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Effects of Concentrated Poultry Operations and Cropland Manure Application on Antibiotic Resistant Escherichia coli and Nutrient Pollution in Chesapeake Bay Watersheds

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Abstract

Manure from poultry operations is typically applied to nearby cropland and may affect nutrient loading and the spread of antibiotic resistance (ABR). We analyzed the concentrations of nitrogen and phosphorus and the occurrence of ABR in *Escherichia coli* (*E. coli*) and extra-intestinal pathogenic *E. coli* isolates from streams draining 15 small (< 19 km^2) watersheds of the Chesapeake Bay with contrasting levels of concentrated poultry operations. Total nitrogen and nitrate plus nitrite concentrations increased with poultry barn density with concentrations two and three times higher, respectively, in watersheds with the highest poultry barn densities compared to

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Author Contributions

Heather Amato led data analysis, manuscript writing and final revisions. Nora Wong, Kishana Taylor and Carey Pelc conducted sampling, data entry, data management and contributed to manuscript writing and revisions. Jay Graham and Thomas Jordan conceived of the study and contributed to the study design, and provided guidance on data analysis and manuscript writing. Dr. Lance Price led genotyping and contributed to manuscript writing and revisions. Mark Altabet led the nutrient analysis.

Supporting Information

Isotope analysis description; figures of linear relationships between barn density and various nutrients/isotopes, and between IMX and cephalosporin resistance; tables of model selection, assay primers and probe sequences for qPCR, and antibiogram and ExPEC status of all 337 *E. coli* isolates (PDF).

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those without poultry barns. Analysis of N and O isotopes in nitrate by mass spectrometry showed an increase in the proportion of ^{15}N associated with an increase in barn density, suggesting that the nitrate associated with poultry barns originated from manure. Phosphorus concentrations were not correlated with barn density. Antibiotic susceptibility testing of putative *E. coli* isolates was conducted using the disk diffusion method for twelve clinically important antibiotics. Of the isolates tested, most were completely susceptible (67%); 33% were resistant to at least one antibiotic, 24% were resistant to ampicillin, 13% were resistant to cefazolin, and 8% were multi-drug resistant. Resistance to three cephalosporin drugs was positively associated with an index of manure exposure estimated from poultry barn density and proportion of cropland in a watershed. The proportion of *E. coli* isolates resistant to cefoxitin, cefazolin, and ceftriaxone, broad-spectrum antibiotics important in human medicine, increased by 18.9%, 16.9%, and 6.2%, respectively, at the highest estimated level of manure exposure compared to watersheds without manure exposure. Our results suggest that comparisons of small watersheds could be used to identify geographic areas where remedial actions may be needed to reduce nutrient pollution and the public health risks of ABR bacteria.

Keywords

concentrated animal feeding operations; poultry litter; nutrient pollution; antibiotic resistance; *E. coli*; Chesapeake Bay

1. INTRODUCTION

Concentrated animal feeding operations (CAFOs) have been linked to both nutrient pollution and the environmental spread of antibiotic resistance (ABR),[1] especially in waterways.[2] Additionally, the continued use of antibiotics in food animal production can increase the spread of antibiotic- resistant bacteria in the environment.[3] The current practice of intensive, high throughput methods for producing broiler chickens (i.e. raised for meat) has a range of potentially negative consequences to human and ecosystem health, many of which are associated with the challenge of managing massive quantities of poultry litter.[4] About 13–26 million metric tonnes of poultry litter (i.e., excreta, feathers, spilled feed, bedding material, and soil) are produced in the United States (U.S.) annually, of which over 90% is applied to land with little or no incorporation into the soil.[5,6] The impacts of poultry production are particularly evident near the Chesapeake Bay on the Delmarva Peninsula, which includes Sussex County, Delaware, a leading U.S. county for poultry sales.[7]

Most poultry litter is applied to cropland near poultry operations, providing more nitrogen and phosphorus than crops can use and thereby increasing nutrient discharges from watersheds.[8, 9] Nutrient discharges can be further augmented when poultry litter is applied near waterways or under conditions such as rainfall, saturated soil, and/or poor soil porosity.[10–12] Increasing watershed discharges of nitrogen and phosphorus have had negative impacts on rivers, lakes, estuaries, and coastal waters globally.[13] Nutrient over-enrichment has a wide range of direct and indirect effects, including depletion of dissolved oxygen and algal blooms that can be toxic to fish and humans.[14] Toxic effects of nutrient over-enrichment can lead to die-off in fish populations, and consumption of fish or shellfish contaminated with

toxins associated with algal blooms have the potential to cause gastrointestinal illness, neurotoxicity, and paralysis in humans.[15] In the Chesapeake Bay, increased nutrient inputs have increased the volume of hypoxic deep water[16] and caused the widespread demise of submerged aquatic vegetation.[17, 18] Several studies have found potential links between watershed nutrient discharges and CAFOs in general[18] and poultry litter applications specifically.[19–21]

Poultry CAFOs can be important emitters of fecal pollution, which includes *Escherichia coli (E. coli)*, a fecal indicator bacterium. Some strains of *E. coli* are of concern to human health due to their ability to cause disease and spread antibiotic resistance.[22] Extra-intestinal pathogenic *E. coli* (ExPEC) is a primary cause of urinary tract infections and urosepsis, and results in an estimated 40,000 deaths each year in the U.S.[23, 24] Chickens are known to be a reservoir of ExPEC,[23–25] but to our knowledge, the presence of ExPEC has not been evaluated in watersheds impacted by concentrated poultry operations.

A major concern of antibiotic use in any setting, including broiler production, is the selection of antibiotic-resistant bacteria and the transfer of resistance genes to pathogenic bacteria. The spread of ABR increases the risk of infections in humans and animals that cannot be effectively treated.[26] Concentrated poultry operations are permitted to use antibiotics for therapy, control, and prevention, but detailed on-farm data on such uses in the U.S. were not publicly available until 2017. The U.S. Food & Drug Administration (FDA) published the 2017 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals (available online at www.fda.gov/media/119332/download), including the drugs with clinical importance in human medicine. However, the report did not include data on the use of clinically-important cephalosporins for specific animal species other than cattle. Antibiotic-resistant pathogenic bacteria and resistance genes have been found in litter from poultry operations,[4, 27–30] and resistant bacteria have been shown to survive in litter for several months.[31]

There is limited evidence on the extent to which concentrated poultry operations potentially increase levels of resistant pathogenic *E. coli* and nutrient pollution in the environment. Stream sediments have been found to be hot spots of bacterial density and activity and a niche that can promote horizontal gene transfer, which plays an important role in the spread of antibiotic resistance. The goal of this study was to quantify both nutrient pollution and the prevalence of antibiotic resistance in waterways near confined poultry operations. To achieve this goal, we collected and analyzed water and sediment samples from tributaries of the Chesapeake Bay in order to: a) quantify average levels of nitrate and phosphorous pollution, b) estimate the prevalence of drug-resistant *E.* coli, including ExPEC, and c) assess associations between nutrient pollution, antibiotic resistance, and the density of poultry farms within watersheds of sampled tributaries.

2. MATERIALS AND METHODS

2.1 Study sites.

We sampled water and sediment from 15 streams draining agricultural watersheds with contrasting levels of concentrated poultry operations (Figure 1). The watersheds, all on the

Delmarva Peninsula, ranged from 0.36 to 18.18 km^2 in area (averaging 7.48 km^2), with 30% −94% cropland (Table 1), mostly used as corn-soybean rotations receiving fertilizer applications. We delineated the watersheds using ESRI's ArcGIS© ArcMap 10.1 software. A 10 meter (1/3 arc) digital elevation model for the Chesapeake Bay region was obtained from the 3D Elevation Project[32] and used to determine upland watershed boundaries. Information about land cover was obtained from the 2006 National Land Cover Data (NLCD) set.[33] Land classified as "pasture/hay" in the 2006 NLCD is included in our definition of cropland for this study since fertilizer can be spread on both types of land[34] and because cropland is sometimes misclassified as pasture.[35]

We used numbers of poultry barns, distinctive long and narrow buildings, as a measure of the intensity of poultry farming. Most poultry farms on the Delmarva Peninsula produce broiler chickens.[36] Virtually all poultry growers work under contract for one of a few companies that integrate the entire production process, including the supply of chicks and feed, and the processing of the meat.[36] Specialized poultry barns are typically 50–66 feet (15–20 m) wide and 600 feet (180 m) long, with 8 foot (2.4 m) high walls, and usually in groups of multiple barns.[36] Poultry barns were counted using ESRI's ArcGIS© world imagery basemap and Google Earth imagery, as done by Fertig et al.[37] The highest density of poultry barns among the watersheds we studied was in Sussex County, DE, a leading U.S. county for poultry sales.[7] Typically, a Delmarva poultry barn houses around 44,000 birds at a time and 5.5 flocks per year for a total of 242,000 birds per year, producing 95 tons (86 tonnes) of poultry litter annually.[36] A long-term commitment to poultry growing is needed to recoup the costs of capital investments in the specialized barns,[36] so the number of barns is generally proportional to the number of birds and tons of poultry manure available for application to cropland.

2.2 Nutrient sampling and processing.

Water samples from the 15 streams were collected during March-May, November, and January during the years of 2012 and 2014 for chemical analysis. Most nutrient samples and all microbiological samples were taken in the spring months when manure was being applied. Samples were collected in acid-washed 1L Nalgene bottles and kept on ice until returned to the lab where they were filtered through 0.45 μm Millipore filters. Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were measured using a Dionex ISC-2000 Ion Chromatography System.[38] The standard concentration range was 0.04 – 5 mg/L for NO_3^--N and 0.02 – 1 mg/L for NO_2^--N. Any samples with NO_3^-- or NO_2^- concentrations above 5 mg/L or 1 mg/L, respectively, were quantitatively diluted with deionized water to fall within the standard concentration range and re-run. Because NO_2^- was usually less than 1% of nitrate plus NO_3^- (median 0.3%), we did not analyze the NO_2^- data separately but instead analyzed patterns of NO_2^- plus NO_3^-, which we abbreviate as NO_x^-.

Total phosphorus (TP) was determined by digestion of both filtered and unfiltered samples to orthophosphate with perchloric acid.[39] Phosphate (PO_4^{3-}) in the digested sample was analyzed by reaction with stannous chloride and ammonium molybdate.[40] Particulate TP was calculated by subtracting filtered TP from whole TP.[41] Total Kjeldahl N (TKN) was determined by digestion of samples to ammonium with sulfuric acid, Hengar granules, and

hydrogen peroxide.[41] The ammonium (NH_4^+) in the digestate was steam distilled and then analyzed using an Astoria Pacific International (API) 300 micro-segmented flow through analyzer with digital detector (API, Clackamas, Oregon, USA) using method A303-S02. Total nitrogen (TN) was calculated as the sum of TKN and NO_x^-. The concentration of total suspended solids (TSS) was measured by filtering unpreserved samples through pre-weighed Nuclepore 0.4 μm filters which were then dried in a vacuum-sealed desiccator, and reweighed. Concentrations were averaged across sampling dates for statistical analyses.

2.3 Isotope analysis.

Nitrogen in manure is enriched with the ^{15}N isotope compared to nitrogen in inorganic fertilizer.[42] Therefore, we analyzed the isotopic composition of NO_3^- in the streams to help assess the importance of manure as its source. Samples taken from 14 sites on February 15, 2013 were sent to the University of Massachusetts Dartmouth Isotope Biogeochemistry Group to measure NO_3^- nitrogen and oxygen isotopic ratios expressed as $\delta^{15}N$ and $\delta^{18}O$ in per mil (‰) units relative to atmospheric N_2 and SMOW (standard mean ocean water), respectively. Our analyses proceeded as described in McIlvin et al[43] with improvements stated in Ryabenko et al[44] that achieved precisions of 0.2 ‰ for $\delta^{15}N$ and 0.5 ‰ for ^{18}O.

When NO_3^- is denitrified, the remaining un-denitrified NO_3^- becomes enriched in both ^{15}N and ^{18}O. Therefore, we used the ^{18}O abundance to estimate the ^{15}N composition before denitrification to obtain an unconfounded isotopic signal of N from manure. For this estimation, we assumed that denitrification increased $\delta^{15}N$ twice as much as $\delta^{18}O$, as generally observed in groundwater.[63] For comparison, we also assumed that the NO_3^- with the lowest $\delta^{18}O$ observed (4.54 ‰) had undergone negligible denitrification. Therefore, we calculated the $\delta^{15}N$ before denitrification as the measured $\delta^{15}N$ minus two times the difference between the measured $\delta^{18}O$ and 4.54 ‰.

2.4 Microbiological sampling and processing.

Water samples (n = 228) and sediment samples (n = 118) were taken from the 15 streams for microbiological analysis on nine different dates from March to May 2014 (Table 1). Approximately 1 liter of water was collected from the surface of streams. When samples sites were accessible, we collected the surface 0 to 5 cm of sediment using a stainless steel scoop at locations close to the bank of each stream. A total of approximately 250 g of sediment was collected. These microbiological samples were taken at the same locations as the samples for nutrient analysis but not always on the same days. Both the water and sediment samples were collected using a telescopic dipper and were placed separately into sterile, polyethylene 1-L Whirl-Pak bags®,[46] and processed within 8 hours of collection. Sampling instruments were decontaminated with sodium hypochlorite solution and then rinsed with sterilized water before and after each use to remove residual sodium hypochlorite.

Standard membrane filtration methods were used to filter the water samples, using 0.45μm membrane filters.[47] Following filtration of 100 mL of each water sample, the filters were placed onto VRBA-MUG agar and incubated at 37°C for 2 hours and 44°C for 22 hours.[48] Up to four suspected *E. coli* colonies—observed as pinkish-purple colonies that fluoresced

under UV light—were selected. Putative *E. coli* isolates were placed on VRBA-MUG plates and incubated at 37°C for 24 hours. Isolates were then streaked onto UTI Chrome agar plates to ensure specificity in identifying putative *E. coli*[49] and were incubated at 37°C for 24 hours. Pinkish-purple colonies were selected again to streak onto LB Agar plates[50] and were incubated at 37°C for 24 hours. For long-term storage, single isolates were preserved in sterile 1.5 mL micro centrifuge tubes containing a mixture of Brucella broth and 20% glycerol and frozen at −80°C.

For each sediment sample, 10 g of sediment were added into 30 mL phosphate-buffered saline solution in a sterile 50 mL conical tube, vortexed for one minute, and allowed to settle for one minute. Twenty-five milliliters of the supernatant were filtered using aseptic filtration processes and plated on selective media following the methods described for the water samples. A positive control (ATCC *E. coli* strain 25922) was processed with each batch of samples, and autoclaved water was used as the negative control in both the lab and field for the microbiological analyses.

2.5 Identification of *E. coli* and specific identification of ExPEC.

Bacterial colonies from a pure culture were suspended in 300 μL of molecular grade water, boiled for ten minutes, centrifuged at 1,000-x g for one minute, and then frozen at −20 °C. A multiplex real-time PCR DNA assay was run for each bacterial isolate to confirm whether the isolate was *E. coli* (Table S2, Supporting Information).[51] The *uidA* gene was used for molecular identification of putative *E. coli* isolates.[52] Multiplex real-time PCR assays were used to identify presumptive ExPEC with the presence of six hallmark virulence genes: *papA, papC, sfaE, afaC, kpsMII*, and *iutA* (Table S2). Reactions consisted of 2 μl template DNA (~200 ng/μl) added to 8 μl 1X QuantaPerfeCTa® Multiplex real-time PCR SuperMix w/ROX (Quanta Biosciences, Gaithersburg, MD) containing primers and probes listed in Table S3 (Supporting Information). For positive controls, an equimolar mix of plasmid-cloned target genes (1 ng/μl) replaced the template DNA. Reactions were run on a Roche Light Cycler 480 (Roche, Pleasanton, CA) with the following conditions: 95°C for 3 min followed by 45 amplification cycles of 95 °C for 15 s, and 55°C for 1 min, followed by a 10 s cooling step at 40°C. Using the referenced methods, putative *E. coli* isolates positive for two or more of the six virulence genes were classified as presumptive ExPEC.[53]

2.6 Antibiotic susceptibility testing.

Antibiotic resistance was assessed by the disk diffusion method using the Clinical and Laboratory Standards Institute (CLSI) guidelines. All *E. coli* antibiotic susceptibility testing was carried out on Mueller Hinton agar and tested for susceptibility to 7 antibiotic classes (12 antibiotics) using the following discs (BD Diagnostic Systems, Sparks, Maryland): aminoglycosides (amikacin- AN 30ug, gentamicin- GM 10 μg), carbapenems (imipenems-IPM 10 μg), cephalosporins (cefazolin - CZ 30 μg, cefoxitin - FOX 30 μg, and ceftriaxone - CRO 30 μg), folate pathway inhibitors (trimethoprim-sulfamethoxazole - SXT 1.25/23.75 μg), aminopenicillins (ampicillin - AMP 10 μg, ampicillin-sulbactam - SAM 10/10 μg), quinolones (nalidixic acid - NA 30 μg, ciprofloxacin - CIP 5 μg), and tetracyclines (tetracycline - TE 30 μg). Zones of inhibition produced by each isolate were measured using a caliper and interpreted into criteria classifications of susceptible, intermediate, or resistant.

[54] Putative *E. coli* isolates resistant to three or more classes of antibiotics were classified as multi-drug resistant (MDR). Per CLSI guidelines, *Escherichia coli* strains ATCC 25922 and ATCC 35218 were used for quality controls, the first with acceptable limits of antibiotic susceptibility and the latter as a positive control resistant to antibiotics used in this study.[55]

2.7 Manure-shed delineation and manure exposure estimate.

Our analysis was designed to distinguish the separate effects of the percentage of cropland and the density of poultry barns on nutrient concentrations in stream water. In a previous study, we found that NO_x^- concentrations in Delmarva streams increase with the percentage of cropland in the watershed.[56] We hypothesized that application of poultry manure to croplands would correlate with further increase in NO_x^- concentrations. We also hypothesized that most poultry manure would be applied to croplands close to the poultry barn of origin and that some poultry litter would be transported across watershed boundaries. Therefore, the “manure-shed” (the area encompassing the poultry barns contributing manure to a watershed) would be larger than the watershed.

To estimate the extent of the manure-shed, we compared three alternate generalized linear regression models of the concentration of NO_x^-. One model assumed manure applied within a watershed comes only from barns within the watershed. The other two models assumed differing transport distances (1.6 km and 8 km) that seemed plausible for the farm vehicles (e.g., tractors) we observed hauling and applying poultry litter. All models included an interaction term for cropland area and barn density to capture the effect of tradeoffs between application of manure and inorganic N fertilizer. We used the Akaike Information Criterion (AIC_c) with a correction for finite sample sizes[57] to identify the best model for predicting NO_x^- concentrations. The separate effects of percent cropland and barn density were distinguishable because there were no correlations between the percentage of cropland and the number of barns within the different areas considered by the alternate models.

While our analysis of nutrient concentrations included effects of cropland in the absence of poultry barns, our analysis of ABR focused on the effects of manure applications in the watershed. Therefore, we created a single index of manure exposure (IMX), which accounts for both the density of poultry barns and the spread of poultry manure in the nearby environment.

2.8 Statistical analysis of ABR.

Univariate generalized linear regression models were used to predict antibiotic resistance in *E. coli* and ExPEC isolates based on IMX. The ABR outcome variable was defined as the percent of isolates at each site with phenotypic resistance to each antibiotic. Isolates found to have “intermediate” resistance were grouped with “resistant” isolates.[58] Multidrug resistance was defined as resistance to three or more classes of antibiotics.

ABR data from *E. coli* isolated from sediment and water samples were aggregated across time points at each sampling site (n=15) for statistical analyses because IMX was fixed (i.e. remained constant throughout the study period). To produce robust 95% confidence intervals and variance estimates, resistance percentages and IMX values for the 15 sites were randomly resampled 1,000 times with replacement to create bootstrapped samples.

Univariate linear regressions were run in SAS 9.4 on the original data (n=15) to calculate parameter estimates and *p*-values and were run a second time on bootstrapped samples (n=15,000) to calculate more precise parameter estimates, confidence intervals and standard errors. All parameter estimates reported in results below are from regressions of bootstrapped samples; *p*-values are from regressions of original data (n=15). Results were considered statistically significant if $p < 0.05$.

3. RESULTS

3.1 Nutrient Concentrations.

Overall mean concentrations of dissolved NO_x^-, total N, whole TKN, particulate TKN, dissolved TKN, whole TP, particulate TP and TSS are reported in Table 2. The mean concentration of dissolved NO_x^- from stream samples was 6.04 mg/L, ranging from 0.54 to 16.30 mg/L at each sampling location, and mean TN was 6.66 mg/L (1.08–16.54 mg/L).

The concentrations of NO_x^- (R^2=0.2136, p=0.05) and total nitrogen (TN) (R^2=0.2754, p=0.03) were weakly correlated with the percentage of cropland in the watershed (Figure 2). The percentage of cropland, however, was not associated with the concentrations of dissolved PO_4^{3-}, TP, whole and dissolved TKN (organic N plus NH_4^+) or total suspended solids (data not shown). In all but one of the streams sampled, most of the TN, often over 90%, was in the form of NO_x^-, but in the stream with the lowest NO_x^- concentration the TN was only 40% NO_x^- (data not shown). Given the small scope of this study and small sample size, it was not possible to assess effect modification due to other environmental factors such as season, rainfall, or temperature.

NO_x^- concentrations increased with the number of barns per area of cropland within 0, 1.6, or 8 km of the watershed (Table S1). AIC_c values of alternate linear models including barn density and the percentage of cropland within the watershed indicated that the best model was the one that accounted for barns per cropland area within 1.6 km of the watershed and included interaction between barn density and percentage of cropland (Table S1, r^2=0.82, p<0.01). This linear model suggests that locally applied manure was typically transported less than 1.6 km from the barn of origin before application to cropland. Therefore, we used barns per cropland within 1.6 km of the watershed to calculate the index of manure exposure (IMX) for each watershed.

IMX was calculated by multiplying the ratio of poultry barns to cropland area within a 1.6 km buffer (based on AIC_c best model for NO_x^-, Table S1) of the watershed to the ratio of cropland area to total area of the watershed:

$$\mathrm{IMX} = \frac{Poultry\ Barns\ within\ 1.6 km\ Buffer}{Cropland\ within\ 1.6\ km\ Buffer\ (km^2)} \times \frac{Cropland\ within\ the\ watershed\ (km^2)}{Total\ area\ of\ watershed\ (km^2)}$$

The first ratio in the IMX equation represents the “manure-shed” - the area encompassing the poultry barns potentially contributing manure to a watershed - which extends beyond the watershed by approximately 1.6 km. The second ratio accounts for the proportion of cropland within the watershed on which manure may be applied.

The R-squared and *p*-values from linear associations between nutrients and the number of poultry barns per cropland area within 1.6 km of the watershed boundaries are reported in Table 2. Unlike NO_x^- and TN, the TKN component (organic N plus NH_4^+) of TN was negatively correlated with barns per cropland within 1.6 km of the watershed (Figure S1, Supporting Information). This was unexpected because TN concentration increases with % cropland (Figure 2), reflecting the effects of applying N fertilizer and manure. As barn density increases, the decrease in TKN is more than offset by the increase in NO_x^-, which results in TN increasing with barn density. Like TKN, the concentrations of TSS, TP, and total particulate P decreased with increasing barn density (Figure S1), although dissolved PO_4^{3-} and total dissolved P had no significant correlation with barn density.

3.2 N and O Isotopes in NO_3^-.

Analysis of stable N and oxygen (O) isotopes in NO_3^- discharged from the watersheds provided further evidence of the connection between manure and the NO_3^- concentration. Manure becomes enriched in ^{15}N isotope due to fractionation in assimilation by poultry and due to faster rates of volatilization of ^{14}N isotope ammonia from manure. Therefore, NO_3^- derived from nitrification of manure has higher ^{15}N abundance than NO_3^- derived from nitrification of inorganic N fertilizer.[59] However, NO_3^- can be further enriched in both ^{15}N and ^{18}O due to denitrification, which preferentially consumes NO_3^- with lighter isotopes.[45]

There was a positive correlation between ^{15}N and ^{18}O abundance in NO_3^- (Figure 3) presumably due to the effects of denitrification. Therefore, we needed to account for the effect of ^{15}N enrichment due to denitrification in order to assess ^{15}N enrichment due to manure application. Estimated $\delta^{15}N\text{-}NO_3^-$ before denitrification increased with the density of barns within 1.6 km of the watershed, though the linear association was only significant with the removal of one outlier (Figure 3). The general pattern in $\delta^{15}N\text{-}NO_3^-$, however, suggests that manure becomes an increasingly important source of NO_3^- N as poultry barn density increases.

3.2 *E. coli*, ExPEC and antibiotic resistance.

Our sample sites had a range of 0–80 poultry barns within 1.6 km of the watershed and IMX ranged from 0–3 (Table 1). In samples from the 15 watersheds, we identified 337 putative *E. coli* isolates (Table 1; Table S3, Supporting Information). Of these, 225 isolates were from water samples and 112 from sediment samples.

Thirty-three percent of the putative *E. coli* isolates were resistant to at least one of the 12 antibiotics we tested. All resistant isolates were resistant to penicillin and susceptible to imipenem and ciprofloxacin. Eighty-three *E. coli* isolates (25%) were resistant to ampicillin, 43 isolates (13%) were resistant to tetracycline, and 43 isolates (13%) were resistant to cefazolin (Table 3). Fewer isolates were resistant to ampicillin-sulbactam (9%), cefoxitin (6%), gentamicin (4%), trimethoprim-sulfamethoxazole (4%), nalidixic acid (3%), ceftriaxone (2%), and amikacin (<1%). *E. coli* isolates from water and sediment samples had similar resistance patterns. Twenty seven isolates (8%) were MDR with resistance combinations primarily including aminopenicillin, tetracycline and cephalosporin classes. Fifty six isolates (17%) were identified as presumptive ExPEC. Of those identified as

ExPEC, 10 isolates (3%) were found to be resistant to at least one antibiotic and 8 (2%) were MDR.

Resistance to cephalosporins was positively associated with IMX (R^2=0.34; p=0.019) (Table 3; Figure S2, Supporting Information). For every one-unit increase in IMX, the percent of cephalosporin resistant isolates increased by 6.43% (Figure 4). At the highest IMX value represented by our watersheds (IMX=3), the resistance to all cephalosporins was 19.3% higher compared to IMX=0 within 1.6 km of the watershed (β=6.43; 95% CI=6.28, 6.57, Table 3). Corresponding increases for specific cephalosporins were 18.9%, 16.9%, and 6.2% for cefoxitin, cefazolin, and ceftriaxone, respectively (Table 3).

There were no other significant associations between IMX and antibiotic resistance in putative *E. coli* isolates, though resistance to ampicillin-sulbactam (penicillin class) had a near-significant association with IMX (R^2=0.24, β=4.21; 95% CI=4.09, 4.33; p=0.054, Table 3). IMX and MDR were not significantly associated (data not shown). An association between IMX and presumptive ExPEC was not estimated due to the limited number of ExPEC-positive isolates identified in this study.

4. DISCUSSION

4.1 Nutrients.

Previous studies on the Delmarva Peninsula have found that TN and NO_x^- concentrations in stream water increased as the percentage of cropland in the watershed increased.[56, 60] One study found NO_x^- or TN concentrations ranging up to 8 and 10 mg N/L, respectively, for watersheds ranging up to 84% cropland.[60] By comparison, we found NO_x^- and TN concentrations ranging up to 16 and 19 mg N/L, respectively, for watersheds ranging up to 94% cropland. Unlike the other studies, we purposely sampled watersheds with contrasting densities of poultry barns.

Our study identified positive correlations of NO_x^- and TN with poultry barn density per cropland area in the manure-shed (Figure 2), which suggests that poultry litter is an important source of N discharge in streams. Studies of watersheds in Texas, Iowa, and Virginia also found that nutrient concentrations increase with number of CAFOs.[11,61–62] CAFO density was positively correlated with concentrations of dissolved inorganic N and phosphate streams draining watersheds in the Shenandoah River Basin in Virginia.[62] This study could not clearly separate the associations with percentage of agricultural land versus with the numbers of CAFOs because those two variables were correlated, though correlations with CAFO densities alone were stronger than those with land use types. Correlations of CAFO densities with estrogenic activity in stream water provided further evidence of the importance of livestock waste.[62] For the watersheds we studied, poultry barn density was not correlated with the percentage of agricultural land, which allowed us to separate the correlations of poultry barns from the correlations of cropland without poultry barns.

To assess the effect of concentrated poultry operation density on TN and NO_x^- discharges, we used the best of the models (Table S1) to calculate concentrations of TN and NO_x^- in

streams draining watersheds with either zero barns or the maximum density observed (3.7 barns/km^2 cropland area). For a watershed with the average percentage of cropland in our study (71.7%), we predicted concentrations of 4.9 mg TN/L and 3.0 mg NO_x^--N/L at zero barns/km^2, and 9.9 mg TN/L and 9.6 mg NO_x^--N/L at 3.7 barns/km^2. Thus, we would expect a watershed with the highest observed barn density to have a TN concentration two times higher and a NO_x^- concentration three times higher than a watershed with a similar percentage of cropland, but no poultry barns.

Our measurements of N and O isotopes in NO_3^- suggested that poultry manure could be an important source of nitrogen, but this interpretation required estimating $\delta^{15}N$ before denitrification based on $\delta^{18}O$ and the assumption that denitrification increases $\delta^{15}N$ at twice the rate that it increases $\delta^{18}O$. Measurements of the relative rate of change in $\delta^{15}N$ and $\delta^{18}O$ with denitrification differ but often follow a 2:1 ratio, particularly in groundwater.[45] In contrast, the ratio has been found to be 1:1 for marine denitrifiers.[63] By comparison, a regression of our $\delta^{15}N$ vs. $\delta^{18}O$ measurements has a slope of 1.7:1, with $\delta^{18}O$ as the independent variable. Regardless of whether we assume the ratio is 2, 1.7, or 1 to 1, our analysis leads to the same conclusion: $\delta^{15}N$ increases with an increase in the density of poultry barns suggesting that manure becomes an increasingly important source of NO_3^- nitrogen. However, one outlier $\delta^{15}N$ measurement contradicts this conclusion and remains unexplained (Figure 3).

We found a negative association between barn density and TKN, whole TP, particulate TP, and TSS (Figure S1), which seems to contradict the link between poultry manure applications and nutrient discharges from watersheds. Hydrologic transport pathways may also be affecting nutrient concentrations. Watersheds with more groundwater flow than surface water flow tend to discharge more NO_x^- and TN.[59] NO_x^- travels primarily via groundwater to streams after leaching into the aquifer while particulate forms of N and P are transported in surface flow. Organic N and NH_4^+ (the components of TKN) are more easily transported in surface flow because both tend to bind to soil particles. Moreover, groundwater flow is much slower than surface water flow. For watersheds on the Delmarva Peninsula, the median time required for groundwater to reach streams has been estimated to range from 20–40 years.[64] Thus, the NO_x^- concentrations we measured in streams likely reflect agricultural practices spanning many years before our study.

Our findings seem to contradict concerns that long-term application of poultry manure will increase watershed discharges of P because the ratio of N:P in manure is much lower than the ratio of N:P uptake by crops.[20, 65] However, our grab sampling of the watersheds under-represents the effects of short-lived episodes of high stream flow, which typically account for most of the discharge of particulate matter and TP from watersheds.[59, 66] It would be necessary to sample high flow events to support conclusions about the effects of poultry manure applications on watershed discharges of TP, which is mostly particulate.

4.2. Antibiotic resistance.

More *E. coli* isolates resistant to one or multiple cephalosporin class antibiotics were found in streams with greater numbers of poultry barns in the watershed area, suggesting that poultry operations are potential emitters of antibiotic resistant *E. coli*. During the sampling

period, we observed cases where the application of poultry litter to cropland occurred within 500 meters of sampled streams. Contamination of rural waterways with resistant *E. coli* from poultry litter may be a route of exposure, posing health risks to people who recreate in the local surface waters or tidal waters of the Chesapeake Bay.[67]

Antibiotic resistance has been associated with increased nutrient concentrations from manure application in agricultural watersheds in Canada[68] and China[69, 70] as we found in our study. High nutrient loads may increase the viability of antibiotic resistant bacteria due to enhanced horizontal gene transfer via mobile genetic elements such as plasmids.[71] Plasmid-mediated horizontal gene transfer facilitates the dissemination of resistance genes among human and animal pathogens transmitted in soil and waterways.[72] Though identifying horizontal transfer of resistance genes was beyond the scope of this study, this mechanism may be partly responsible for the spread of cephalosporin resistance in our study site.

Our results suggest that poultry litter applied to croplands may be a source of cephalosporin resistance in the environment (Table 3), which is consistent with previous findings.[73] In 2012, U.S. FDA prohibited the extra label and prophylactic use of cephalosporins for major food animal species, in efforts to protect the drug's effectiveness in humans.[74] Evidence suggests that cephalosporin resistance in *E. coli* from poultry decreases sharply following the cessation of cephalosporin use.[75] Despite the lack of data on antibiotic use in our study area, we would not expect to find cephalosporin-resistant *E. coli* from poultry two years after the prohibition of prophylactic use of cephalosporins. Other studies have shown that cephalosporin-resistant *E. coli* is persistent in soil and manure near CAFOs.[76] Our findings suggest that cephalosporin-resistant *E. coli* may persist in the poultry production environment despite low or no cephalosporin use, or potentially that cephalosporin use for disease treatment by the poultry industry continues and is selecting for resistance in *E. coli*.

Among cephalosporins, the highest prevalence of resistance was that of cefazolin (12.8% of isolates), a first-generation cephalosporin, followed by cefoxitin (6.2%) and ceftriaxone (2.0%) resistance, second- and third-generation cephalosporins, respectively. Interestingly, cefoxitin and ceftriaxone had stronger positive correlations with poultry barn density (Table 3). This may suggest that while environmental transmission of first-generation cephalosporin resistance has been circulating for a longer period of time, second- and third-generation cephalosporins used more recently in poultry operations are more localized and highly correlated with recent application of poultry litter.

The prevalence of ampicillin-resistant putative *E. coli* isolates in all stream sites was higher than resistance to any other antibiotic observed in this study. This may reflect the development of ampicillin resistance occurring naturally[77] or from historical use in poultry operations[78] as suggested by the near-significant correlation between ampicillin-sulbactam resistance and poultry barn density in this study (Table 3).

Several limitations of this study are a result of limited available data on CAFO practices. At the time of the study, there was no publicly available data on antibiotic use in Delmarva poultry operations, the number of chickens per barn, or the amounts of poultry litter applied

to croplands in the watersheds. We observed piles of poultry litter throughout the crop fields in preparation for spreading, and directly observed litter application in progress on some farms during sampling events. Poultry barns on the Delmarva Peninsula have been reported to produce 86 tonnes of litter annually.[36] Therefore, we used numbers of poultry barns per area of cropland as a surrogate measure of the relative rate of poultry manure application. The intensification of manure application remains a concern when livestock production is geographically concentrated.[79, 80] Though not ideal, spatial correlation of sampling locations is difficult to avoid when studying the effects of geographically concentrated CAFOs. Geographically specific information on farming practices is generally considered proprietary, which limits our ability to estimate the true effects of livestock farming on emissions of nutrients and antibiotic resistant bacteria. Additionally, this study was limited in scope and did not include an analysis of the genotypes and resistance determinants in the recovered *E. coli* isolates.

5. CONCLUSION.

We found increased concentrations of nitrogen and increased prevalence of cephalosporin resistant *E. coli* in streams associated with higher poultry barn density within 1.6 km of the streams' watersheds. Though additional research is needed to better understand the mechanisms linking nutrient load to ABR, our approach to sampling and analysis, which focuses on relatively small watersheds, could be used to identify geographic areas where remedial actions may be needed to reduce nutrient pollution as well as the spread of ABR bacteria in aquatic environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Nitrogen concentrations in watersheds increased with numbers of poultry barns in and around the watershed
- Cephalosporin-resistant *E. coli* in streams was positively correlated with the estimated poultry manure exposure
- Studying small watersheds has the potential to reveal sources of antibiotic resistant bacteria and nutrient pollution

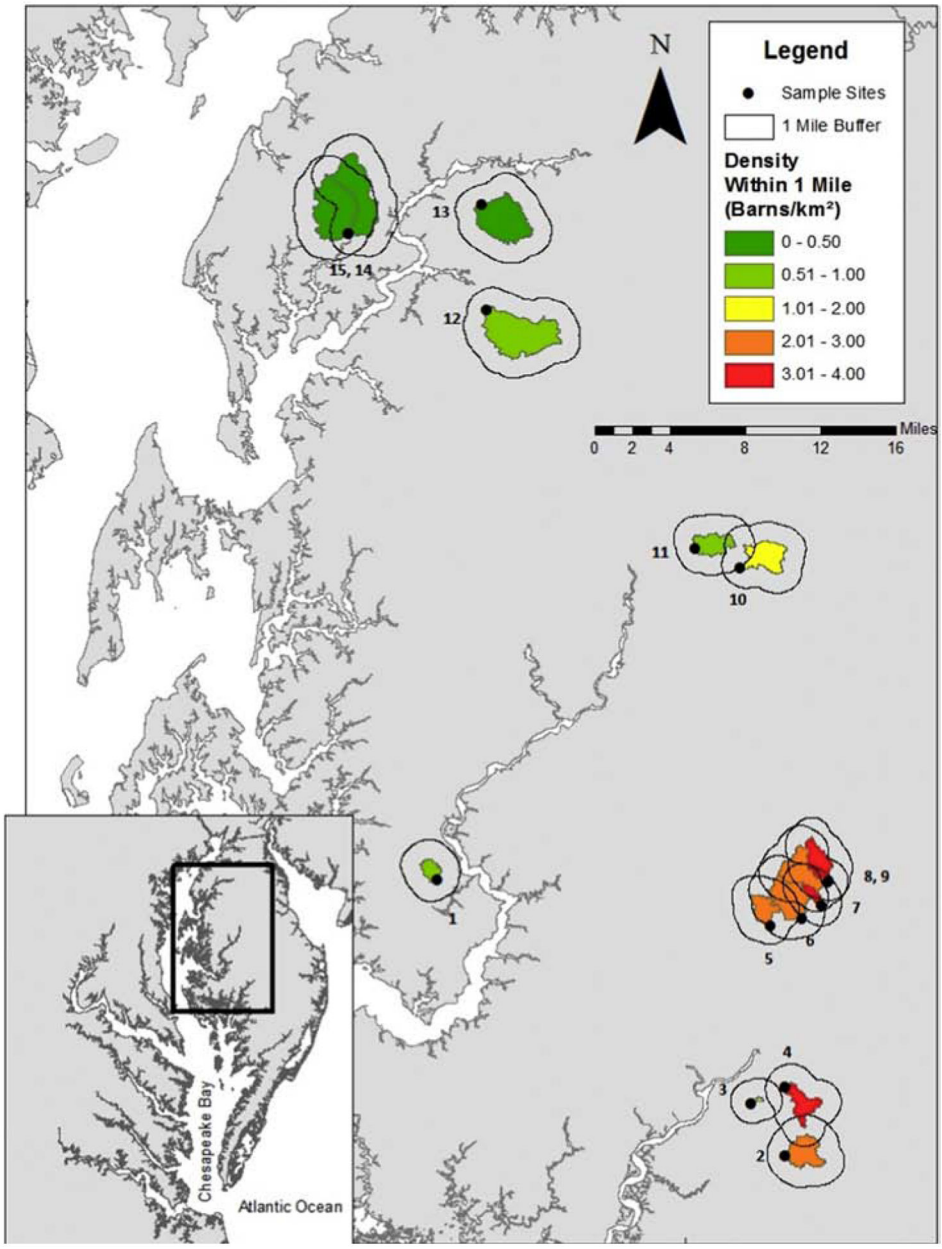

Figure 1.
Delmarva Peninsula sampling sites and poultry barn density within watersheds.

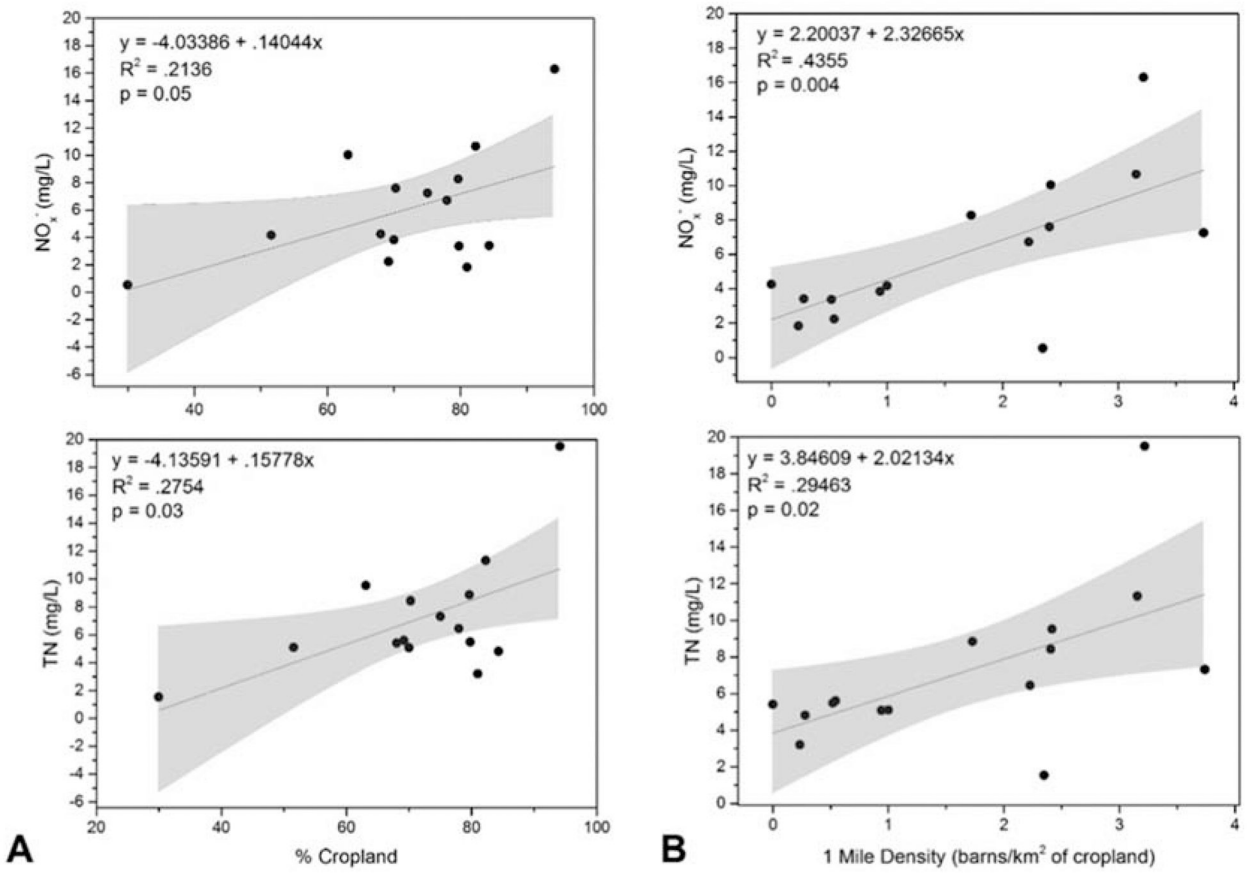

Figure 2.

Relationship between NOx-, TN and the percentage of cropland within the watershed (A) and the barn density per km^2 of cropland within a 1-mile (1.6 km) buffer of the watershed (B). Shaded areas represent the 95% confidence limits of the univariate linear regressions.

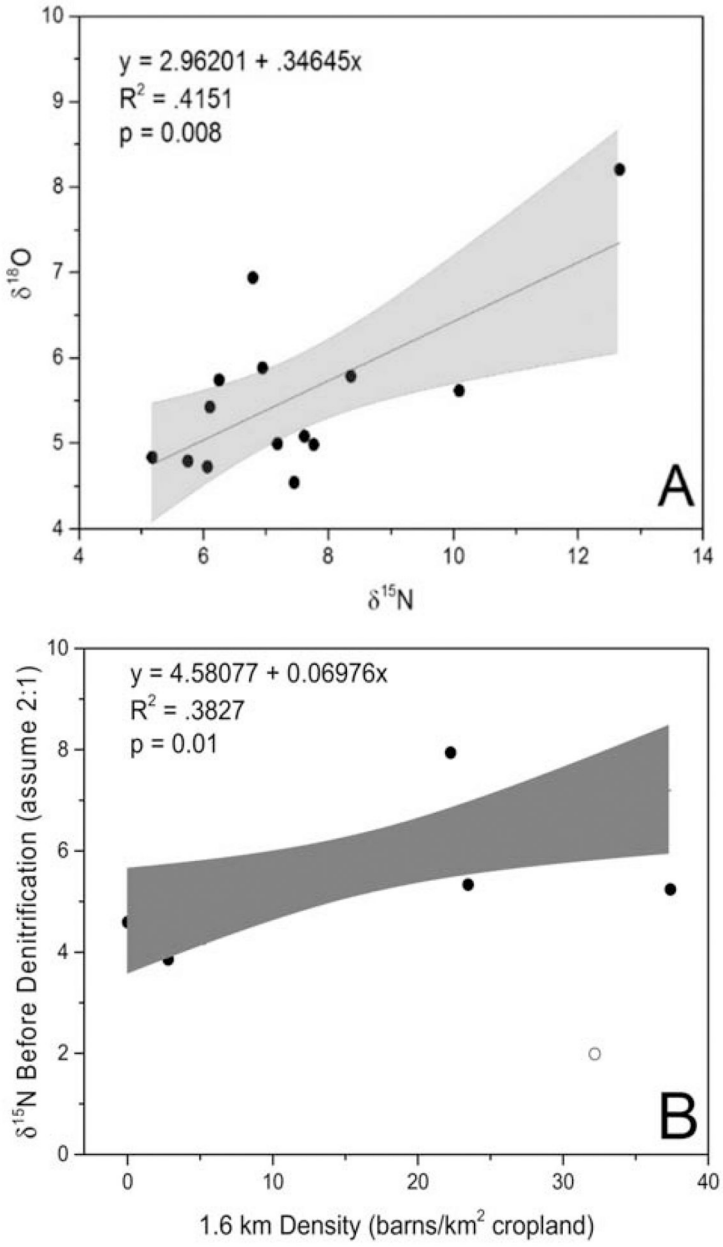

Figure 3.

Linear relationship between isotopes $\delta^{18}O$ and $\delta^{15}N$ in NO_3^- (A) and estimated $\delta^{15}N$ in NO_3^- before denitrification vs. poultry barn density within the manure-shed (B). Hollow data point represents an outlier in the data that was removed to visualize linear relationship between poultry barn density and additional enrichment of $\delta^{15}N$. Statistical information on graph is for a regression omitting the outlier. The relationship is not significant if the outlier is included. Shaded areas represent the 95% confidence limits of the linear regressions.

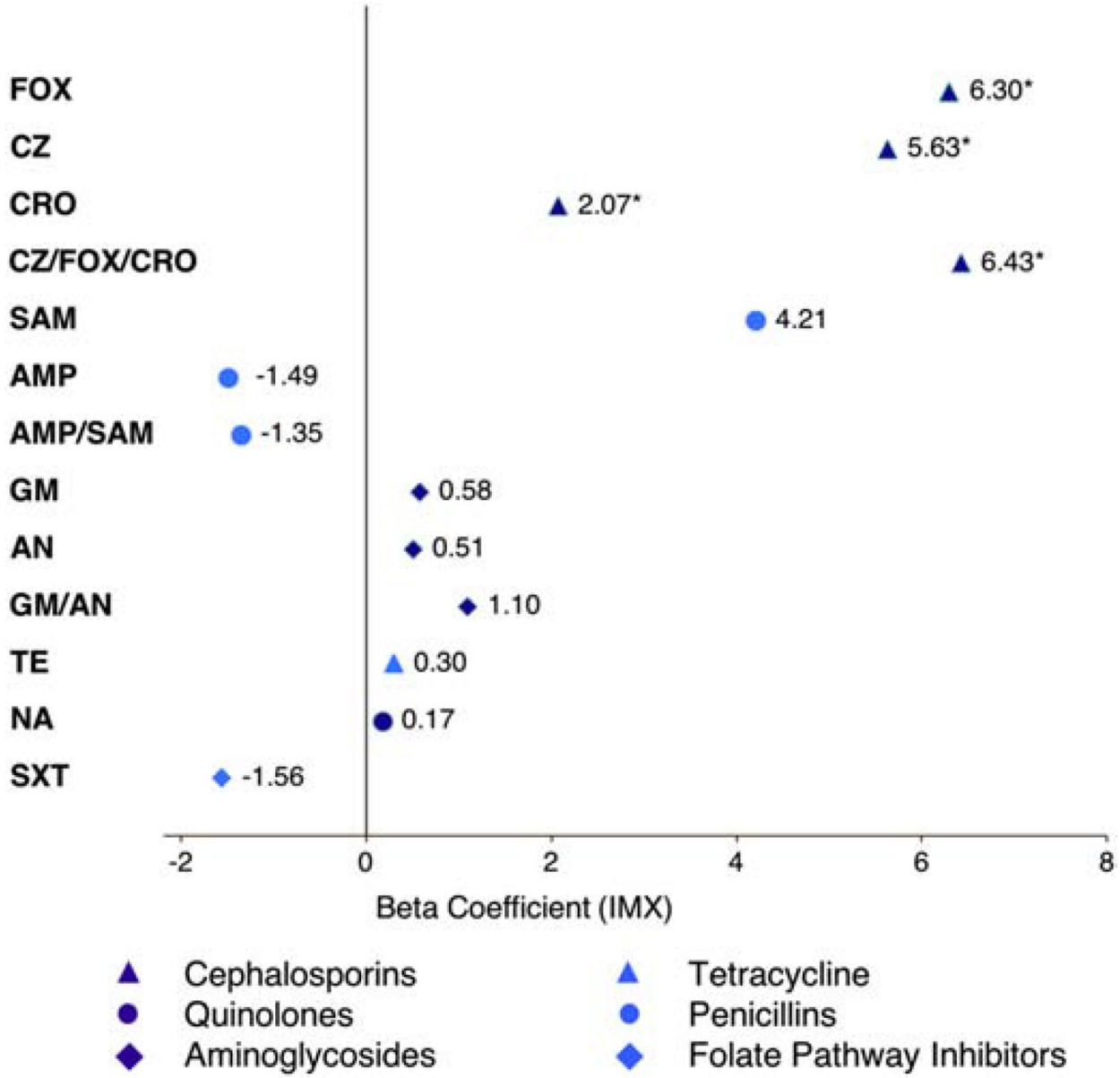

Figure 4.

Predicted effect of IMX on prevalence of resistance among *E. coli* isolates by sample site. Estimates are beta coefficients from univariate linear regressions of bootstrapped samples. Beta coefficients represent the change in percent of resistant isolates per one-unit increase of IMX, which ranged from 0–3 for each sample site. Confidence intervals, R-squared and *p*-values are reported in Table 2. Linear regressions of original data (not bootstrapped, n=15) in Supporting Information (Figure S3). *Estimates are significant at the α=0.05 level.

Table 1.

Description of sample sites and summary results of microbiologic and nutrient analyses.

Site	Watershed area (km^2)	Area of cropland / pasture in watershed (km^2)	cropland / pasture in 1.6 km buffer (km^2)	No. poultry barns in 1.6 km buffer	IMX	No. water, sediment samples[1]	No. (%) samples positive for *E. coli*	No. (%) samples positive for ExPEC	No. (%) MDR *E. coli* isolates	Nutrient samples[2]	Average dissolved NO_x^--N(mg/L)[3]
1	2.30	1.83	13.48	7	0.41	17, 12	29 (100)	4 (14)	1 (3.4)	8	3.37
2	6.17	1.85	15.77	37	0.70	17, 10	27 (100)	2 (7)	9 (34.6)	5	0.54
3	0.36	.29	9.25	16	1.38	18, 14	31 (97)	13 (41)	6 (19.4)	5	8.28
4	4.99	3.75	21.40	80	2.81	18, 8	26 (100)	4 (15)	6 (23.1)	5	7.26
5	3.90	3.04	16.18	36	1.73	17, 10	27 (100)	3 (11)	9 (33.3)	5	6.73
6	8.45	5.93	27.44	66	1.69	17, 6	22 (96)	0 (0)	6 (30)	5	7.61
7	1.54	1.45	13.98	45	3.03	17, 11	25 (89)	2 (7)	4 (15.4)	5	16.30
8	7.38	4.66	27.31	66	1.52	21, 3	24 (100)	2 (8)	5 (20.8)	8	10.05
9	5.00	4.11	20.92	66	2.60	6, 0	6 (100)	1 (17)	2 (33.3)	8	10.68
10	7.09	3.65	19.99	20	0.52	13, 8	21 (100)	2 (10)	8 (38.1)	5	4.18
11	5.03	3.48	20.25	11	0.38	18, 10	26 (93)	7 (26)	7 (25.9)	9	2.24
12	18.18	12.72	38.28	36	0.66	6, 0	6 (100)	0 (0)	1 (16.7)	7	3.84
13	13.40	9.11	29.87	0	0.00	14, 6	20 (100)	1 (5)	8 (40)	7	4.26
14	13.57	10.99	38.57	9	0.19	16, 7	23 (100)	0 (0)	7 (30.4)	6	1.83
15	14.83	12.51	39.33	11	0.24	13, 13	24 (92)	5 (19)	5 (20)	7	3.41

Note: IMX = Index of Manure Exposure, MDR = Multi-drug resistant (indicated by phenotypic resistance to 3 or more antimicrobials).

[1] Samples for microbiological analyses only (i.e. identification of *E. coli*, ExPEC, and antibiotic resistance).

[2] Additional water samples were taken separately to test for various forms of nitrogen and phosphorus.

[3] NO_X-N indicates dissolved Nitrate plus Nitrite.

Table 2.

Mean, minimum and maximum average concentrations (mg L^{-1} N, P, or TSS) of nutrient analytes at each sampling location, and R^2 and *p* values for linear regressions of average concentration and the number of barns per cropland area within 1.6 km of the watershed boundaries for each sampling location.

Analyte	Mean	Min	Max	R^2	*P*
Dissolved NO_x^-	6.04	0.54	16.30	0.44	***0.004***
Total N	7.18	1.54	19.51	0.29	***0.02***
Whole TKN	1.03	0.36	2.54	0.49	***0.002***
Particulate TKN	0.33	0.06	0.85	0.62	***<0.001***
Dissolved TKN	0.7	0.24	1.7	0.29	***0.02***
Whole TP	0.14	0.014	0.47	0.45	***0.004***
Particulate TP	0.09	0.006	0.43	0.43	***0.005***
TSS	25.24	2.26	151.88	0.29	***0.02***

Note: All mean, minimum and maximum values are in mg/L. Bolded *p*-values are significant at the α=0.05 level.

Table 3.

Prevalence of antimicrobial resistance in water and sediment samples and univariate regression results from bootstrapped samples of IMX and percentage of samples with resistant *E. coli* at each sampling location.

Drug Class	Type	Total Resistant Isolates N=337 (%)	R^2	MSE	β (95% CI)	*p*-value
Aminoglycosides	AN	1 (0.3)	0.26	0.91	0.51 (0.49, 0.52)	*0.0551*
	GM	13 (3.9)	0.02	1.96	0.58 (0.51, 0.64)	*0.6241*
	GM/AN	14 (4.1)	0.07	1.97	1.10 (1.04, 1.16)	*0.3602*
Carbapenems	IMP	0	-		-	-
Cephalosporins	CRO	7 (2.0)	0.50	1.43	2.07 (2.04, 2.11)	***0.0046***
	FOX	21 (6.2)	0.51	3.00	6.30 (6.20, 6.40)	***0.0025***
	CZ	43 (12.8)	0.27	2.46	5.63 (5.49, 5.78)	***0.0386***
	CZ/FOX/CRO	46 (13.6)	0.34	2.95	6.43 (6.28, 6.57)	***0.0187***
Folate Pathway Inhibitors	SXT	13 (3.9)	0.04	2.68	−1.56 (−1.68, −1.44)	*0.4562*
Penicillins	AMP	83 (24.6)	0.02	3.06	−1.49 (−1.65, −1.34)	*0.6421*
	SAM	29 (8.6)	0.24	2.72	4.21 (4.09, 4.33)	*0.0542*
	AMP/SAM	84 (24.9)	0.02	3.09	−1.35 (−1.51, −1.19)	*0.6849*
Quinolones	CIP	0	-	-	-	-
	NA	10 (3.0)	0.00	2.13	0.17 (0.10, 0.25)	*0.8393*
	CIP/NA	10 (3.0)	0.00	2.13	0.17 (0.10, 0.25)	*0.8393*
Tetracyclines	TE	43 (12.8)	0.00	3.47	0.30 (0.11, 0.50)	*0.8639*

Note: Beta coefficients represent the change in percent of resistant isolates per one-unit increase of IMX. Bolded *p*-values are significant at the α=0.05 level. MSE = mean squared error, CI = confidence interval, IMX = Index of Manure Exposure.