

UC Berkeley

UC Berkeley Previously Published Works

Title

Effects of concentrated poultry operations and cropland manure application on antibiotic resistant *Escherichia coli* and nutrient pollution in Chesapeake Bay watersheds

Permalink

<https://escholarship.org/uc/item/4zx9p9t2>

Authors

Amato, Heather K

Wong, Nora M

Pelc, Carey

et al.

Publication Date

2020-09-01

DOI

10.1016/j.scitotenv.2020.139401

Peer reviewed



Effects of concentrated poultry operations and cropland manure application on antibiotic resistant *Escherichia coli* and nutrient pollution in Chesapeake Bay watersheds

Heather K. Amato^a, Nora M. Wong^b, Carey Pelc^c, Kishana Taylor^d, Lance B. Price^b, Mark Altabet^e, Thomas E. Jordan^c, Jay P. Graham^{a,*}

^a Division of Environmental Health Sciences, University of California, Berkeley School of Public Health, 2121 Berkeley Way, Berkeley, CA 94704, United States of America

^b Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, 950 New Hampshire Ave NW, Washington, D.C. 20052, United States of America

^c Smithsonian Environmental Research Center, 647 Contees Wharf Rd, Edgewater, MD 21037, United States of America

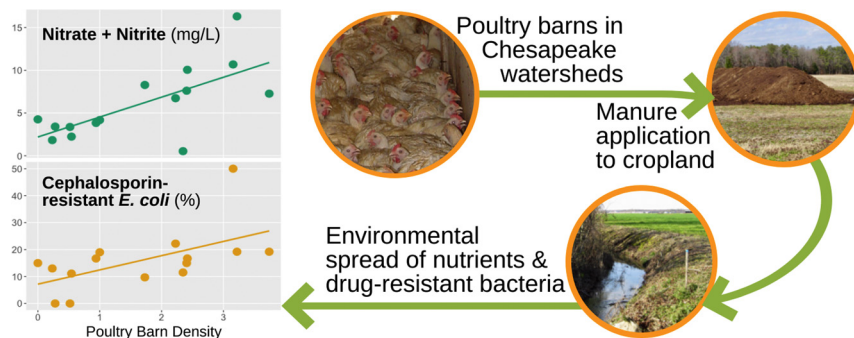
^d Department of Microbiology and Molecular Genetics, University of California, Davis, One Shields Ave, Davis, CA 95616, United States of America

^e Department of Estuarine and Ocean Sciences, School for Marine Science and Technology, University of Massachusetts Dartmouth, 836 S Rodney French Blvd, New Bedford, MA 02744, United States of America

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.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 13 March 2020

Received in revised form 10 May 2020

Accepted 11 May 2020

Available online 13 May 2020

Editor: Damia Barcelo

Keywords:

Concentrated animal feeding operations

Poultry litter

Nutrient pollution

Antibiotic resistance

E. coli

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 km²) 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 those without poultry barns. Analysis of N and O isotopes in nitrate by mass spectrometry showed an increase in the proportion of ¹⁵N 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

* Corresponding author at: 2121 Berkeley Way West, Berkeley, CA 94720-7358, United States of America.
E-mail address: jay.graham@berkeley.edu (J.P. Graham).

exposure estimated from poultry barn density and proportion of cropland in a watershed. The proportion of *E. coli* isolates resistant to ceftiofur, 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.

© 2020 Elsevier B.V. All rights reserved.

1. Introduction

Concentrated animal feeding operations (CAFOs) have been linked to both nutrient pollution and the environmental spread of antibiotic resistance (ABR) (Graham and Nachman, 2010), especially in waterways (Howarth et al., 2002). Additionally, the continued use of antibiotics in food animal production can increase the spread of antibiotic-resistant bacteria in the environment (Durso and Cook, 2014). 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 (Graham et al., 2009a). 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 (Moore et al., 1995; Paudel et al., 2004). 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 (USDA, 2012).

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 (Astor and Denver, 2015; Delaware, U. o., 2012). 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 (USEPA, n.d.; Weldon and Hornbuckle, 2006; Hodne, 2005). Increasing watershed discharges of nitrogen and phosphorus have had negative impacts on rivers, lakes, estuaries, and coastal waters globally (Nixon, 1995). 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 (Cloern, 2001). 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 (Marine Environments, 2018). In the Chesapeake Bay, increased nutrient inputs have increased the volume of hypoxic deep water (Hagy et al., 2004) and caused the widespread demise of submerged aquatic vegetation (Orth et al., 2010; Orth and Moore, 1983). Several studies have found potential links between watershed nutrient discharges and CAFOs in general (Orth and Moore, 1983) and poultry litter applications specifically (McBroom and Young, 2009a; Kleinman et al., 2015; Vories et al., 2001).

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 (Richmond, 1972). 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. (Johnson and Russo, 2002; Russo and Johnson, 2003) Chickens are known to be a reservoir of ExPEC, (Johnson and Russo, 2002; Russo and Johnson, 2003; Bergeron et al., n.d.) 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 (Mellata, 2013). 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, (Graham et al., 2009a; Silbergeld et al., 2008; Price et al., 2007; Hayes et al., 2004; McEwen and Fedorka-Cray, 2002) and resistant bacteria have been shown to survive in litter for several months (Graham et al., 2009b).

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 (Fig. 1). The watersheds, all on the Delmarva Peninsula, ranged from 0.36 to 18.18 km² in area (averaging 7.48 km²), 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 m (1/3 arc) digital elevation model for the Chesapeake Bay region was obtained from the 3D Elevation Project (USGS, n.d.-a) and used to determine upland watershed boundaries. Information about land cover was obtained from the 2006 National Land Cover Data (NLCD) set (USGS, n.d.-b). 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 (Parker and Li, 2006) and because cropland is sometimes misclassified as pasture (Wickham et al., 2013).

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 (Rhodes et al., 2011). Virtually all poultry growers work under

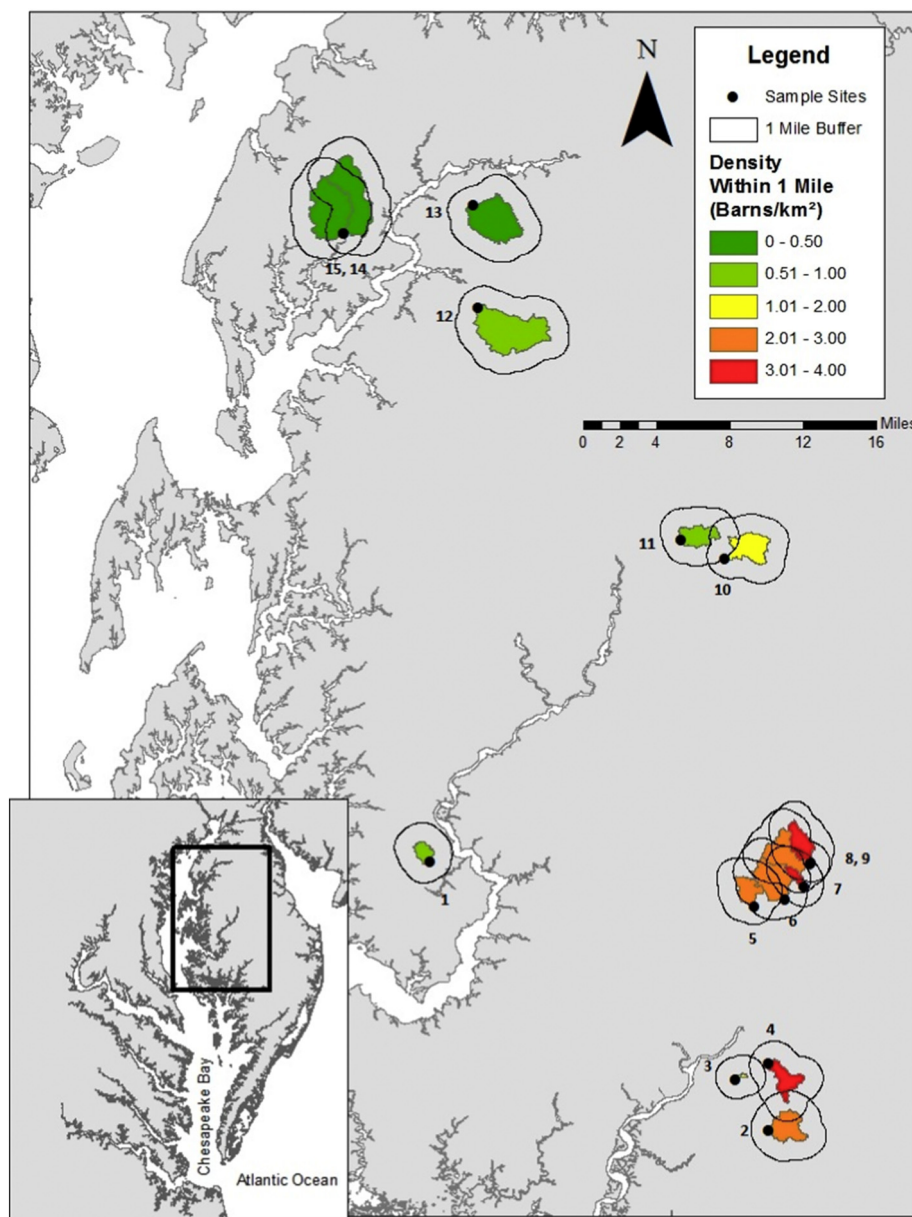


Fig. 1. Delmarva Peninsula sampling sites and poultry barn density within watersheds.

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 (Rhodes et al., 2011). Specialized poultry barns are typically 50–66 ft (15–20 m) wide and 600 ft (180 m) long, with 8 ft (2.4 m) high walls, and usually in groups of multiple barns (Rhodes et al., 2011). Poultry barns were counted using ESRI's ArcGIS© world imagery basemap and Google Earth imagery, as done by Fertig et al. (2014). The highest density of poultry barns among the watersheds we studied was in Sussex County, DE, a leading U.S. county for poultry sales (USDA, 2012). 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 t) of poultry litter annually (Rhodes et al., 2011). A long-term commitment to poultry growing is needed to recoup the costs of capital investments in the specialized barns (Rhodes et al., 2011) 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 1 L 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 (ThermoScientific, 2006). 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 <1% of nitrate plus NO_3^- (median 0.3%), we did not analyze the NO_2^- data

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)	Area of cropland/pasture in 1.6 km buffer (km ²)	No. poultry barns in 1.6 km buffer	IMX	No. water, sediment samples ^a	No. (%) samples positive for <i>E. coli</i>	No. (%) samples positive for ExPEC	No. (%) MDR <i>E. coli</i> isolates	Nutrient samples ^b	Average dissolved NO _x ⁻ -N (mg/L) ^c
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	0.29	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).

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

^b Additional water samples were taken separately to test for various forms of nitrogen and phosphorus.

^c NO_x⁻N indicates dissolved Nitrate plus Nitrite.

separately but instead analyzed patterns of NO₂⁻ plus NO₃⁻, which we abbreviate as NO_x⁻.

Total phosphorus (TP) was determined by digestion of both filtered and unfiltered samples to orthophosphate with perchloric acid (King, 1932). Phosphate (PO₄³⁻) in the digested sample was analyzed by reaction with stannous chloride and ammonium molybdate (APHA, 1992). Particulate TP was calculated by subtracting filtered TP from whole TP (Martin, 1972). Total Kjeldahl N (TKN) was determined by digestion of samples to ammonium with sulfuric acid, Hengar granules, and hydrogen peroxide (Martin, 1972). The ammonium (NH₄⁺) 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 ¹⁵N isotope compared to nitrogen in inorganic fertilizer (Karr et al., 2001a). Therefore, we analyzed the isotopic composition of NO₃⁻ 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 NO₃⁻ nitrogen and oxygen isotopic ratios expressed as δ¹⁵N and δ¹⁸O in per mil (‰) units relative to atmospheric N₂ and SMOW (standard mean ocean water), respectively. Our analyses proceeded as described in McIlvin and Altabet (2005) with improvements stated in Ryabenko et al. (2009) that achieved precisions of 0.2‰ for δ¹⁵N and 0.5‰ for ¹⁸O.

When NO₃⁻ is denitrified, the remaining un-denitrified NO₃⁻ becomes enriched in both ¹⁵N and ¹⁸O. Therefore, we used the ¹⁸O abundance to estimate the ¹⁵N composition before denitrification to obtain an unconfounded isotopic signal of N from manure. For this estimation, we assumed that denitrification increased δ¹⁵N twice as much as δ¹⁸O, as generally observed in groundwater (Granger et al., 2008). For comparison, we also assumed that the NO₃⁻ with the lowest δ¹⁸O observed (4.54‰) had undergone negligible denitrification. Therefore, we calculated the δ¹⁵N before denitrification as the measured δ¹⁵N minus two times the difference between the measured δ¹⁸O 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 L 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® (Nasco, 2006) and processed within 8 h 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 0.45 μm membrane filters (APHA 9222 B, 1999). Following filtration of 100 mL of each water sample, the filters were placed onto VRBA-MUG agar and incubated at 37 °C for 2 h and 44 °C for 22 h (Neogen Violet Red Bile Agar w/MUG (7359), n.d.). 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 h. Isolates were then streaked onto UTI Chrome agar plates to ensure specificity in identifying putative *E. coli* (Chromagar CHROMagar ECC, n.d.) and were incubated at 37 °C for 24 h. Pinkish-purple colonies were selected again to streak onto LB Agar plates (Teknova LB Agar Plates, n.d.) and were incubated at 37 °C for 24 h. 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 1 min, and allowed to settle for 1 min. 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 10 min, centrifuged at 1000 $\times g$ for 1 min, and then frozen at $-20\text{ }^{\circ}\text{C}$. 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) (Liu et al., 2012). The *uidA* gene was used for molecular identification of putative *E. coli* isolates (Millman et al., 2013). Multiplex real-time PCR assays were used to identify presumptive ExPEC with the presence of six hallmark virulence genes: *papA*, *papC*, *sfaE*, *afaC*, *kpsMIII*, and *iuta* (Table S2). Reactions consisted of 2 μL template DNA ($\sim 200\text{ ng}/\mu\text{L}$) 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 $\text{ng}/\mu\text{L}$) replaced the template DNA. Reactions were run on a Roche Light Cycler 480 (Roche, Pleasanton, CA) with the following conditions: 95 $^{\circ}\text{C}$ for 3 min followed by 45 amplification cycles of 95 $^{\circ}\text{C}$ for 15 s, and 55 $^{\circ}\text{C}$ for 1 min, followed by a 10 s cooling step at 40 $^{\circ}\text{C}$. Using the referenced methods, putative *E. coli* isolates positive for two or more of the six virulence genes were classified as presumptive ExPEC (Johnson et al., 2003).

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 30 μg , gentamicin- GM 10 μg), carbapenems (imipenem- 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 (Clinical and Laboratory Standards Institute, 2015). 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 (Center for Medicare and Medicaid Services, 2003).

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 (Jordan et al., 1997). 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 (Burnham and Anderson, 2002) 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 (Magiorakos et al., 2012). 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 1000 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).

Table 2

Mean, minimum and maximum average concentrations (mg/L 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 $\alpha = 0.05$ level.

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 (Fig. 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 <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:

$$\text{IMX} = \frac{\text{Poultry Barns within 1.6 km Buffer}}{\text{Cropland within 1.6 km Buffer (km}^2\text{)}} \times \frac{\text{Cropland within the Watershed (km}^2\text{)}}{\text{Total Area of Watershed (km}^2\text{)}}$$

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 (Fig. S1, Supporting Information). This was unexpected because TN concentration increases with % cropland (Fig. 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 (Fig. 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 (Karr et al., 2001b). 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 (Chen and MacQuarrie, 2005).

There was a positive correlation between ^{15}N and ^{18}O abundance in NO_3^- (Fig. 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.

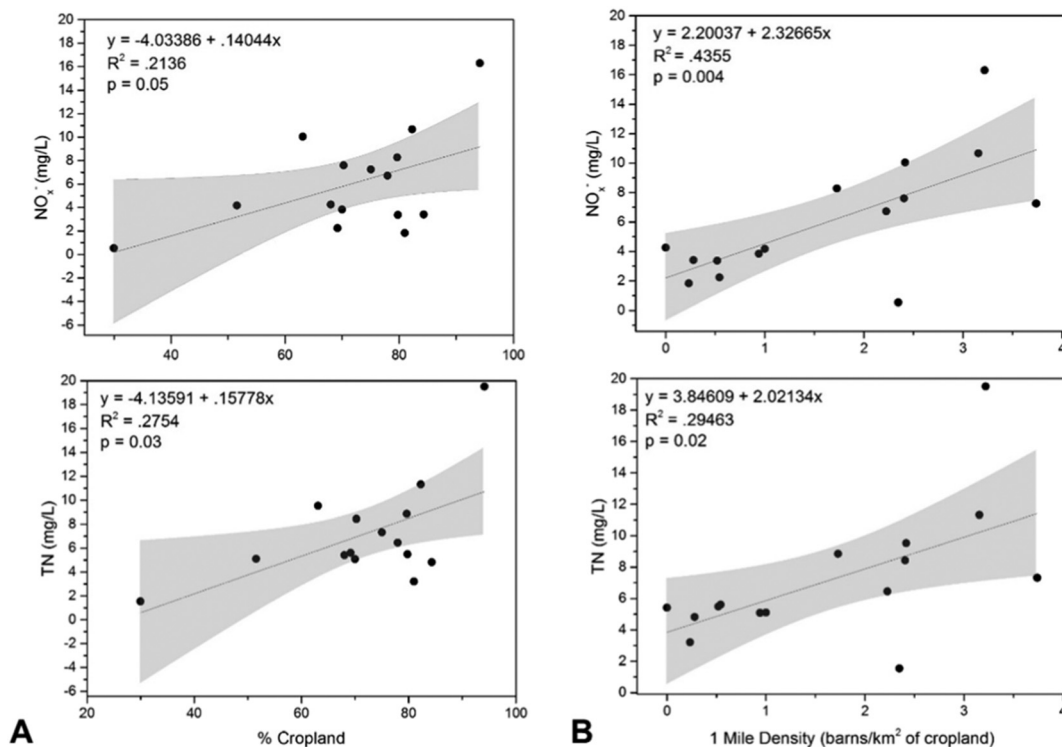


Fig. 2. Relationship between NO_x^- , TN and the percentage of cropland within the watershed (A) and the barn density per km^2 of cropland within a 1-mile (1.6 km) buffer of the watershed (B). Shaded areas represent the 95% confidence limits of the univariate linear regressions.

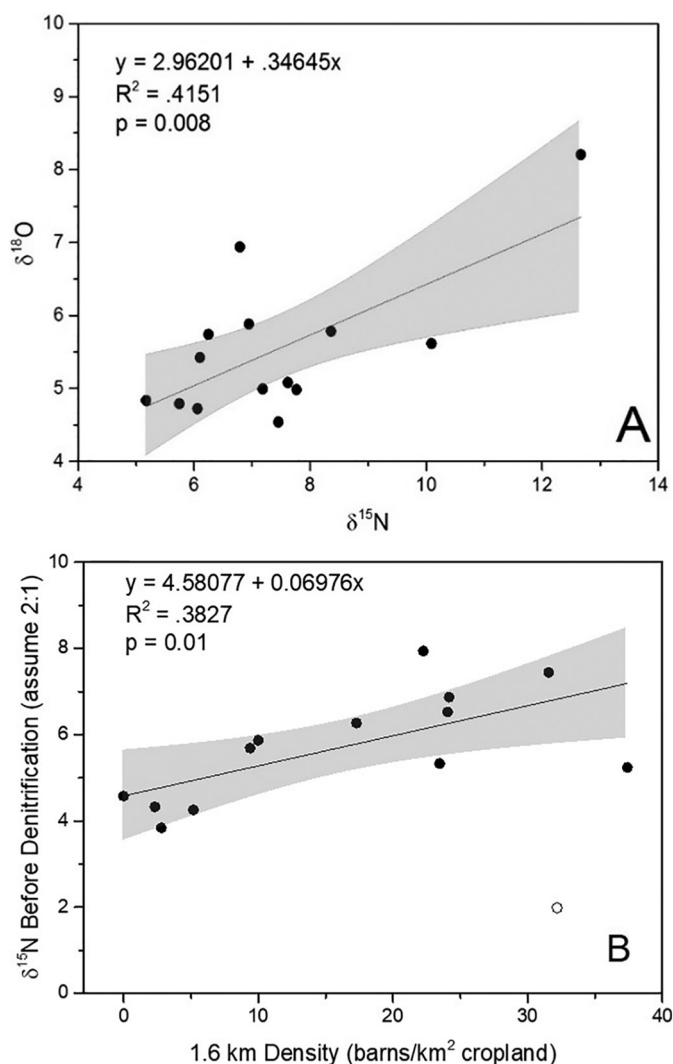


Fig. 3. Linear relationship between isotopes $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ in NO_3^- (A) and estimated $\delta^{15}\text{N}$ in NO_3^- before denitrification vs. poultry barn density within the manure-shed (B). Hollow data point represents an outlier in the data that was removed to visualize linear relationship between poultry barn density and additional enrichment of $\delta^{15}\text{N}$. 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.

Estimated $\delta^{15}\text{N}\text{-NO}_3^-$ 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 (Fig. 3). The general pattern in $\delta^{15}\text{N}\text{-NO}_3^-$, however, suggests that manure becomes an increasingly important source of NO_3^- N as poultry barn density increases.

3.3. *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 to 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%), ceftiofloxacin (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; Fig. S2, Supporting Information). For every one-unit increase in IMX, the percent of cephalosporin resistant isolates increased by 6.43% (Fig. 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 ($\beta = 6.43$; 95% CI = 6.28, 6.57, Table 3). Corresponding increases for specific cephalosporins were 18.9%, 16.9%, and 6.2% for ceftiofloxacin, 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 ($R^2 = 0.24$, $\beta = 4.21$; 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 (Jordan et al., 1997; Sutton et al., 2009). 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 (Sutton et al., 2009). 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 (Fig. 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 (Weldon and Hornbuckle, 2006; McBroom and Young, 2009b; Ciparis et al., 2012). CAFO density was positively correlated with concentrations of dissolved inorganic N and phosphate streams draining watersheds in the Shenandoah River Basin in Virginia (Ciparis et al., 2012). 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 (Ciparis et al., 2012). 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 b/km² 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/km², and 9.9 mg TN/L and 9.6 mg NO_x^- -N/L at 3.7 b/km². Thus, we would expect a watershed with the highest

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
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 $\alpha = 0.05$ level. MSE = mean squared error, CI = confidence interval, IMX = Index of Manure Exposure.

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 $\delta^{15}\text{N}$ before denitrification based on $\delta^{18}\text{O}$ and the assumption that denitrification increases $\delta^{15}\text{N}$ at twice the rate that it increases $\delta^{18}\text{O}$. Measurements of the relative rate of change in $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ with denitrification differ but often follow a 2:1 ratio, particularly in groundwater (Chen and MacQuarrie, 2005). In contrast, the ratio has been found to be 1:1 for marine denitrifiers (Granger et al., 2008). By comparison, a regression of our $\delta^{15}\text{N}$ vs. $\delta^{18}\text{O}$ measurements has a slope of 1.7:1, with $\delta^{18}\text{O}$ 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: $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ measurement contradicts this conclusion and remains unexplained (Fig. 3).

We found a negative association between barn density and TKN, whole TP, particulate TP, and TSS (Fig. 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 (Karr et al., 2001b). 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 to 40 years (Sanford and Pope, 2013). 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 (Kleinman et al., 2015; Staver and Brinsfield, 2001). 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 (Karr et al., 2001b; Correll, 1999). 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 m 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 (USEPA, 2012).

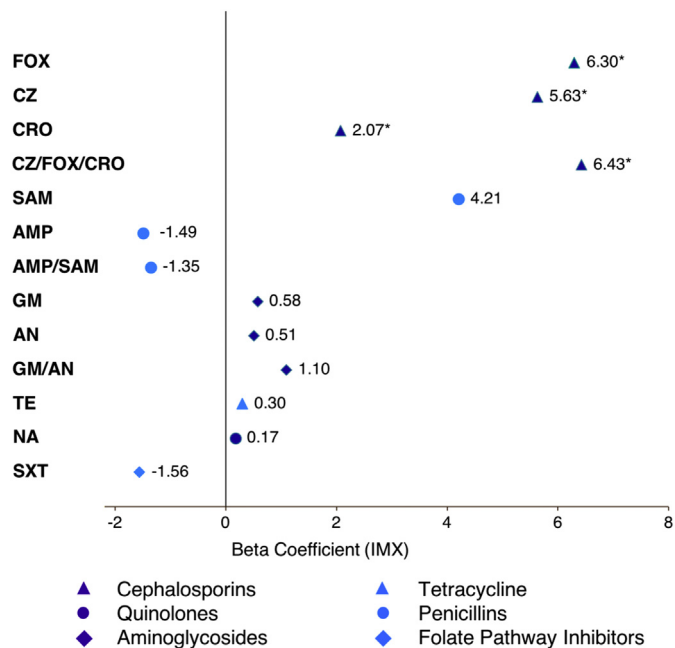


Fig. 4. Predicted effect of IMX on prevalence of resistance among *E. coli* isolates by sample site. Estimates are beta coefficients from univariate linear regressions of bootstrapped samples. Beta coefficients represent the change in percent of resistant isolates per one-unit increase of IMX, which ranged from 0 to 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 (Fig. S3). *Estimates are significant at the $\alpha = 0.05$ level.

Antibiotic resistance has been associated with increased nutrient concentrations from manure application in agricultural watersheds in Canada (Maal-Bared et al., 2013) and China (Zhang et al., 2014; Chen et al., 2017) 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 (Schlüter et al., 2007). Plasmid-mediated horizontal gene transfer facilitates the dissemination of resistance genes among human and animal pathogens transmitted in soil and waterways (von Wintersdorff et al., 2016). 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 (Sayah et al., 2005). 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 (USFDA, n.d.). Evidence suggests that cephalosporin resistance in *E. coli* from poultry decreases sharply following the cessation of cephalosporin use (Dutil et al., 2010). 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 (Hartmann et al., 2012). 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 (Pleydell et al., 2007) or from historical use in poultry operations (Tadesse et al., 2012) 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 t of litter annually (Rhodes et al., 2011). 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 (Ribaud et al., 2011; Carrel et al., 2016). 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.

Funding sources

This study was funded by grants from the Smithsonian Institution, the George Washington University Opportunity Fund, and support for JG and HA was covered in part by the NIH, under Award Numbers: K01 TW 009484 and R01AI135118.

Credit authorship contribution statement

Heather K. Amato: Formal analysis, Writing - review & editing. **Nora M. Wong:** Investigation, Writing - review & editing. **Carey Pelc:** Investigation, Writing - review & editing. **Kishana Taylor:** Investigation, Writing - review & editing. **Lance B. Price:** Investigation, Writing - review & editing. **Mark Altabet:** Formal analysis. **Thomas E. Jordan:** Conceptualization, Methodology, Formal analysis, Writing - original draft. **Jay P. Graham:** Conceptualization, Methodology, Formal analysis, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful for the expertise and input of Dr. Gregg Davis, Dr. Jeanne Jordan, Mr. Matthew McCarroll, Mr. Nitin Sukumar and Dr. Alan Hubbard for their valuable contributions to the methodology, microbial testing process, and data analysis. We also thank the volunteers who devoted their time to collecting samples.

Appendix A. Supplementary data

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). Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2020.139401>.

References

- APHA, 1992. Standard Methods for the Examination of Water and Wastewater. 1995. APHA 9222 B, 1999. Standard Total Coliform Membrane Filter Procedure.
- Ator, S., Denver, J., 2015. Understanding Nutrients in the Chesapeake Bay Watershed and Implications for Management and Restoration—The Eastern Shore. U.S. Geological Survey, U.S. Department of the Interior, Reston, Virginia.
- Bergeron, C. R.; Prussing, C.; Boerlin, P.; Daignault, D.; Dutil, L.; Reid-Smith, R. J.; Zhanel, G. G.; Manges, A. R., Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada, Emerg. Infect. Dis. 2012, 18 (2).
- Burnham, K., Anderson, D.R., 2002. Model Selection and Multimodel Inference. Second edition. Springer-Verlag, New York.

- Carrel, M., Young, S.G., Tate, E., 2016. Pigs in space: determining the environmental justice landscape of swine concentrated animal feeding operations (CAFOs) in Iowa. *Int. J. Environ. Res. Public Health* 13 (9).
- Center for Medicare & Medicaid Services. Acceptable Limits for Quality Control Strains Used to Monitor Accuracy of Disk Diffusion Testing of Nonfastidious Organisms (Using Mueller-Hinton Medium Without Blood or Other Supplements) ASPEN Survey Explorer Update- Attachment 3 [Online], 2003. (accessed January 24, 2015).
- Chen, D.J.Z., MacQuarrie, K.T.B., 2005. Correlation of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in NO_3^- during denitrification in groundwater. *J. Environ. Eng. Sci.* 4, 221–226.
- Chen, Z., Yu, D., He, S., Ye, H., Zhang, L., Wen, Y., Zhang, W., Shu, L., Chen, S., 2017. Prevalence of antibiotic-resistant *Escherichia coli* in drinking water sources in Hangzhou city. *Front. Microbiol.* 8, 1133.
- Chromagar CHROMagar ECC, d. http://www.chromagar.com/fichiers/1402908186LF_EXT_003_ECC_V6_Siteweb.pdf.
- Ciparis, S., Iwanowicz, L.R., Voshell, J.R., 2012. Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams. *Sci. Total Environ.* 414, 268–76.55.
- Clinical & Laboratory Standards Institute, 2015. Performance Standards for Antimicrobial Disk Susceptibility Tests. 12th edition. <https://clsi.org/standards/products/microbiology/m02/> (accessed January).
- Cloern, J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. *Mar. Ecol. Prog. Ser.* 210, 223–253.
- Correll, D.L., 1999. Phosphorus: a rate limiting nutrient in surface waters. *Poult. Sci.* 78 (5), 674–682.
- Delaware, U. o., 2012. Phosphorous in Poultry Litter: Guidelines from the University of Delaware. University of Delaware College of Agriculture and Natural Resources, Newark, DE.
- Durso, L.M., Cook, K.L., 2014. Impacts of antibiotic use in agriculture: what are the benefits and risks? *Curr. Opin. Microbiol.* 19, 37–44.
- Dutil, L., Irwin, R., Finley, R., et al., 2010. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. *Emerg. Infect. Dis.* 16 (1), 48–54. <https://doi.org/10.3201/eid1601.090729>.
- Fertig, B., Carruthers, T.J.B., Dennison, W.C., 2014. Oyster $\delta^{15}\text{N}$ as a bioindicator of potential wastewater and poultry farming impacts and degraded water quality in a subestuary of Chesapeake Bay. *J. Coast. Res.* 30 (5), 881–892.
- Graham, J., Nachman, K., 2010. Managing waste from confined animal feeding operations in the United States: the need for sanitary reform. *J. Water Health* 8 (4), 646–670.
- Graham, J.P., Price, L.B., Evans, S.L., Graczyk, T.K., Silbergeld, E.K., 2009a. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. *Sci. Total Environ.* 2701–2710.
- Graham, J.P., Evans, S.L., Price, L.B., Silbergeld, E.K.J.E.r., 2009b. Fate of antimicrobial resistant enterococci and staphylococci and resistance determinants in stored poultry litter. *Environ. Res.* 109 (6), 682–689.
- Granger, J., Sigman, D.M., Lehmann, M.F., Tortell, P.D., 2008. Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria. *Limnol. Oceanogr.* 53 (6), 2533–2545.
- Hagy, J.D., Boynton, W.R., Keefe, C.W., Wood, K.V., 2004. Hypoxia in Chesapeake Bay, 1950–2001: long-term change in relation to nutrient loading and river flow. *Estuaries* 27, 634–658.
- Hartmann, A., Amoureux, L., Locatelli, A., Depret, G., Jolivet, C., Gueneau, E., Neuwirth, C.J.F. i.m., 2012. Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy region). *Front. Microbiol.* 3, 83.
- Hayes, J.R., English, L.L., Carter, P.J., Proescholdt, T., Lee, K.Y., 2004. Multiple-antibiotic resistance of *Enterococcus* spp. isolated from commercial poultry production environments. *Appl. Environ. Microbiol.* 70 (7), 6005–6011.
- Hodne, C.J., 2005. Concentrating on Clean Water: The Challenge of Concentrated Animal Feeding Operations; the Iowa Policy Project: Mount Vernon.
- Howarth, R.W., Sharpley, A., Walker, D., 2002. Sources of nutrient pollution to coastal waters in the United States: implications for achieving coastal water quality goals. *Estuaries* 2002 (25), 656–676.
- Johnson, J.R., Russo, T.A., 2002. Extraintestinal pathogenic *Escherichia coli*: “the other bad *E. coli*”. *J. Lab. Clin. Med.* 139 (3), 155–162.
- Johnson, J.R., Murray, A.C., Gajewski, A., Sullivan, M., Snippes, P., Kuskowski, M.A., Smith, K.E., 2003. Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products. *Antimicrob. Agents Chemother.* 47 (7), 2161–2168.
- Jordan, T.E., Correll, D.L., Weller, D.E., 1997. Effects of agriculture on discharges of nutrients from coastal plain watersheds of Chesapeake Bay. *J. Environ. Qual.* 26, 836–848.
- Karr, J.D., et al., 2001a. Tracing nitrate transport and environmental impact from intensive swine farming using delta nitrogen-15. *J. Environ. Qual.* 30 (4), 1163–1175.
- Karr, J.D., Showers, W.J., Gilliam, J.W., Andres, A.S., 2001b. Tracing nitrate and environmental impact from intensive swine farming using delta nitrogen-15. *J. Environ. Qual.* 30, 1163–1175.
- King, E.J., 1932. The colorimetric determination of phosphorus. *Biochem. J.* 26, 292–297.
- Kleinman, P.J., Church, C., Saporito, L.S., McGrath, J.M., Reiter, M.S., Allen, A.L., Tingle, S., Binford, G.D., Han, K., Joern, B.C., 2015. Phosphorus leaching from agricultural soils of the delmarva peninsula, USA. *J. Environ. Qual.* 44 (2), 524–534.
- Liu, C.M., Aziz, M., Kachur, S., Hsueh, P., Huang, Y., Keim, P., Price, L.B., 2012. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiol.* 12, 56.
- Maal-Bared, R., Bartlett, K.H., Bowle, W.R., 2013. Phenotypic antibiotic resistance of *Escherichia coli* and *E. coli* O157 isolated from water, sediment and biofilms in an agricultural watershed in British Columbia. *Sci. Total Environ.* 443, 315–323.
- Magiorakos, A.P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C., Harbarth, S., Hindler, J., Kahlmeter, G., Olsson-Liljequist, B.J.C.m., 2012. Infection, multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18 (3), 268–281.
- Marine Environments, 2018. Harmful algal blooms | CDC. <https://www.cdc.gov/habs/illness-symptoms-marine.html>.
- Martin, D.F., 1972. *Marine Chemistry, v.1, Analytical Methods*. 1. Marcel Dekker, New York.
- McBroom, M.W., Young, J.L., 2009a. The Poultry Litter Land Application Rate Study – Assessing the Impacts of Broiler Litter Applications on Surface Water Quality. Nova Publishers Hauppauge, New York.
- McBroom, M.W., Young, J.L., 2009b. Assessing the Impacts of Broiler Litter Applications on Surface Water Quality. Faculty Publications.
- McEwen, S.A., Fedorka-Cray, P.J., 2002. Antimicrobial use and resistance in animals. *Clin. Infect. Dis.* 34 (3), S93–S106.
- McIlvin, M.R., Altabet, M.A., 2005. Chemical conversion of nitrate and nitrite to nitrous oxide for nitrogen and oxygen isotopic analysis in freshwater and seawater. *Anal. Chem.* 77 (17), 5589–5595 (Referenced in Supporting Information).
- Mellata, M., 2013. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. *Foodborne Pathog. Dis.* 10 (11), 916–932.
- Millman, J.M., Waits, K., Grande, H., Marks, A.R., Marks, J.C., Price, L.B., Hungate, B.A., 2013. Prevalence of antibiotic-resistant *E. coli* in retail chicken: comparing conventional, organic, kosher, and raised without antibiotics. *F1000Research* 2, 155.
- Moore, P., Daniel, T., Sharpley, A., Wood, C., 1995. Poultry manure management: environmentally sound options. *J. Soil Water Conserv.* 50 (3), 321–327.
- Nasco, 2006. Instruction Sheet for Whirl-Pak Sample Pags.
- Neogen Violet Red Bile Agar w/MUG (7359), d. http://www.neogen.com/Acumentia/pdf/ProdInfo/7359_Pl.pdf.
- Nixon, S.W., 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia* 41, 199–219.
- Orth, R.J., Moore, K.A., 1983. Chesapeake Bay: an unprecedented decline in submerged aquatic vegetation. *Science* 222, 51–53.
- Orth, R.A., Williams, M.R., Marion, S.R., Wilcox, D.J., Carruthers, T.J., Moore, K.A., Kemp, W.M., Dennison, W.C., Rybicki, N., Bergstrom, P., Barui, R.A., 2010. Long-term trends in submersed aquatic vegetation (SAV) in Chesapeake Bay, USA, related to water quality. *Estuar. Coasts* 33, 1144–1163.
- Parker, D., Li, Q., 2006. Poultry Litter Use and Transport in Caroline, Queen Anne's, Somerset and Wicomico Counties in Maryland: A Summary Report; Mid-Atlantic Regional Water Program.
- Paudel, K.P., Adhikari, M., Martin, N.R., 2004. Evaluation of broiler litter transportation in northern Alabama, USA. *J. Environ. Manag.* 73 (1), 15–23.
- Pleydell, E.J., Brown, P.E., Woodward, M.J., Davies, R.H., French, N.P., 2007. Sources of variation in the ampicillin-resistant *Escherichia coli* concentration in the feces of organic broiler chickens. *Appl. Environ. Microbiol.* 203–210.
- Price, L.B., Graham, J.P., Lackey, L.G., Roess, A., Vailes, R., Silbergeld, E., 2007. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. *Environ. Health Perspect.* 115 (12), 1738–1742.
- Rhodes, J.L., Timmons, J., Nottingham, J.R., Musser, W., 2011. In: Extension, U.o.M.C. (Ed.), *Broiler Production Management for Potential and Existing Growers*. University of Maryland Cooperative Extension Poultry, pp. 1–23.
- Ribaudo, M.G.N., Aillery, M., Kaplan, J., Johansson, R., Agapoff, J., Christensen, L., Breneman, V., Peters, M., 2011. *Manure Management for Water Quality: Costs to Animal Feeding Operations of Applying Manure Nutrients to Land*. U.S. Department of Agriculture-Economic Research Service.
- Richmond, M., 1972. Third Stenhouse-Williams memorial lecture: some environmental consequences of the use of antibiotics: or ‘what goes up must come down’. *J. Appl. Bacteriol.* 35 (2), 155–176.
- Russo, T.A., Johnson, J.R., 2003. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes Infect.* 449–456.
- Ryabenko, E., Altabet, M.A., Wallace, D.W., 2009. Effect of chloride on the chemical conversion of nitrate to nitrous oxide for $\delta^{15}\text{N}$ analysis. *Limnol. Oceanogr. Methods* 7 (7), 545–552 (Referenced in Supporting Information).
- Sanford, W.E., Pope, J.P., 2013. Quantifying Groundwater's role in delaying improvements to Chesapeake Bay water quality. *Environ. Sci. Technol.* 47, 13330–13338.
- Sayah, R.S., Kaneene, J.B., Johnson, Y., Miller, R., 2005. Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Environ. Microbiol.* 71, 1394–1404.
- Schlüter, A., Szczepanowski, R., Pühler, A., Top, E.M., 2007. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* 31 (4), 449–477.
- Silbergeld, E.K., Graham, J.P., Price, L.B., 2008. Industrial food animal production, antimicrobial resistance, and human health. *Annu. Rev. Public Health* 29, 151–169.
- Staver, K.W., Brinsfield, R.B., 2001. Agriculture and water quality on the Maryland Eastern Shore: where do we go from Here?: long-term solutions to accelerated eutrophication must provide mechanisms for redistributing nutrients flowing into concentrated animal-producing regions. *BioScience* 51 (10), 859–868.
- Sutton, A.J., Fisher, T.R., Gustafson, A.B., 2009. Historical changes in water quality at German branch in the Choptank River basin. *Water Air Soil Pollut.* 199 (1), 353–369.
- Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Aparna, S., Bartholomew, M.J., McDermott, P.F., 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. *Emerg. Infect. Dis.* 18 (5), 741–749.
- Teknova LB Agar Plates, d. <http://www.teknova.com/LB-Plates-s/45.htm>.
- ThermoScientific, ThermoScientific (Eds.), 2006. *ICS-2000 Ion Chromatography System Operator's Manual (Vol. Document No. 031857)*.

- USDA, 2012. Census of agriculture county profile Sussex county Delaware. https://www.agcensus.usda.gov/Publications/2012/Online_Resources/County_Profiles/Delaware/cp10005.pdf.
- USEPA, 2012. 2012 Recreational Water Quality Criteria. USEPA- Office of Water, Washington, D.C, pp. 1–2.
- USEPA, d. Nutrient pollution-sources and solutions <http://www2.epa.gov/nutrientpollution/sources-and-solutions>.
- USFDA, d. Cephalosporin order of prohibition questions and answers <https://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/ucm421538.htm>.
- USGS, d. 3D elevation program (3DEP) <http://nationalmap.gov/3DEP/>.
- USGS, d. National land cover database 2006 (NLCD 2006). <http://www.mrlc.gov/nlcd2006.php>, Accessed date: 12 September 2016.
- von Wintersdorff, C.J.H., et al., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* 7.
- Vories, E.D., Costello, T.A., Glover, R.E., 2001. Runoff from cotton fields fertilized with poultry litter. *Trans. ASAE* 44 (6), 1495–1502.
- Weldon, M.B., Hornbuckle, K.C., 2006. Concentrated animal feeding operations, row crops, and their relationship to nitrate in eastern Iowa Rivers. *Environ. Sci. Technol.* 40 (10), 3168–3173.
- Wickham, J.D., Stehman, Stephen V., Gass, Leila, Dewitz, Jon, Fry, Joyce A., Wade, Timothy G., 2013. Accuracy assessment of NLCD 2006 land cover and impervious surface. *Remote Sens. Environ.* 130, 294–304.
- Zhang, X., Li, Y., Liu, B., Wang, J., Feng, C., Gao, M., Wang, L., 2014. Prevalence of veterinary antibiotics and antibiotic-resistant *Escherichia coli* in the surface water of a livestock production region in northern China. *PLoS One* 9 (11), e111026.