neomycin[f]	NEO	16-128	R ≥ 32
Gentamicin[d]	GEN	4-256	R ≥ 16; S ≥ 4
Ampicillin[d]	AMP	8-128	R ≥ 32; S ≥ 8
Florfenicol[c]	FFN	4-128	R ≥ 16
Tylosin Base[f]	TYLB	5-80	R ≥ 16
Clindamycin[h]	CLI	1-32	R ≥ 4

f:Approved for use in swine feed (growth promotion), d: Approved for use as injection or water soluble (disease treatment), h: Approved for use in humans, c: Approved for use in cattle

Of the 19 *E. coli* isolates from swine farm #1, two isolates were resistant to 2 antimicrobials, three were resistant to 1 antimicrobial and the rest were non-resistant. Of the 71 *E. coli* isolates from swine farm #2, nine isolates were resistant to 4 to 6 antimicrobials, forty-seven were resistant to 2 to 3 antimicrobials, and fifteen of the isolates were non-resistant. One of the 10 *E. coli* isolates from the reference site #1 and the only *E. coli* isolate from reference site #2 displayed 4 antimicrobial resistance traits (Figure 5).

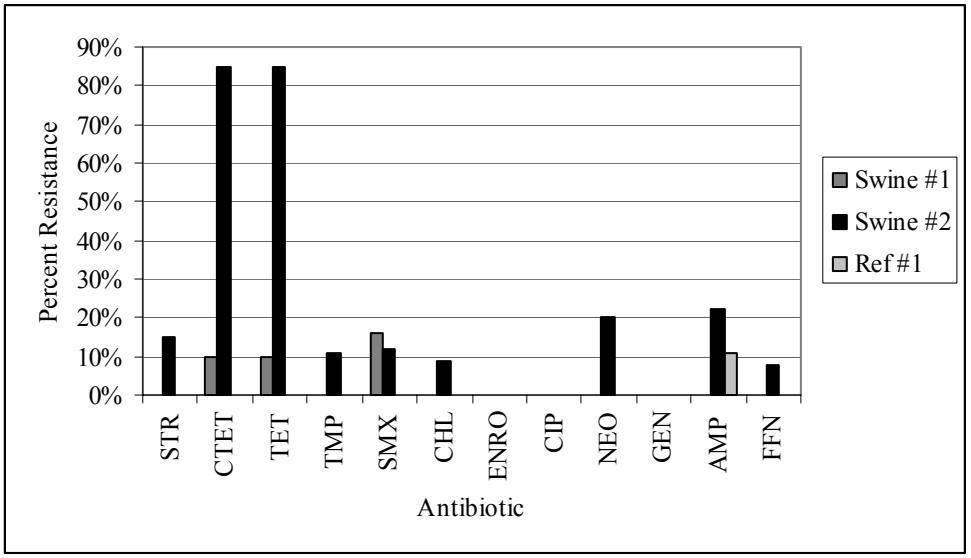
Figure 5. Frequency Distribution of Antibiotic Resistance of Groundwater *E. coli* isolates



The Wilcoxon Exact Scores test was used to compare frequencies of antimicrobial resistance in the *E. coli* isolates. The null hypothesis for this analysis is that there would be no difference in the antimicrobial resistance frequencies in *E. coli* regardless of the site it was isolated from. The results show that there are no statistically significant differences between the *E. coli* antimicrobial resistance frequency between the swine farms ($p = 0.06$) and that there are statistically significant differences between the swine farms pooled together versus those at the references sites (#1: $p = 0.01$; #2: $p = 0.003$). There are also statistically significant differences between the swine farm #2 sites and the reference sites (#1: $p = 0.03$; #2: $p = 0.009$). There was no statistically significant difference in frequency of *E. coli* resistance between the swine farm #1 site and the reference sites (#1: $p = 0.29$; #2: $p=0.11$) and also between the reference sites ($p = 0.37$). Overall, the frequency of antimicrobial resistance traits per *E. coli* isolate at the swine farms was significantly higher than at the reference sites studied.

Minimum inhibitory concentrations (MIC) were generated for 17 antimicrobials of which it is expected that the *E. coli* isolates will be inherently resistant to vancomycin, erythromycin, tiamulin, tylosin base, and clindamycin (Table 1). The *E. coli* isolates found at the swine farm #1 site show resistance to chlortetracycline, tetracycline and sulfamethoxazole; all of which are approved for use in swine operations (Figure 6).

Figure 6. Drug Resistance (%) of Biochemically Speciated Groundwater *E. coli* Isolates



The *E. coli* bacteria isolated from the swine farm #2 site had predominant resistance to tetracycline and chlortetracycline, which are both approved for use in swine feed. The isolates were also resistant to ampicillin, streptomycin, chloramphenicol, sulfamethoxazole and trimethoprim, and fewer of them were also resistant to florfenicol and neomycin. Chloramphenicol is the only resistance trait present that is not an antimicrobial permitted for use in swine feed or to treat swine disease, and it is considered to be a drug of last resort for human purposes. However, florfenicol is a derivative of chloramphenicol that is approved for use in cattle by the FDA-CVM (Purdue, 1996). Cross-resistance between florfenicol and chloramphenicol has been seen in *E. coli* isolated from bovines. The *E. coli* isolates in the study were resistant to florfenicol mediated by the flo gene, which specifies non-enzymatic cross-resistance to both florfenicol and chloramphenicol (White et al., 2001). Trimethoprim can be used for treatment of sick animals, but is also used for humans, and sulfamethoxazole is primarily available in combination with trimethoprim which might explain the presence of this resistance trait in bacteria associated with this swine farm. One out of the ten *E. coli* isolates found at the reference #1 site was resistant to only ampicillin. The reference #2 site had only one *E. coli* isolate recovered from groundwater and it was resistant to four antimicrobials: tetracycline, chlortetracycline, trimethoprim and ampicillin. This isolate came from the well located next to a surface water sampling site that is under reducing and low pH conditions.

CONCLUSIONS

The information collected from this study reveals that *E. coli* were found in groundwater on or near Eastern North Carolina swine farms that have either the conventional anaerobic lagoon and sprayfield land application system for swine waste management or an alternative technology of separating swine waste into solid and liquid fraction, compacting the swine waste solids and applying them as bio-solids. These results clearly indicate fecal contamination of the groundwater. The swine farm #1 site had low levels of *E. coli* bacteria in groundwater compared to the ground water levels of the other swine farm. However, there were very high levels of nitrates detected in the same water samples from swine farm #1. The swine farm #2 site had higher concentrations of *E. coli* in the groundwater than swine farm #1, however the nitrate levels were lower than at the swine farm #1 site. The swine farm #2 site is a smaller operation (design capacity for 1500) that separates and compacts the swine waste solids and applies them as bio-solids to the land application field while returning the separated liquid to the lagoon. One study has shown that the practice of removing wet manure solids prior to land application reduces nitrate concentrations in the waste (Lazarus et al., 1999), which may explain why the second swine farm has lower nitrate concentrations present in the groundwater when compared to the first swine farm where liquid waste from the lagoon is applied to the land. Specifically, elevated levels of nitrates (40 mg/L) were found in the groundwater from shallow wells under the land application fields at swine farm #1, and there were statistically significant differences between the nitrate concentrations in the up gradient wells and those that were impacted by swine waste in the land application fields and down gradient from the lagoon. There were lower concentrations of nitrate found in the oxygen-rich wells at the swine farm #2 (8 mg/L) and reference agricultural sites (4 -16 mg/L). The reference #1 agricultural site had chemical fertilizers applied to the land for additional nitrogen inputs that may be the source of the nitrate groundwater concentrations at this site. The reference #2 site may also be affected by the application of chemical fertilizers. There are elevated levels of nitrate at all of the agricultural sites in this study compared to expected background levels (0.05 – 0.5 mg/L) (Novotny and Olem, 1994). However, the majority of the samples were below the maximum contaminant level for nitrate in drinking water (10 mg/L) except in the shallow wells in the middle of the land application field at the first swine farm. The swine farm #1 site has sandy soils while the swine farm #2 site has well drained loamy sandy soils with moderate permeability. These soil

differences also may cause differences between bacteria and nutrient transport through the soil matrix to groundwater on the different study sites. Because fewer bacteria were found at the swine farm #1 site, perhaps other factors that were not systematically investigated in this study, such as timing and magnitude of land application, soil type and soil moisture content, may further explain these differences. There were only very low levels of indicator bacteria found in one well each during the first round of sampling at the reference sites. However, there were detectable levels of nitrates (3.5-16.4 mg-N/L) found in the groundwater at these sites. There were statistically significant differences among the *E. coli* concentrations between the swine farm #2 site and the other three sites (swine farm #1, references #1 and #2), and temporal variability was seen among the different sampling periods.

The results from this study also show that the frequency of antimicrobial resistance per *E. coli* isolate from the groundwater at the swine farms was significantly higher than at the reference sites studied. Multi-drug resistance is present in the *E. coli* isolates from groundwater near swine farm sites having lagoons and sprayfields, with isolates resistant to up to 4 to 5 antimicrobials beyond the expected range, and not in *E. coli* isolates at the reference sites. The antimicrobials to which resistance was observed in bacteria were largely consistent with the antimicrobials approved for use in swine feeding operations. Therefore, groundwater on or near swine farms may be a potential environmental reservoir for antimicrobial resistance genes in bacteria and it is clear that swine farms are negatively impacting the groundwater quality in Eastern North Carolina.

Overall, the results of this study demonstrated that antibiotic-resistant *E. coli* are being found in groundwater associated with commercial swine farms that have the lagoon and land application system or an alternative solids separation-land application system for waste management. Nutrients are more commonly measured than bacteria as an indicator of water quality related to organic waste sources, and it is often assumed that if nutrients are present then fecal bacteria would also be present. This study reveals that nitrate concentrations can not be used as predictor variables for bacteria densities in groundwater. It was commonly found that the bacteria were present in the groundwater from wells were no detectable levels of nitrate were found and that high concentrations of nitrate were found in groundwater in which there were no or only low levels of *E. coli*. Hence, the presence and concentrations of one can not be used as a predictor of the other.

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