Occurrence, Spatiotemporal Variability, and Sources of Dissolved Organic Nitrogen (DON) in Low-relief Streams on the Eastern Shore of Virginia

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Dedication

For my children.

Abstract

Intensive agriculture and widespread fertilizer use on the Eastern Shore of Virginia has fueled concerns and investigations into the upland-derived nitrate (NO_3^-) in low-relief streams draining into the shallow, seaside coastal lagoons. However, a thorough understanding of the dynamics of dissolved organic nitrogen (DON) in freshwater sources that may also contribute to nitrogen loading to the lagoons is lacking. This study quantified concentrations of DON, NO_3^-, and total dissolved nitrogen (TDN) under baseflow conditions in 15 streams varying in watershed size and cropland use on the Eastern Shore of Virginia across a one-year period to examine spatial and seasonal patterns. Mean concentrations of DON in streams ranged from 0.328 to 2.14 mg N L^{-1} and represented 12 to 70% of the TDN pool. In 14 of the 15 streams, NO_3^- was the principal form of nitrogen ranging in mean concentrations from 0.094 to 6.06 mg N L^{-1}. Instream DON concentrations were independent of NO_3^- concentrations, watershed area, and cropland use. Unlike NO_3^-, DON varied seasonally with maximal DON concentrations observed in spring. DON ranged from 6 to 41% of the TDN in groundwater with concentrations from 0.776 to 2.12 mg N L^{-1}. These concentrations were lower than the respective concentrations determined in surface-water samples (0 to 0.773 mg N L^{-1}) collected concurrently. In a laboratory experiment, DON was eluted in the effluent (1.02 mg N L^{-1}) from an intact sediment core using artificial groundwater influent containing NO_3^- only and represented nearly 60% of the TDN in the core effluent sample. The data from this study establish DON as an important and dynamic constituent of the TDN pool in freshwater systems on the Eastern Shore of Virginia.

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1. Introduction

1.1 Background Literature

Industrial conversion of gaseous nitrogen (N_2) to ammonia (NH_3) by way of the Haber-Bosch process gave rise to widespread use of synthetic fertilizers for industrial-scale food production, which has resulted in the over-enrichment of nitrogen in ecosystems and altered the global nitrogen cycle (Galloway and Cowling 2009). Coastal ecosystems, many of which are nitrogen-limited, are particularly susceptible to the consequences of nitrogen enrichment, including eutrophication, harmful algal blooms, hypoxia, and loss of seagrass habitats (Seitzinger and Harrison 2008). These economically and ecologically important ecosystems primarily receive land-derived nitrogen inputs through freshwater transport including rivers, streams, and groundwater (Boyer et al. 2006). Global riverine export of nitrogen is estimated to be 59 Tg N yr^{-1}, most of which is delivered to coasts (~48 Tg N yr^{-1}, 80%) and, to a lesser extent, to inland systems (11 Tg N yr^{-1}) by large rivers (Boyer et al. 2006).

Research aimed at determining nitrogen loading to coastal aquatic ecosystems has predominantly focused on the dissolved inorganic forms of reactive nitrogen (nitrite [NO_2^-], nitrate [NO_3^-], and ammonium [NH_4^+]) which are considered to be biologically available to primary producers (Kroeger et al. 2006, Webster et al. 2019). Despite representing a significant fraction of the total dissolved nitrogen (TDN) pool, dissolved organic nitrogen (DON) has been excluded from such investigations. DON is a mixture of nitrogen-containing organic compounds such as urea (a common constituent of fertilizer), amino and nucleic acids, proteins and humic-like substances, and other unidentified nitrogenous substances (Bronk 2002, Sipler and Bronk 2014). In rivers, autochthonous sources of DON include the release of nitrogenous organic compounds by living organisms such as bacteria, phytoplankton, macrophytes, and higher trophic organisms (Jørgensen 2009, Johnson et al. 2013). Other allochthonous sources of DON include inputs from groundwater and sediments as well as atmospheric precipitation, release from soil, and municipal and residential sewage (Seitzinger and Sanders 1999, Murphy et al. 2000, Pehlivanoglu-Mantas and Sedlak 2006). The exclusion of DON from many studies of nitrogen occurrence in fresh and coastal waters is due in part to its complex, heterogeneous composition and the requirement of a multi-chemical analysis approach for quantification instead of direct measurement. Moreover, DON has been historically ignored from nutrient budget studies based on the assumption that DON is unavailable for use by phytoplankton and therefore unlikely to contribute to eutrophication. The assumption was based on repeated observations of high concentrations of DON in nitrogen-limited aquatic ecosystems (Bronk et al. 2007). However, studies have demonstrated that a portion of DON may be utilized by phytoplankton and bacteria in freshwater, estuarine, and marine ecosystems (Seitzinger and Sanders 1997, 1999, Seitzinger et al. 2002, Bronk et al. 2007, Mulholland and Lomas 2008, Petrone et al. 2009, Fiedler et al. 2015, Jani and Toor 2018). Consequently, the DON pool can be divided into two fractions: the biologically labile (or readily available) fraction and the refractory (or less biologically available) fraction. These labile and refractory fractions are commonly defined in the literature by their molecular weights (Berman and Chava 1999, Bronk et al. 2007). Low-molecular-weight compounds in the DON pool, including urea and amino and nucleic acids, are considered biologically available. In contrast, humic-like substances with higher molecular weights are considered to be more refractory and consequently less biologically available (See et al. 2006, Bronk et al. 2007). Quantifying the magnitude of the labile and refractory fractions of DON has been the subject of several studies with estimates of the biologically available DON ranging from 10 to 70% (Seitzinger and Sanders 1997, 1999, Stepanauskas et al. 1999, Seitzinger et al. 2002, Stepanauskas et al. 2002, Bronk et al. 2007, Petrone et al. 2009, Jani and Toor 2018).

The biological availability (bioavailability or utilization) of DON can vary both by source and season. For instance, Seitzinger et al. (2002) demonstrated that the overall mean bioavailability of DON using estuarine biological communities was greatest for urban/suburban stormwater runoff (59%), followed by agricultural pasture runoff (30%). The utilization of DON in forest runoff samples was the lowest among the three sources (23%). The study by Seitzinger et al. (2002) revealed that bacterial and phytoplankton production by treatment with DON and dissolved inorganic nitrogen (DIN) were enhanced relative to treatment with DIN alone, although the relationship between DON utilization and phytoplankton growth was not well correlated. Utilization of DON varied seasonally by source. In agriculture pasture runoff, DON utilization was greatest in spring and lowest in fall, whereas DON from the urban/suburban runoff was high in spring and fall but not summer, and DON utilization in forest runoff was highest in summer (Seitzinger et al. 2002). Seasonal changes in bioavailability of DON was also examined over the course of one-year in a eutrophic estuary and an oligotrophic estuary (Knudsen-Leerbeck et al. 2017), and the investigators determined that the bioavailability of DON was low in winter and high during summer.

Beyond bioavailability, autochthonous sources of DON also can vary seasonally (Bronk et al. 1998). There are many sources of autochthonous DON in the water column. These include passive (diffusion) and active releases from bacteria (e.g., exoenzymes) and phytoplankton (exudation), sloppy feeding by heterotrophic grazing, decay and dissolution of fecal matter from zooplankton, viral-induced lysis of bacteria and phytoplankton, and bacterial transformation of detritus and associated release of DON (Bronk 2002). In the mesohaline portion of the Chesapeake Bay, DON release was greatest during May and lower in August and October due to autotrophic and heterotrophic processes (Bronk et al. 1998).

Both the importance and impact of DON bioavailability is dependent on whether or not DON is present at appreciable concentrations. DON has been shown to be the dominant form of nitrogen in several freshwater riverine systems (Wiegner et al. 2006, Petrone et al. 2009). Wiegner et al. (2006) investigated the concentrations of TDN and DON as well as the percent of TDN that is DON (%DON) in nine rivers along the eastern coast of the United States. Each river was characterized according to land use. Concentrations of TDN and DON ranged from 0.090 to 0.924 mg N L^{-1} and 0.015 to 0.486 mg N L^{-1}, respectively. In the nine freshwater rivers studied, %DON was highly variable with a range of 8 to 94% (Table 1). Pocomoke and Choptank, two rivers with the largest fraction of agricultural land use, exhibited high concentrations of DON

Table 1. Percent land cover and TDN and DON concentrations in nine rivers on the eastern coast of USA (Wiegner et al. 2006).

River	% Urban	% Agriculture	% Forest	% Wetland	% Other	TDN[1] mg N L^{-1}	DON[1] mg N L^{-1}	%DON
Forest 17a	0	0	100	0	0	0.188 ± 0.003	0.015 ± 0.003	8 ± 1
Bass	2.03	0	82.7	14.04	1.23	0.090 ± 0.001	0.052 ± 0.001	58 ± 1
Delaware	3.3	16.5	74.6	2.5	3	0.924 ± 0.017	0.109 ± 0.017	12 ± 1
Hudson	6.16	19.89	64.43	NA	9.42	0.581 ± 0.027	0.167 ± 0.027	28 ± 4
Altamaha	3.3	26.4	64.2	4.8	1.3	0.427 ± 0.028	0.179 ± 0.028	35 ± 0
Savannah	5.42	25.02	52.28	NA	17.28	0.596 ± 0.022	0.118 ± 0.022	20 ± 0.4
Pocomoke	1.1	44.9	56	14.4	2.4	0.519 ± 0.004	0.486 ± 0.004	94 ± 0
Choptank	1.9	55.3	26	14.4	2.4	0.440 ± 0.038	0.365 ± 0.038	83 ± 2
Peconic	33.33	10.42	18.75	NA	37.5	0.363 ± 0.011	0.214 ± 0.011	59 ± 1

[1] TDN and DON values were originally expressed by the authors of the study in units of µmol $L^{-1,}$ but were converted to mg N L^{-1} for the context of this study

relative to TDN, 94% and 83%, respectively. In four of the nine rivers surveyed, DON was the predominant form (i.e., >50% of TDN).

Land use is considered an important driver of nitrogen inputs into the environment (Causse et al. 2015). Only a few studies have evaluated land use as a driver for variation in

DON concentrations in freshwater systems (Pellerin et al. 2004, Kroeger et al. 2006, Aitkenhead-Peterson et al. 2009). Pellerin et al. (2004) examined the relationship between DON concentrations and developed land use in subcatchments of the Ipswich River located in northeastern Massachusetts. The 39 subcatchments were dominated by urban/developed land use consisting of residential areas as well as land purposed for commercial, industrial, and transportation uses with less than 10% as agricultural land. In the 39 subcatchments of the Ipswich River, DON concentrations were weakly correlated ($r^2 = 0.09$, $p = 0.04$) with developed land use. By expanding the dataset to include DON concentrations and land-use data from 117 additional sites from other studies, the correlation between DON and land use was slightly stronger compared to the original dataset for Ipswich River, but overall remained weak ($r^2 = 0.27$, $p < 0.01$). In contrast, the variation of %DON exhibited a much stronger relationship with developed land use ($r^2 = 0.63$, $p = 0.04$), which was weaker upon inclusion of the additional 117 sites (Pellerin et al. 2004). Similarly, no significant relationship was determined between DON and land use or population density in Texas watersheds with varying degrees of agriculture and urbanization (Aitkenhead-Peterson et al. 2009).

Kroeger et al. (2006) examined several factors influencing DON concentrations and %DON in groundwater for a group of watersheds in the northeastern United States, including land use and population density. The results of Kroeger et al. (2006) indicated that agricultural land use was not related to DON concentrations, but that DON was positively correlated with population density. The significant and positive relationship between DON concentration and population density contrasted with other studies that indicated DON is largely derived from natural sources.

Under baseflow conditions, groundwater is a major source of flow to nontidal streams draining to the Chesapeake Bay (Bachman et al. 1998). The widespread application of fertilizers on the Eastern Shore of Maryland and Virginia has fueled investigations into NO_3^- contamination in streams and groundwater (Denver et al. 2004, Gu et al. 2007, 2008, Flewelling et al. 2012, 2014, Ator and Denver 2015). These investigations revealed concentrations of NO_3^- in groundwater from the unconfined Columbia aquifer are notably higher compared to those concentrations found in stream waters and can exceed the U.S. Environmental Protection Agency (US EPA) drinking water limit of 10 mg N L^{-1} (US EPA 2004). Streambed sediments containing organic matter are considered to be important filters of NO_3^- in groundwater (Mills 2008). Akin to the body of research for DON in surface water, only a few studies have given attention to DON's presence in groundwater and related controlling factors (Kroeger et al. 2006). Furthermore, the findings by Kroeger et al. (2006) indicate that DON was a dominant form of nitrogen in groundwater within watersheds consisting of mostly forested and residential land use.

Streambed sediments play an essential role in the biogeochemical cycling of nitrogen (Burdige and Zheng 1998). This may be especially true for low-relief streams where rates of sediment and organic matter accumulation may be more appreciable relative to larger rivers. The roles of aquatic sediments in the removal of NO_3^- by denitrification to form gaseous N_2 have been well studied (Seitzinger 1990, Mills 2008). However, the release of DON from freshwater sediments through which NO_3^--rich groundwater passes is unresolved.

The primary objectives of this study were to:

- quantify the concentrations of DON and TDN in low-relief streams that discharge to the seaside lagoons of the Eastern Shore of Virginia;

- evaluate among-stream variability and seasonal patterns of DON, NO_3^-, and TDN concentrations;
- explore land-use drivers of among-stream variability; and
- investigate sources of DON during baseflow conditions in the Eastern Shore's seaside watersheds.

The data collected and presented in this study on DON (1) add to the body of evidence that NO_3^- alone may inadequately estimate biologically available nitrogen loading into seaside lagoons on the Eastern Shore and (2) provide a greater understanding of the spatiotemporal variability and sources of DON in low-relief streams on the Eastern Shore. The relationship between DON concentrations and land use as well as the extent of fertilizer application was examined to gain insights into anthropogenic activities related to industrial agriculture that may contribute to DON loading to the coastal lagoons. Furthermore, this study sought to determine whether groundwater or streambed sediments serve as potential allochthonous sources of DON to the study streams.

1.2 Research Questions

1. What are the concentrations of DON and TDN in low-relief streams on the Eastern Shore and how do the concentrations of DON compare to NO_3^- concentrations?
2. What are the seasonal and among-stream variabilities of DON, NO_3^-, and TDN concentrations in low-relief streams of mixed-use watersheds characterized predominantly by cropland agriculture?
3. Is groundwater an important source for DON in streams on the Eastern Shore and do streambed sediments through which NO_3^--rich groundwater is passed have the potential to contribute to DON concentrations in surface water?

2. Methods

2.1 Site Description

The study was conducted on the Eastern Shore of Virginia located at the southern end of the Delmarva Peninsula. The Delmarva Peninsula is situated on the mid-Atlantic coast of the United States (Figure 1) and is the most eastern region of Virginia's Coastal Plain. The western shore of the Delmarva Peninsula is bounded by the Chesapeake Bay while Virginia's portion of the Eastern Shore is bordered by coastal lagoons that serve collectively as an interface between land and the Atlantic Ocean located further east. These coastal lagoons are positioned within the Virginia Coast Reserve Long-Term Ecological Research (VCR-LTER) site.

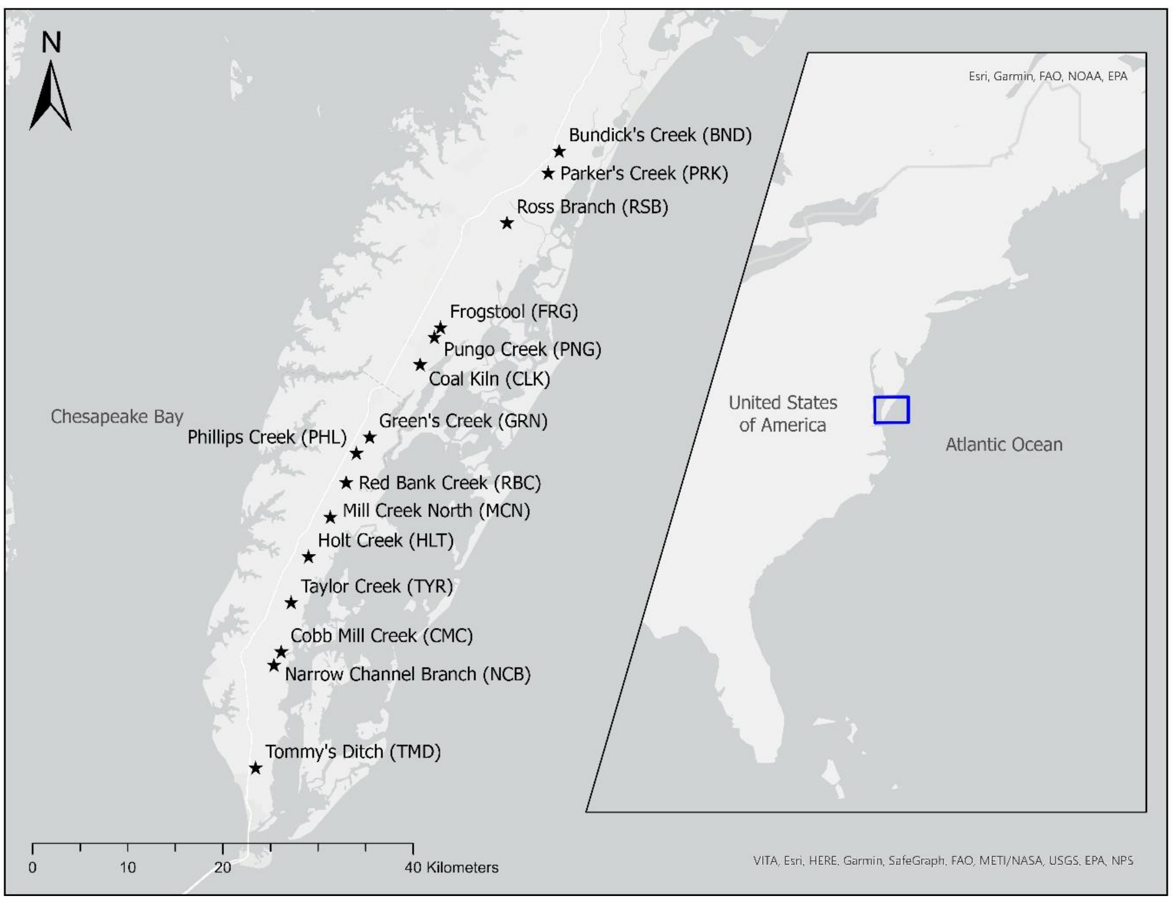

Figure 1. Map of Eastern Shore of Virginia along the mid-Atlantic coastline of the United States of the America with locations of 15 stream sites.

The shallow subsurface of the Eastern Shore of Virginia is constituted of sedimentary deposits that form the unconfined Columbia Aquifer as the topmost stratified layer underlain by additional shallow confined aquifers. The deposits of clay, silt, sand, gravel, and shell material that make up the Columbia aquifer are unconsolidated and highly permeable. The permeability of the overlying sandy soils enables freshwater recharge of the unconfined aquifer following local precipitation. The typically gaining streams on Virginia's Eastern Shore primarily receive groundwater discharge from the Columbia aquifer directly into the stream channels (Richardson 1995).

On the seaside of the Eastern Shore of Virginia, 15 streams ranging from the northern section of Accomack County to the southern portion of Northampton County were selected for this study (Figure 1, Table 2).

Table 2. Identification, location, and watershed area (ha) for 15 streams sites in the study area.

Stream Name	**Stream Name Abbreviation**	**Latitude**	**Longitude**	**Watershed Area (ha)**
Bundick's Creek	BND	37° 45' 57.7" N	75° 36' 23.5" W	30.5
Parker's Creek	PRK	37° 44' 46.3" N	75° 36' 39.8" W	49.8
Ross Branch	RSB	37° 41' 50.7" N	75° 40' 4.6" W	142
Frogstool	FRG	37° 35' 52.7" N	75° 44' 47.5" W	448
Pungo Creek	PNG	37° 35' 20.1" N	75° 45' 13.5" W	398
Coal Kiln	CLK	37° 33' 47.2" N	75° 46' 12.5" W	482
Green's Creek	GRN	37° 29' 37.8" N	75° 49' 47.2" W	139
Phillips Creek[a]	PHL	37° 27' 27.7" N	75° 51' 11.7" W	---
Red Bank Creek	RBC	37° 27' 2.3" N	75° 51' 25.4" W	140
Mill Creek North	MCN	37° 25' 2.8" N	75° 52' 33.2" W	135
Holt Creek	HLT	37° 23' 20.5" N	75° 53' 18.8" W	117
Taylor Creek	TYR	37° 20' 12.8" N	75° 55' 15.7" W	134
Cobb Mill Creek [Culvert] [a] [Hillslope][b]	 CMC-1 CMC-2	 37° 17' 28.9" N 37° 17' 27.2" N	 75° 55' 52.3" W 75° 55' 46.2" W	627
Narrow Channel Branch	NCB	37° 16' 37.9" N	75° 56' 27.0" W	235
Tommy's Ditch	TMD	37° 10' 47.3" N	75° 57' 41.1" W	541

[a] Watershed area not available for Phillips Creek
[b] Culvert site of Cobb Mill Creek was sampled on all sampling dates (Table 3)
[c] Hillslope site of Cobb Mill Creek was sampled only on 29 June 2020 for groundwater and surface water collection.

All streams selected for this study drain eastward toward the coastal lagoons. These freshwater streams are first- or second-order streams with low gradients and are surrounded by agricultural areas that undergo yearly cultivation and receive nitrogen-based fertilizer applications. Water samples were collected at one consistent location for each stream, typically where the stream emerged from a culvert under SR 600, and that position is the latitude-longitude value reported in Table 2.

2.2 Surface-water and Groundwater Sample Collection

Grab samples of surface water were collected upstream of the tidal influence in acid-washed, deionized water (DIW)-rinsed, stream-water-rinsed HDPE bottles between May 2019 and February 2020 during baseflow conditions (Table 3). All bottles were completely filled and capped. At least one sampling campaign of all stream sites with active flow was conducted every three months to evaluate spatial and seasonal variations in dissolved nitrogen species.

Table 3. Surface-water and groundwater sampling dates. Number of samples is reported in parentheses.

DATE COLLECTED	SAMPLES COLLECTED
11 May 2019	Surface Water (15)
29 June 2019	Surface Water (15) + Groundwater (5)
25 August 2019	Surface Water (15)
16 November 2019	Surface Water (15)
16 February 2020	Surface Water (15)
22 February 2020	Surface Water (8)

Two sample collections were conducted in February 2020. The first sample collection (16 February 2020) included all 15 streams. During sample collection, however, higher than typical stream flows were observed visually in the stream channels along with standing water in surrounding areas as a consequence of precipitation in the region several days before

(12 February 2020). To evaluate if flow conditions may have had an effect on dissolved nitrogen concentrations, a second sampling with a subset of streams was performed the following week (22 February 2020) during which no precipitation occurred.

During the June sampling campaign, groundwater samples were collected immediately following surface-water collection at Bundick's Creek, Coal Kiln, Phillips Creek, Cobb Mill Creek (Hillslope), and Tommy's Ditch. Groundwater collection was performed using a steel drive-point (internal diameter: 2.54 cm) and peristaltic pump. The steel drive-point was pounded using a mallet through streambed sediments in the center of the stream to an approximate depth of one meter. The vinyl tubing (Nalgene) was inserted into the drive-point, several volumes of the drive-point were pumped in order to rinse the equipment, and then a groundwater sample was collected as described above.

Following surface-water and groundwater collection, each sample was immediately placed on ice until all samples had been collected and could be transported to the Aqueous Geochemistry Laboratory at the University of Virginia. Upon arrival, samples were stored under refrigerated conditions (4°C) until filtration. Surface-water samples were filtered within two days of sample collection to minimize microbial activity during storage and to remove suspended material using 0.45-µm (pore diameter) membrane filters (Whatman Metricel®). Filtered samples were stored under refrigerated conditions to inhibit biological alteration of the nitrogen species by bacteria. Groundwater samples were centrifuged prior to filtration to remove large suspended material from the sample. Following centrifugation at approximately 5000 x *g*, the supernatant was decanted and refrigerated prior to filtration (0.45-µm pore diameter) within two days of sample collection. The compact, pelletized material at the bottom of the centrifuge tube was discarded.

2.3 Analysis of Dissolved Nitrogen Species

Concentrations of NO_3^- and NO_2^- in filtered samples were quantified using single-channel ion chromatography. Chromatographic separation of ionic species in aqueous samples was performed on a Dionex ICS-2100 ion chromatograph (IC) equipped with a Dionex IonPac AS18 anion-exchange column. Concentrations of NH_4^+ in surface water and groundwater samples were determined on a Lachat QuikChem® 8500 Flow Injection Autoanalyzer in accordance with QuikChem® Method 31-107-06-1-B.

Total dissolved nitrogen (TDN) was analyzed using the persulfate oxidation method (Nydahl 1978, Valderrama 1981, Bronk et al. 2000). Aliquots of 5 or 10 mL of samples were diluted prior to digestion in glass ampules to ensure the oxidizing reagent was in excess relative to the organic matter contributed by the sample aliquot. The efficiency of TDN analyses was assessed by inclusion of at least two dissolved organic nitrogen standards (i.e., urea, leucine, and/or glycine) in each digestion. Dissolved organic nitrogen standards were prepared in duplicate and processed in the same manner as the field samples. Because the persulfate reagent is alkaline, glass ampules were sealed immediately after the addition of the persulfate reagent to minimize the loss of the NH_3 by volatilization. The samples were autoclaved at 121°C at 15 PSI for approximately 40 minutes to promote the chemical oxidation of organic matter. Subsequent to digestion in the autoclave, the TDN samples were analyzed for NO_3^- on the autoanalyzer by the QuikChem® Method 31-104-04-1-E.

2.4 Land-Use Analysis of Watersheds

Land-use parameters, such as total watershed area, total cropland area, cropland area as a percentage of the total watershed area, and annual fertilizer application rate (expressed as kg N ha^{-1} $year^{-1}$) for each corresponding stream except for Phillips Creek (PHL) were determined via spatial analysis. No watershed delineation was available for PHL. Delineation of the watershed for each stream was digitized previously based on digital elevation models (DEMs) (A. L. Mills, unpublished data, 2019). The point from which the contributing watershed area was delineated was the point at which each stream was sampled in this study. Individual watershed delineations were merged into a single data layer within ArcGIS 2.3 Pro for Windows. The total area for each watershed was calculated using ArcGIS 2.3 Pro (ESRI 2020).

The two primary land uses in the study area are forested cover and agriculture for crop cultivation (Sanford et al. 2009). The USDA estimated that >70% of the non-pasture cropland in Accomack and Northampton counties is treated with fertilizer (chemical and manure), with a small fraction (14 to 24%) attributed to manure (USDA NASS 2017). Active agricultural fields used for crop cultivation on the Eastern Shore of Virginia were identified and manually delineated from high resolution aerial photography from the Virginia Base Mapping Program in a previous study aimed at estimating reactive nitrogen loads from fertilizer applications rates (Johnson 2018). In that study, a crop rotation and a fertilizer application rate based on nitrogen content (expressed as kg N ha^{-1} yr^{-1}) for each active agricultural field were assigned using 2013-2016 data from CropScape (USDA NASS 2016) and recommended fertilizer application rate from the Virginia Cooperative Extension, respectively (Johnson 2018).

The merged watershed data layer and agricultural fields data layer were overlaid in ArcGIS 2.3 Pro using a common projected coordinate system. An intersection analysis was then performed that generated a new data layer with whole or portions of the active agricultural fields that were positioned within the boundary of each watershed. The geospatial area of each intersected field was determined and aggregated within each watershed. Furthermore, the intersected field areas were multiplied by the recommended fertilizer application rate specific to each type of crop or crop rotation to determine the estimated amount of reactive nitrogen from fertilizer applied annually to each field. The estimated amount of reactive nitrogen applied annually in agricultural fields was then aggregated for each watershed. The percent of cropland within each watershed was calculated as the fraction of total area of agricultural fields normalized to the total watershed area and multiplied by 100%.

2.5 Sediment Core Experiment

To assess the production of DON within streambed sediments from the Eastern Shore of Virginia, a laboratory experiment was performed by pumping an artificial groundwater solution (AGW) through a sediment core collected from Coal Kiln on 27 April 2019 in conjunction with another investigator’s study assessing denitrification in the stream sediments (E. A. Cronin, personal communication, July 24, 2019). The core was collected by hammering a single, intact polyvinyl chloride (PVC) pipe with an internal diameter of 7.6 cm into the center of the streambed of Coal Kiln. The PVC pipe was carefully removed and capped before being transported to the laboratory and stored upright under frozen conditions.

For the sediment core study, the core was removed from frozen storage and mounted upright in the laboratory under ambient room-temperature conditions. The intact sediment core was then connected to a peristaltic pump via vinyl tubing (Nalgene). Once the core was sufficiently thawed, artificial groundwater (AGW) was prepared and pumped through the core for several weeks to ensure steady-state flow conditions. The AGW was prepared by diluting 60 mg $MgSO_4 \cdot 7H_2O$, 20 mg KNO_3, 36 mg $NaHCO_3$, 36 mg $CaCl_2$, 25 mg $CaSO_4 \cdot 2H_2O$ in 1 L of deionized water (Bolster 2000).

A sample of influent artificial groundwater and of core outflow were collected contemporaneously and stored under refrigerated conditions. The samples were filtered within two days of sample collection using 0.45-µm membrane filters and stored further under refrigerated conditions until TDN, NO_3^-, NO_2^-, and NH_4^+ analyses were performed as described in Section 2.3.

2.6 Data Analysis

Dissolved organic nitrogen (DON) was computed as the difference between the sum of the three major dissolved inorganic nitrogen species (NO_3^-, NO_2^-, and NH_4^+) and TDN, each of which were measured independently (methods described above):

$$DON = TDN - (NO_3^- + NO_2^- + NH_4^+) \qquad \text{(Equation 1)}$$

In cases where the calculated DON concentration was negative as a consequence of the measured TDN concentration being less than sum of the concentration of NO_3^-, NO_2^- and NH_4^-, the DON concentration was reported as zero. In addition, measured TDN concentrations were not adjusted based on the recovery of the organic standards since the composition of DON species among samples from different streams may not be uniform. Concentrations of nitrogen species measured below the lowest calibration standard but greater than zero are reported as quantified and the values were used to calculate DON. The value of %DON was the percentage of DON with respect to TDN (Equation 2).

$$\%DON = (DON / TDN) \times 100\% \qquad \text{(Equation 2)}$$

Calculations and data visualization were performed in R version 4.02 (The R Code Team 2020). Statistical analysis and data transformations were conducted in SAS version 9.4 (SAS Institute 2016). Unless otherwise specified, all tests used a significance level (α) of 0.05. Normality and homoscedasticity of the data were evaluated using the Shapiro- Wilk Test and Brown-Forsythe Test. Data were transformed as needed to meet the assumptions of parametric hypothesis testing including normality of residuals and equal variances. One-way analysis of variance (ANOVA) was then performed to determine differences among streams and sampling dates for each major dependent variable: NO_3^-, TDN, DON, and %DON. Correlations between dependent variables were evaluated using Pearson's correlation coefficient (r). Single linear regressions were performed to evaluate the relationships between land-use parameters. Other statistical analyses are described as appropriate in Chapter 3 (Results).

2.7 Analytical Error

Sources of error in this study are related to the chemical analysis of TDN and the multi-chemical approach for quantifying DON. The process of quantifying TDN in this study includes the oxidation of the dissolved organic matter in the filtered stream samples and measuring the resultant NO_3^- concentration as mg N L^{-1}. However, the yields of various organic nitrogen compounds through the digestion process can vary and are known to be incomplete (i.e., <100%). For example, oxidation yield of aliphatic amino acids and proteins by the persulfate oxidation method has been reported as low as 92% (Nydahl 1978). Also, the double bonds between two nitrogen atoms appear to be highly resistant to full oxidation into NO_3^- (Nydahl 1978). Therefore, TDN and DON concentrations may be underestimated. This would be particularly true for samples containing more refractory nitrogenous compounds. This source of error is considered relatively insignificant because the motivation for understanding nitrogen budgets and the dynamics of DON is related to the more bioavailable forms of DON which are expected to be readily oxidized to NO_3^- during the digestion process. Because DON is calculated and not measured directly, error in its measurement is propagated through error in the measurements of each chemical component used to determine DON (i.e., TDN, NO_3^-, NO_2^-, and NH_4^+).

3. Results

3.1 Differences between Two Closely-timed Sample Collections

Two sample collections were performed in February 2020, the first of which included 15 streams and the second set included eight streams. The second set of samples collected on 22 February 2020, which were used to verify the higher than normal stream flows observed during the initial sample collection on 16 February 2020 were not significantly different from samples six days earlier with respect to concentrations of NO_3^-, DON, and TDN as well as %DON. The nitrogen speciation results for the eight streams that were sampled twice in February are reported in Table A-1 (see Appendix A). A paired *t*-test was performed to evaluate the null hypothesis that the concentrations of NO_3^-, DON, and TDN as well as %DON were not significantly different from each other. No significant differences were identified for NO_3^-, $t(14) = -0.26$, $p = 0.797$, or TDN, $t(14) = 0.08$, $p = 0.797$ (Table A-2, Appendix A). DON concentrations were at the threshold level of statistical significance $t(14) = 2.13$, $p = 0.051$, and were more varied than NO_3^- and TDN concentrations. Lastly, %DON values were not significantly different between the two samples dates, $t(14) = 1.11$, $p = 0.288$. Due to the lack of significant differences in concentrations of dissolved nitrogen species between the two sets of samples, the samples collected on 16 February 2020 were considered appropriate for use in this study, and the samples from 22 February 2020 were not utilized further in this study.

3.2 Nitrogen Speciation in Surface Water

In 75 samples of surface water collected from low-relief streams on the Eastern Shore of Virginia over the course of a year, the most frequently detected nitrogen species in the TDN pool were NO_3^- and DON (Figure 2A-C). Ammonia was present in more than 50% of the surface-

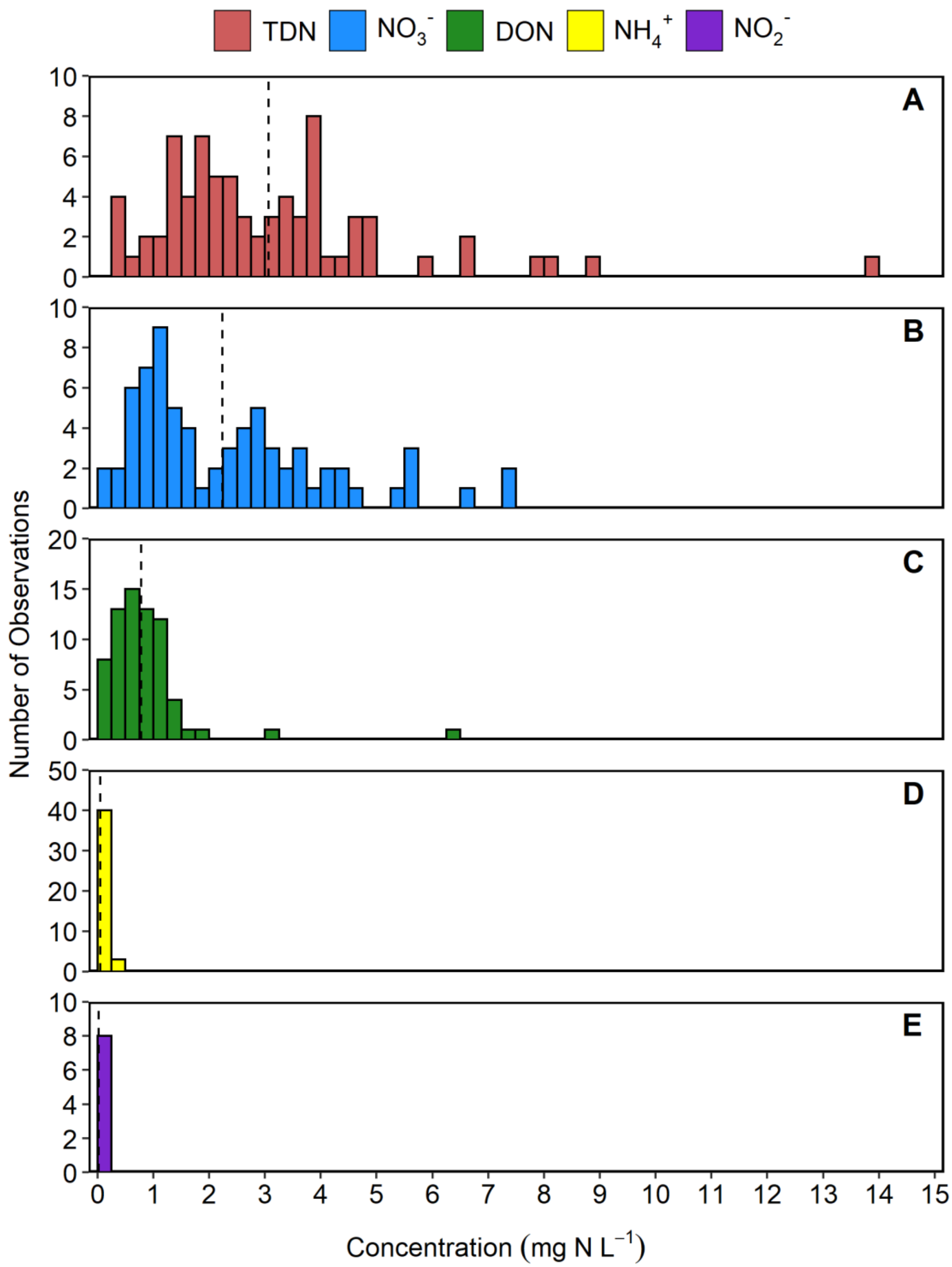

Figure 2. Number of observations of the various N species and their concentrations (bin widths = 0.25 mg N L^{-1}) in surface water collected from low-relief streams on the Eastern Shore of Virginia over the entire course of this study: **(A)** TDN, **(B)** NO_3^-, **(C)** DON, **(D)** NH_4^+, and **(E)** NO_2^-. Dotted lines represent the overall mean of all measured concentrations

water samples, but concentrations (<0.500 mg L^{-1}; Figure 2D) were negligible in comparison to NO_3^- and DON. NO_2^- was rarely quantified in samples and, when present, always occurred at low concentrations (<0.250 mg L^{-1}; Figure 2E). The results of this study, therefore, focus primarily on the more important forms of dissolved nitrogen, NO_3^- and DON, and their contribution to the TDN pool. The speciation data for all individual samples are reported in Appendix B.

The concentrations of NO_3^- ranged from 0 to 7.40 mg N L^{-1}, with an overall mean of 2.23 mg N L^{-1} (Figure 2B). The mean NO_3^- concentrations for each stream ranged from 0.094 to 6.06 mg N L^{-1} (Figure 3 and Appendix C). The streams with the lowest and highest mean NO_3^- concentrations were Mill Creek North (MCN) and Tommy's Ditch (TMD), respectively (Figure 3 and Appendix C). Excluding MCN, mean NO_3^- concentration in each stream was greater than the corresponding mean DON concentration (Figure 3).

To determine differences in NO_3^- concentrations among streams, the suitability of a parametric one-way ANOVA was assessed. This assessment included an examination of normality for residuals using the Shapiro-Wilk test and of homogeneity of variances using a Brown–Forsythe test . The residuals of NO_3^- concentrations were negatively skewed ($W = 0.940$, $p = 0.001$) and subsequently transformed using a square-root function to fit a more normal distribution ($W = 0.973$, $p = 0.107$) (Appendix D).

The variances of NO_3^- concentrations from the median value of each stream were homogenous, $F(14,60) = 0.95$, $p = 0.510$). Therefore, the among-stream differences in NO_3^- were compared using a one-way ANOVA. There were significant differences in NO_3^- concentrations, $F(14,60) = 12.54$, $p < 0.001$) (Appendix D). A post-hoc Ryan-Einot-Gabriel-

Welsch Multiple Range (REGWQ) Test was performed to identify specific differences among streams (Appendix B). The mean NO_3^- concentration of MCN was significantly lower than all other streams ($p < 0.05$). In contrast, TMD had the highest mean NO_3^- concentration, which was significantly greater than all but two other streams: Narrow Channel Branch (NCB) and Coal Kiln (CLK).

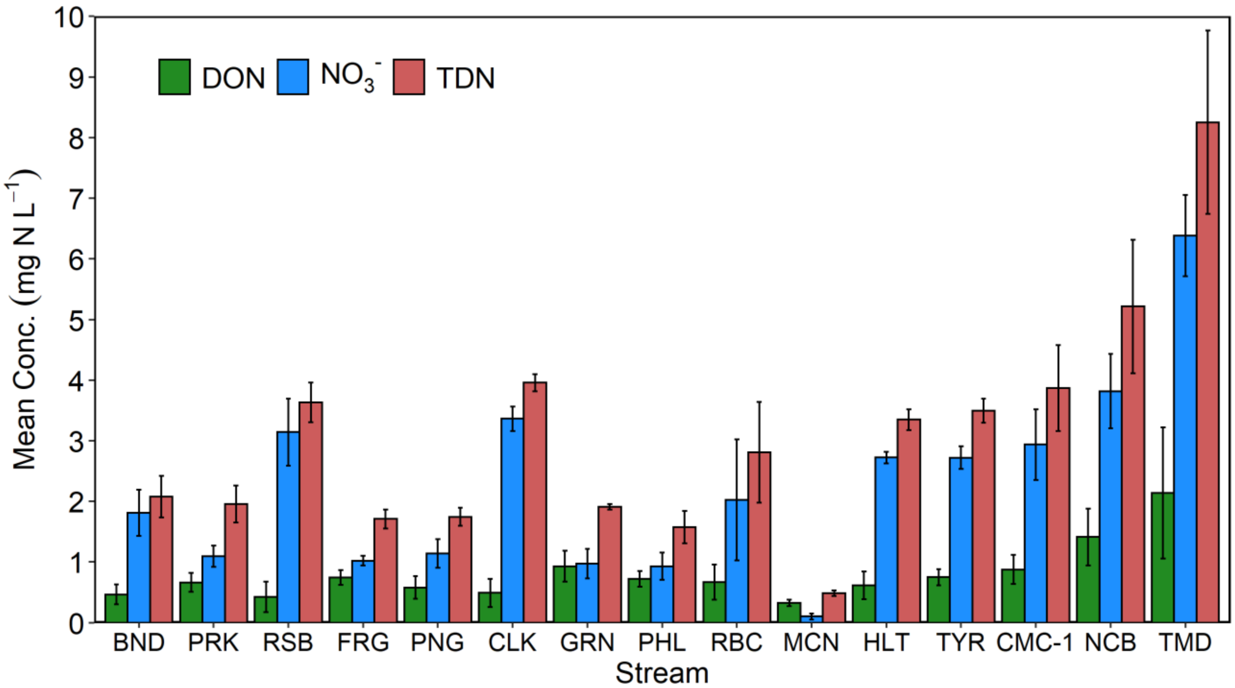

Figure 3. Means and standard errors of DON, NO_3^- , and TDN concentrations in surface water from 15 streams ordered by geographic location (north to south proceeds left to right). All stream name abbreviations are listed in Table 2.

DON concentrations from surface water ranged from 0 to 6.45 mg N L^{-1}, with an overall mean of 0.787 mg N L^{-1} (Figure 2C). With the exception of two samples (NCB and TMD, both collected on 11 May 2019), measured DON concentrations were less than 2 mg N L^{-1}. For DON, the mean measured concentration by stream ranged from 0.328 to 2.14 mg N L^{-1} (Figure 3). The lowest and highest mean DON concentrations were also measured at MCN and TMD, respectively (Figure 3 and Appendix C).

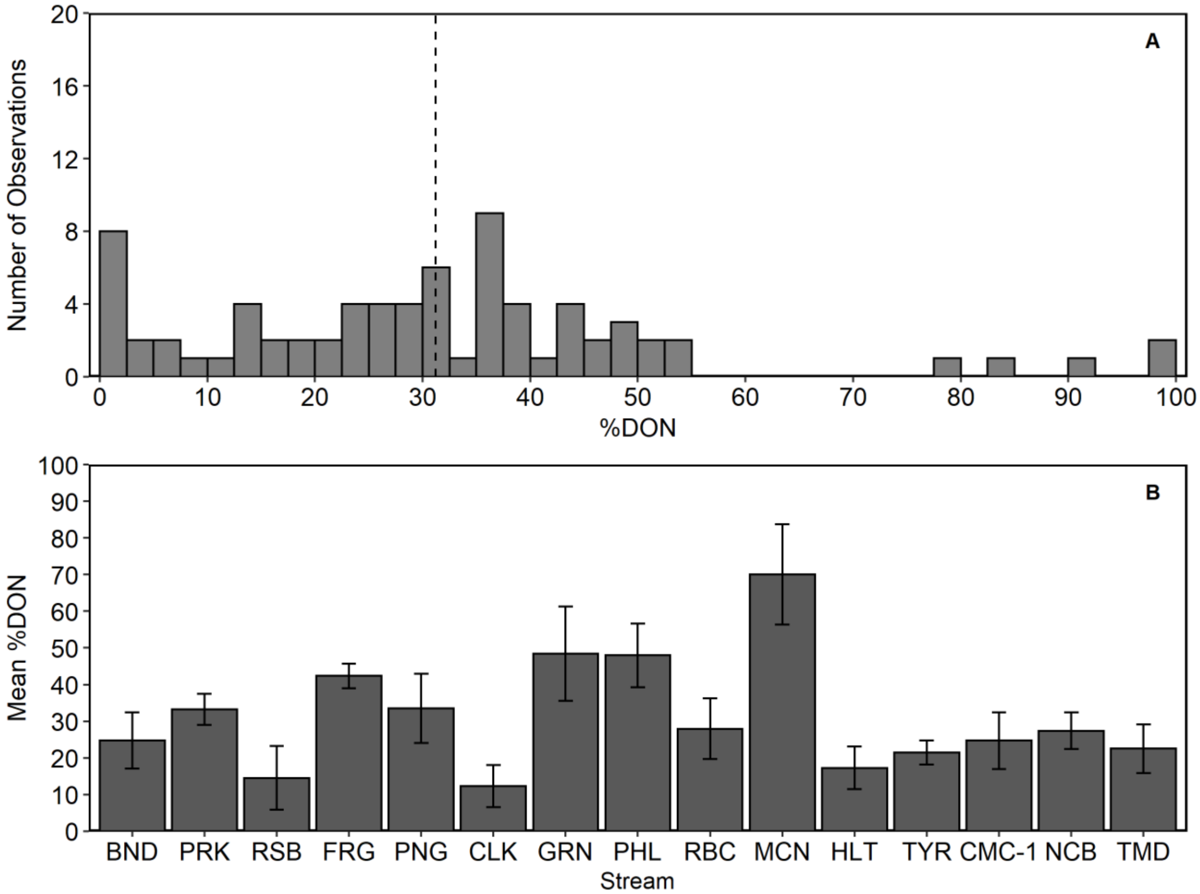

Figure 4. DON as a percent of TDN **(A)** Number of times values were observed across all 15 streams and sample dates (bin widths: 2.5%). **(B)** The mean and standard error %DON for 15 low-relief streams ordered by geographic location (north to south).

DON as a percentage of TDN (%DON) was highly variable, spanning from 0 to 100% across all samples, but DON typically (66 out of 75 samples) constituted less than 50% of TDN (Figure 4A and Appendix C). In MCN, DON was the prevailing form of dissolved nitrogen in all but one sample (June 2019), while the %DON in the stream for the other sample dates ranged from 45 to 100%. In a few streams, in one or two samples DON was the most prevalent form of nitrogen in the TDN pool (Frogstool (FRG), Pungo Creek (PNG), Green’s Creek (GRN), Red Bank Creek (RBC), and Phillips Creek (PHL)). Samples in these streams that had >50% DON were collected in May (2019), November (2019), and February (2020). The stream and sample date combination with the highest %DON value was MCN on 11 May 2019. The mean measured %DON values for the 15 streams ranged from 12 to 70%. Only one (MCN) of the 15 streams had a mean %DON value of >50% (Figure 4B). The overall mean %DON across all samples was 31%.

Variations in DON concentration and %DON were examined using a one-way ANOVA following transformation as needed to allow residuals to conform to a normal distribution and to ensure homoscedasticity. DON concentrations transformed using a square-root function were normally distributed residuals ($W = 0.980$, $p < 0.237$). Variances from the median DON concentrations were found to be homogeneous following the square-root transformation using the Brown-Forsythe test, $F(14,60) = 0.64$, $p = 0.823$. The variations of mean DON concentrations were not significantly different among streams. Values of %DON did not require transformation prior to performing ANOVA as the residuals were not significantly skewed ($W = 0.990$, $p = 0.837$) and variances of %DON from group median were homogenous, $F(14,60) = 0.55$, $p = 0.894$. Differences among mean %DON were significant as determined by the one-way ANOVA, $F(14,60) = 3.71$, $p < 0.001$ (Appendix D). The only differences among streams for %DON as determined by the REGWQ Test were attributed to MCN whose %DON value was significantly greater than RBC, NCB, CMC-1 (Cobb Mill Creek, culvert), BND (Bundick's Creek), TMD, TYR (Taylor Creek), HLT (Holt Creek), RSB (Ross Branch) and CLK, but was not significantly different from GRN, PHL, FRG, and PNG. These streams are listed in order of highest to lowest mean %DON values (Appendix B).

TDN concentrations varied between 0.412 and 13.8 mg N L^{-1} (Figure 2A), with measured mean values for each stream between 0.485 and 8.25 mg N L^{-1} (Figure 3). Across all stream samples, the mean measured TDN concentration was 3.07 mg N L^{-1} (Figure 2A and Appendix B). The highest individual and mean concentrations of NO_3^-, DON, and TDN were exhibited by TMD. The stream with lowest mean TDN content (0.412 mg N L^{-1}) was MCN (Figure 2).

Differences in TDN concentrations among streams were compared using a one-way ANOVA after assessing normality for residuals using the Shapiro-Wilk test and the homogeneity of variances using a Brown–Forsythe test. Similar to NO_3^-, residuals of TDN concentrations were negatively skewed ($W = 0.816$, $p < 0.001$). TDN concentrations were log-transformed to fit a more normal distribution ($W = 0.966$, $p = 0.042$). Variances from the median TDN value were only slightly different following natural log-transformation, $F(14,60) = 1.87$, $p = 0.048$. Although the transformed TDN concentrations did not meet the criteria of homogeneous variances and normally distributed residual, these deviations were slight with respect to the significance level (α) of 0.05. Therefore, a one-way ANOVA was utilized to maintain consistency with the evaluation of differences among streams for NO_3^-, DON, and %DON. TDN concentrations between streams were significantly different from one another, $F(14,60) =18.00$, $p < 0.001$. A REGWQ test revealed differences in TDN concentrations among streams that were generally reflective of those differences in mean NO_3^- concentrations. The mean TDN concentration for MCN was significantly lower than all other streams ($p < 0.05$) whereas for TMD the mean TDN concentration was significantly greater than all other streams except NCB (Appendix D).

TDN concentrations were significantly and positively correlated with NO_3^- concentrations ($r = 0.923$, $p < 0.001$) (Figure 5A). DON and NO_3^- were only slightly correlated in a positive manner ($r = 0.337$, $p = 0.003$) (Figure 5B).

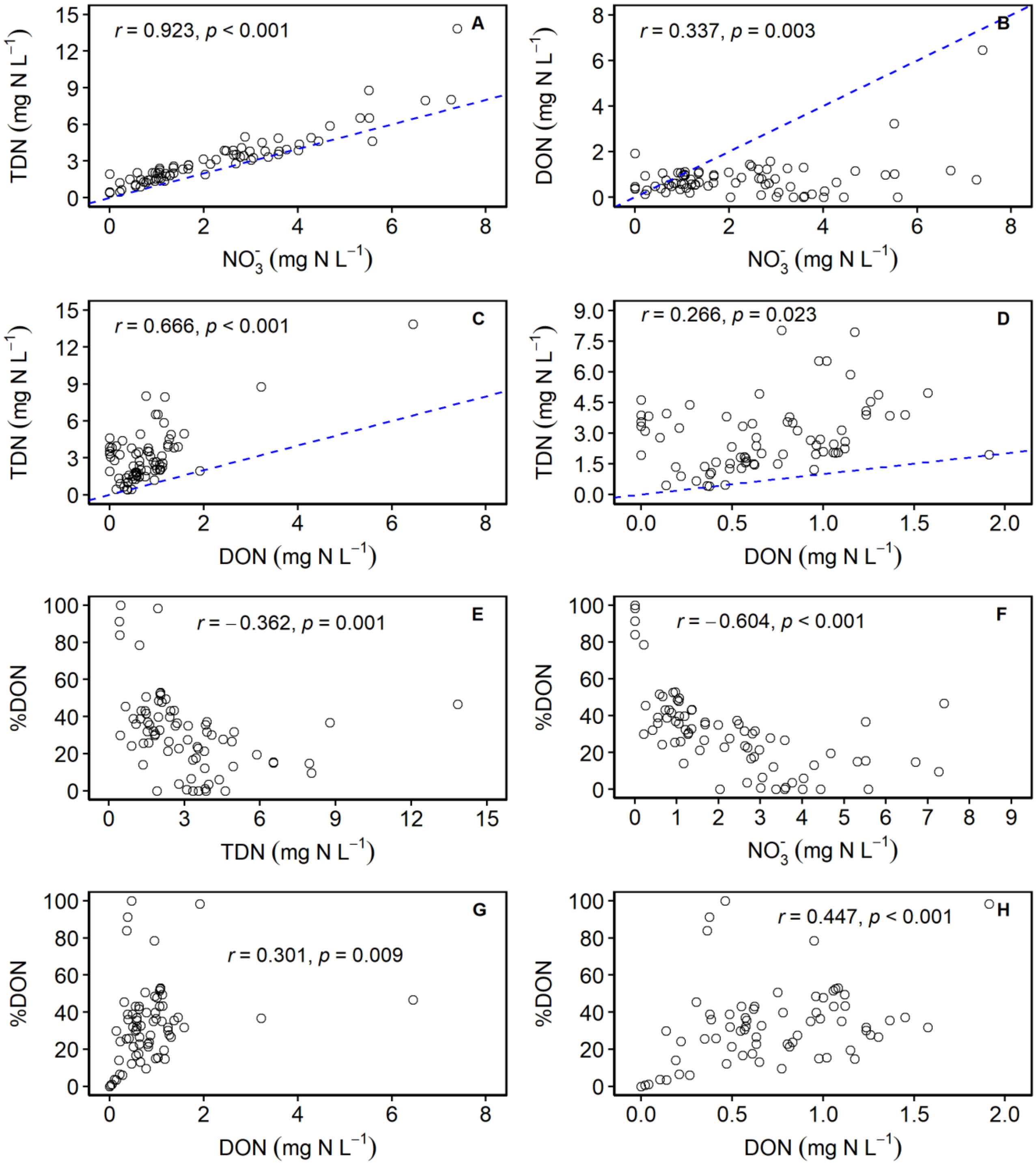

Figure 5. Pearson correlation analyses between (A) TDN and NO_3^-, (B) DON and NO_3^-, (C) TDN and DON, and (D) TDN and DON without May samples for NCB and TMD. Pearson correlation analyses for between %DON and (E) TDN, (F) NO_3^-, (G) DON, and (H) DON without May samples for NCB and TDN. Blue dashed line in plot (A-D) shows the 1:1 line.

The relationship between TDN and DON ($r = 0.666$, $p < 0.001$) (Figure 5C) was stronger than the relationship between NO_3^- and DON (Figure 5B), but it was still much weaker than the relationship between TDN and NO_3^- (Figure 5A). The positive correlation between TDN and DON concentrations was largely driven by the NCB (8.78 and 3.22 mg N L^{-1}, respectively) and TMD (13.8 and 6.44 mg N L^{-1}, respectively) samples collected on 11 May 2020. These were the two highest DON concentrations of all samples. Excluding these samples from the dataset, the correlation between TDN and DON is much weaker ($r = 0.266$, $p = 0.023$) (Figure 5D). %DON was negatively correlated with TDN concentrations ($r = -0.362$, $p = 0.001$) and NO_3^- concentrations ($r = -0.604$, $p < 0.001$) (Figure 5E-F). The correlation coefficients for %DON and DON concentrations (with and without the two high DON samples) were low, indicating a weak relationship between the two measures (Figure 5G-H, respectively).

3.3 Seasonality

Seasonal differences within and among streams were investigated based on the observed concentrations of NO_3^-, DON, and TDN in samples collected periodically over the year. In most streams, a similar pattern across sampling dates was observed when sorted chronologically (Figure 6). For this seasonal pattern, a peak or near-peak TDN concentration was observed in May followed by a decrease in TDN concentration generally in Jun and August and subsequently followed by a resurgence of TDN in November or February. The concentrations when plotted against time plot look like a trough, and this pattern is referred to herein as a “trough pattern.” This general trough pattern was observed in 8 of the 15 streams: BND, PRK (Parker’s Creek), FRG, PHL, HLT, RBC, NCB and TMD. In two streams (CLK and GRN), there was a level or flat pattern whereby the TDN concentrations were generally consistent across sampling dates.

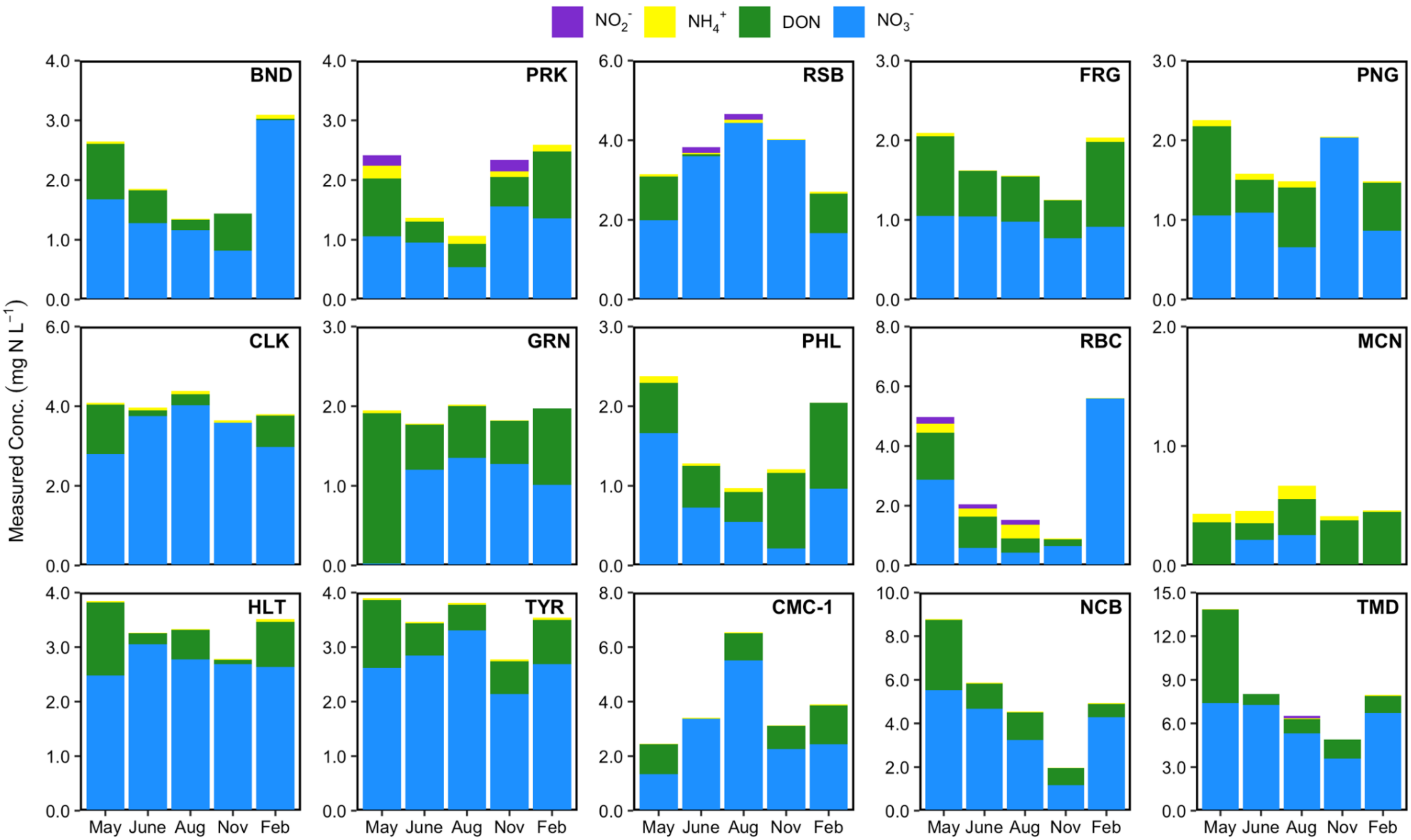

Figure 6. Concentrations of NO_2^-, NH_4^+, DON, and NO_3^- expressed as mg N L^{-1} in surface water collected from streams by geographic location (north to south proceeds left to right, top to bottom). The top of the bar represents the TDN concentration. Note that the scales on y-axis vary by plot. The x-axis is ordered in chronological sequence of the sampling events.

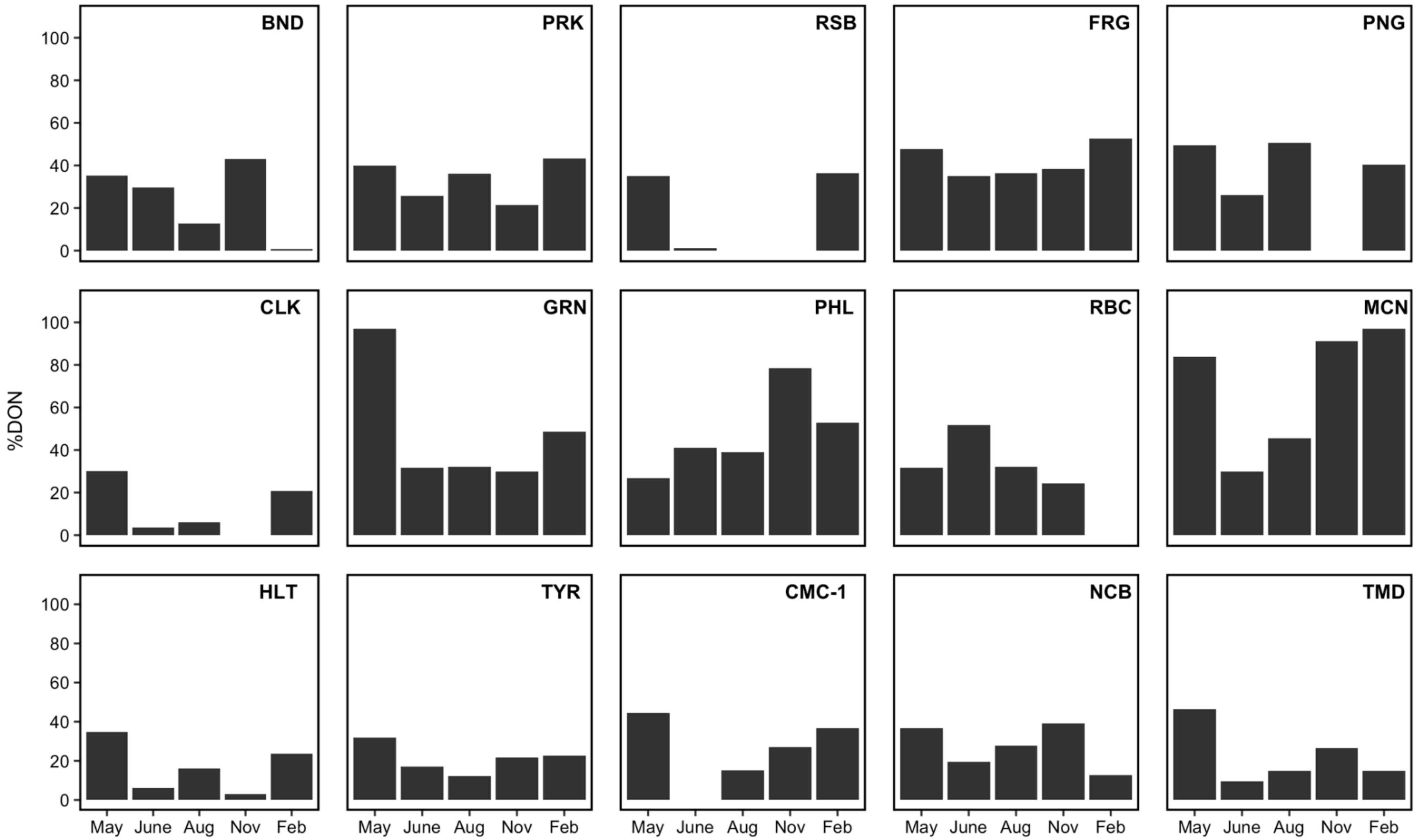

Figure 7. %DON in surface water collected from low-relief streams. Plots are ordered by geographic location (north to south proceeds left to right, top to bottom). The x-axis is ordered in chronological sequence of the sampling events.

Additionally, an inverted trough or "crest" pattern for which TDN concentrations increased from May to August and generally decreased thereafter was observed in three streams: RSB, MCN, and CMC-1. The seasonal pattern of TDN concentrations observed in PNG and TYR did not appear similar to other streams.

As the major component of the TDN pool, the seasonal changes in NO_3^- concentration were commonly reflected in seasonal changes in TDN concentration (Figure 6 and Appendix E). However, in other cases, the seasonal changes were driven by variation in DON concentrations. Of the eight streams that exhibited a trough pattern for TDN concentrations, six of these streams (BND, PRK, PHL, RBC, NCB, and TMD) also exhibited a trough pattern of NO_3^- concentrations. For the other two streams, HLT and FRG, the NO_3^- concentrations were relatively stable across the sample dates, while the variation in TDN was more directly influenced by the variation in the DON concentrations.

An overall common pattern for DON concentrations (Figure 6) and %DON (Figure 7) spanning across all sample dates was not clearly identified. In a few cases, streams exhibited patterns that were similar to a few others. For example, DON concentrations generally decreased from May to February in RBC and NCB. In May and February, FRG, GRN, CLK, and TYR had higher concentrations, while generally lower but consistent DON concentrations were observed in June, August, and November. However, it did appear that DON concentrations and %DON were highest in May and February.

The mean concentrations of NO_3^-, DON, and TDN on each sampling date were calculated to understand general seasonal differences for these nitrogen forms on the Eastern Shore of Virginia (Figure 8). For NO_3^-, the mean concentration did not vary significantly across sampling

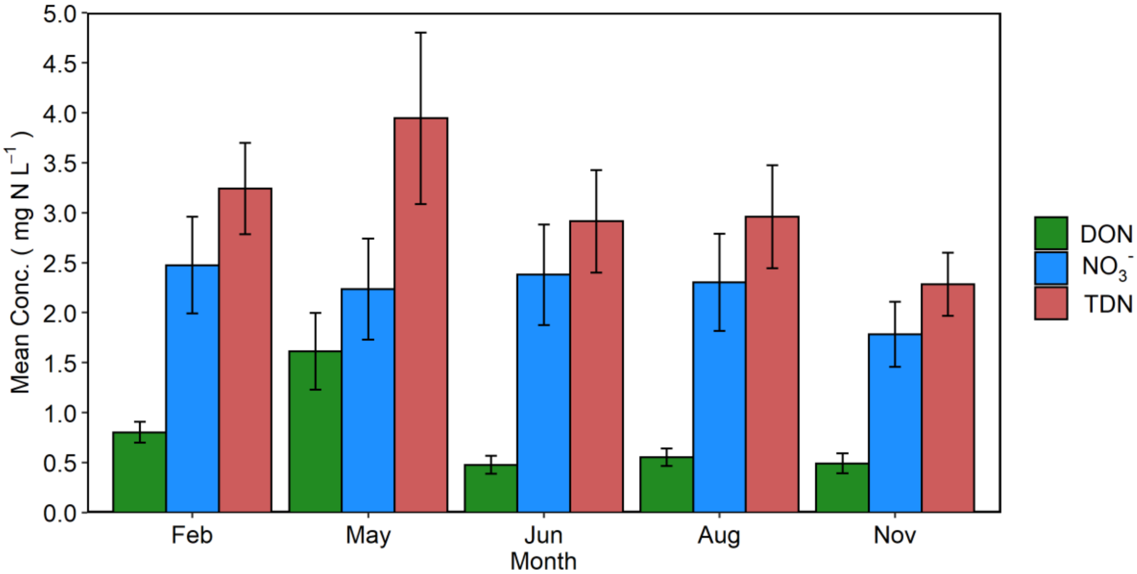

Figure 8. Mean and standard errors of DON, NO_3^-, and TDN concentrations in surface water aggregated by sample date and ordered by calendar year for low-relief streams on the Eastern Shore of Virginia.

months and ranged from 1.78 to 2.47 mg N L^{-1}. In contrast, the mean DON concentrations spanned from 0.476 to 1.61 mg N L^{-1} and were slightly more variable compared to NO_3^-. The sampling month with the lowest mean NO_3^- concentration was in November, whereas the lowest mean DON concentration was in June. February exhibited the highest mean concentration of NO_3^- concentration, while May was associated with the highest mean concentration of DON. The variations of DON concentration within seasons were smaller compared to variations observed for NO_3^-. The month with the highest mean concentration of TDN was May, largely attributed to the higher concentrations of DON observed that month. Mean %DON values for each sampling followed a similar pattern to the mean DON concentrations (Figure 9).

Differences in DON, NO_3^-, and TDN concentrations among sample dates were assessed using a one-way ANOVA (Appendix F). The Shapiro-Wilk test was used to evaluate residuals for normality and the Brown-Forsythe test was used to evaluate homoscedasticity.

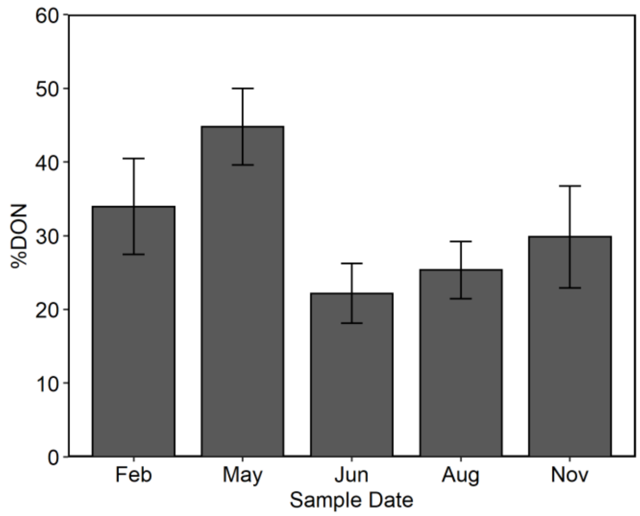

Figure 9. Mean and standard errors of %DON in surface water aggregated by sample date and ordered by calendar month.

The residuals of DON, NO_3^-, and TDN concentrations were all negatively skewed (DON: $W = 0.670$, $p < 0.001$; NO_3^-: $W = 0.918$, $p < 0.001$; TDN: $W = 0.854$, $p < 0.001$). NO_3^- concentrations were transformed using a square-root function so the residuals would fit a more normal distribution ($W = 0.987$, $p = 0.663$). DON concentrations and %DON were transformed using natural log function with a value shift of plus one to accommodate values of zero (0 mg L^{-1} and 0%). Following transformation, residuals of DON concentrations still deviated from a normal distribution ($W = 0.953$, $p = 0.008$), while residuals for %DON conformed to a normal distribution ($W = 0.972$, $p = 0.094$). Despite the deviation from a normal distribution for transformed DON concentrations, the transformed data were considered adequate for ANOVA-based hypothesis testing. The TDN concentrations were transformed using a natural log function. The distribution of residuals for natural log-transformed TDN concentrations was more aligned with a normal distribution ($W = 0.968$, $p = 0.053$). The Brown-Forsythe test indicated that variances of DON, NO_3^-, and TDN concentrations (post-transformation) from the median value by samples date were homogeneous. No differences among sampling dates were identified for NO_3^- concentrations, $F(4,70) = 0.029$, $p = 0.886$) or TDN concentrations, $F(4,70) = 1.15$, $p = 0.340$). However, differences in DON concentrations were significant, $F(4,70) = 7.10$, $p < 0.001$), whereby the mean DON concentration in May was significantly greater than all other sampling dates (Appendix F).

%DON residuals were similarly skewed ($W = 0.930$, $p = 0.001$). For %DON, the data were transformed using the natural log with a plus one shift (log (PDON + 1)) to adjust for zero values. After transformation, residuals were normally distributed ($W = 0.971$, $p = 0.083$) and variances from the median were homogeneous, $F(4,70) = 0.95$, $p = 0.441$. A one-way ANOVA indicated a significant effect between sample dates for %DON, $F(4,70) = 2.75$, $p = 0.035$. The only difference in %DON among sampling dates based on the REGWQ test was between May and June, for which May was significantly greater than June ($p < 0.05$).

3.4 Watershed and Land-use Characteristics

Through use of aerial imagery and geospatial analysis, the amount of cropland within each watershed (excluding PHL) was determined and ranged from 10.7 to 241 hectares (Figure 10A) in watersheds ranging in area from 30.5 to 627 hectares (Table 2). Cropland use, calculated as the fraction of cropland area divided by watershed area, for each stream and expressed as a percentage, was 8 to 58% (Figure 10B). Watersheds for 11 of the 14 streams exhibited cropland use between 35% and 58%, while the four remaining streams had markedly less cropland use relative to the watershed area (8-20%). Those watersheds with less relative amounts of cropland included TYR (8%), GRN (13%), MCN (14%), and HLT (20%).

Watershed and agricultural field boundaries for TMD are illustrated in Figure 11; results for all other streams are included in Appendix I.

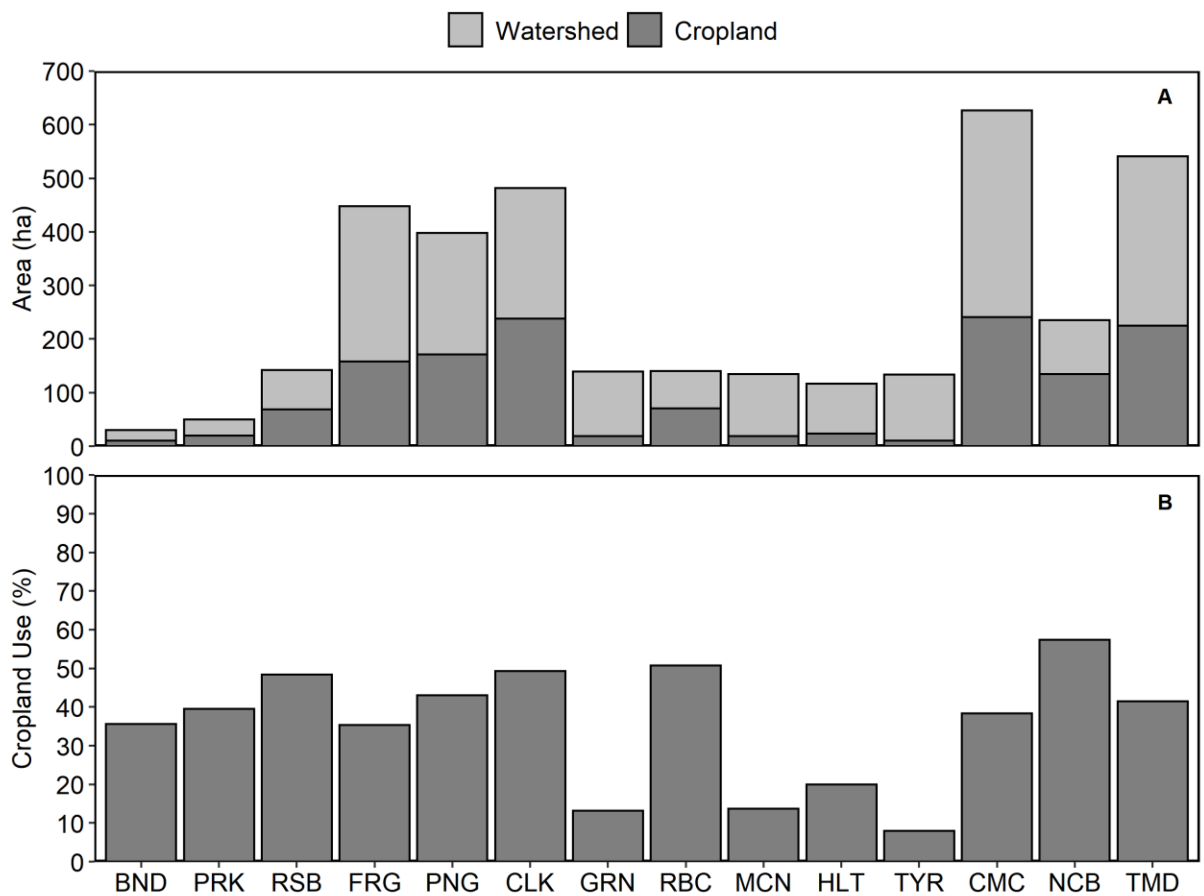

Figure 10. Cropland and watershed areas **(A)** in hectares (ha) and **(B)** percent of cropland use for 14 watersheds of Virginia's Eastern Shore.

The amount of reactive nitrogen introduced annually to the study watersheds was determined by calculating the amount of nitrogen applied annually for each field as the product of field area (within the watershed boundary) and the recommended fertilizer application rate for crop rotation for that field. The amounts of nitrogen from each field in the watershed were summed across fields to quantify the total applied nitrogen from fertilizer for the whole watershed. The amounts of annually applied nitrogen varied from 1280 to 29,400 kg N year^{-1} (Figure 12A).

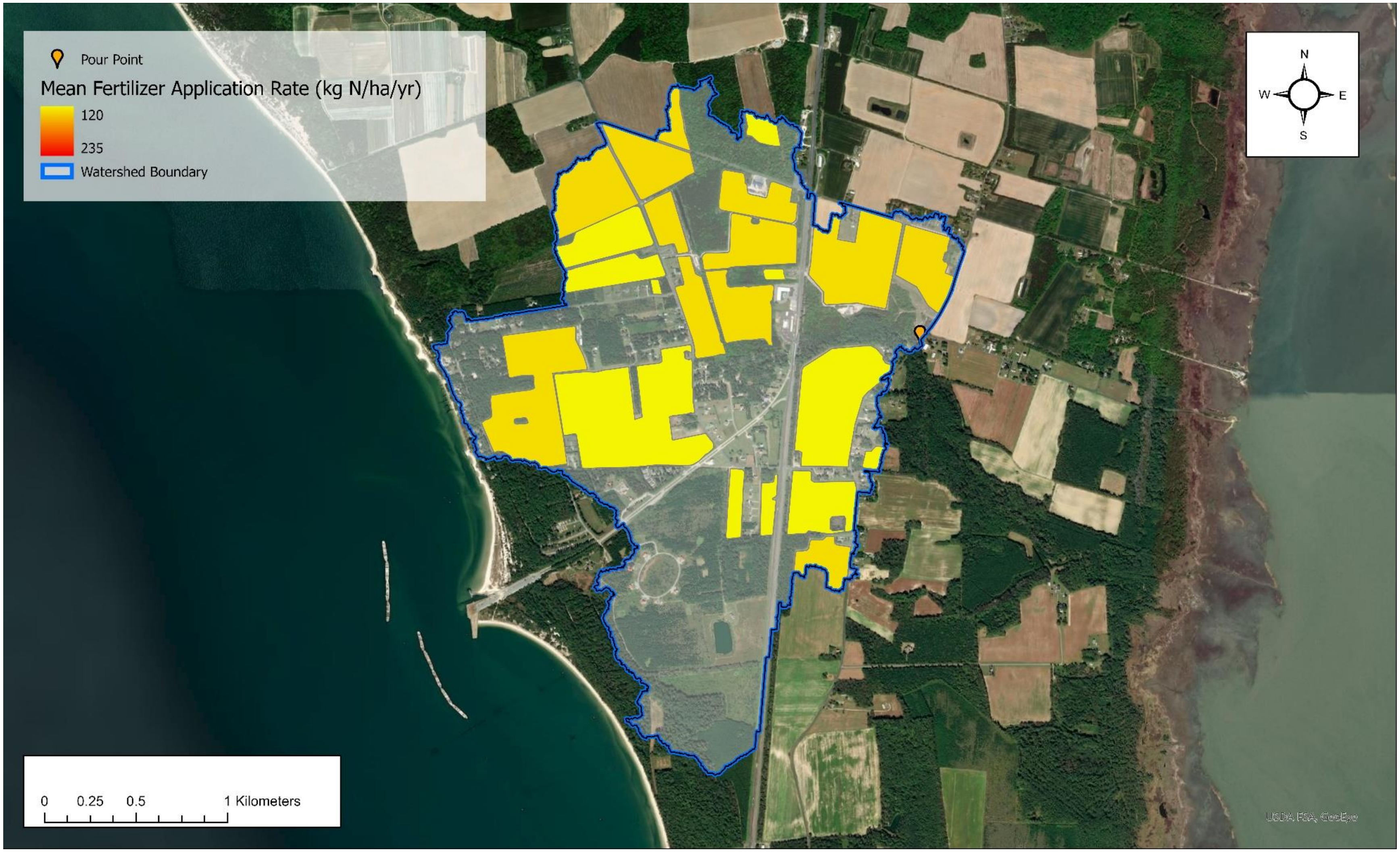

Figure 11. Watershed and agricultural field boundaries for Tommy's Ditch. The mean nitrogen application rates from fertilizer (kg N ha^{-1} $year^{-1}$) in each field are illustrated using a gradient scale.

The amount of applied reactive nitrogen averaged across the 14 watersheds was 12,411 kg N year^{-1}. The collective annual applied load of reactive nitrogen in the studied watersheds excluding Phillips Creek was 173,750 kg N year^{-1}. Generally, the variation in the amounts of applied reactive nitrogen when divided by watershed area was similar to that of the variation in percent cropland use (i.e., comparing Figure 10B and Figure 12B). The one exception to this observation was the watershed for PRK in which a relatively large fraction of the crop land use was associated with a higher fertilizer application rate (Figure I-2). For all watersheds, the amount of reactive nitrogen applied when normalized to per unit watershed area ranged from 9.55 to 73.7 kg N year^{-1} ha^{-1} with a mean of 48.1 kg N year^{-1} ha^{-1} (Figure 12B).

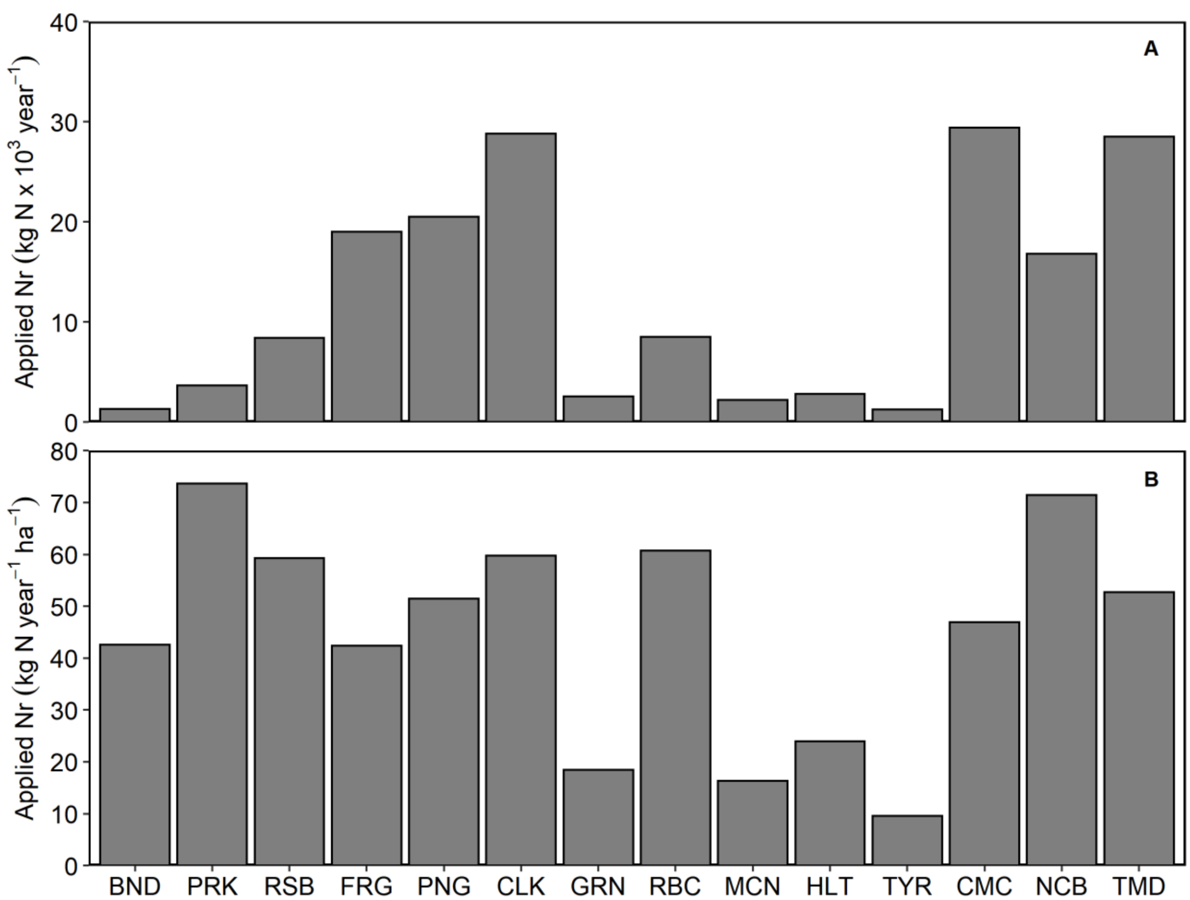

Figure 12. Applied reactive nitrogen (Nr) estimates from fertilizer **(A)** per watershed and **(B)** normalized per unit area (1 hectare) of watershed.

The relationships of DON, NO_3^-, and TDN concentrations to the predictive factors of watershed identity, nature of land use, and rates of fertilizer application were examined using linear regression analysis (Figure 13). Concentrations of DON were weakly correlated with cropland area ($r^2 = 0.187$, $p = 0.122$), watershed area ($r^2 = 0.184$, $p = 0.125$), percent cropland use ($r^2 = 0.044$, $p = 0.473$), total applied reactive nitrogen ($r^2 = 0.211$, $p = 0.099$), or applied reactive nitrogen per area of watershed ($r^2 = 0.042$, $p = 0.480$). Compared to DON, concentrations of NO_3^- and TDN were more strongly correlated these with watershed and land-use parameters. Significant positive regressions were determined for NO_3^- and TDN with cropland area (ha) (NO_3^-: $r^2 = 0.285$, $p = 0.049$; TDN: $r^2 = 0.282$, $p = 0.050$) and total applied reactive nitrogen (NO_3^-: $r^2 = 0.301$, $p = 0.042$; TDN: $r^2 = 0.304$, $p = 0.041$) (Figure 13).

3.5 Comparison of Groundwater and Surface Water

The concentrations of DON, NO_3^-, and TDN were measured in groundwater and surface-water samples collected contemporaneously from Bundick's Creek (BND), Coal Kiln (CLK), Cobb Mill Creek (hillslope) (CMC-2), Phillips Creek (PHL), and Tommy's Ditch (TMD) on 25 June 2019. DON concentrations in groundwater samples ranged between 0.776 to 2.12 mg N L^{-1}, which, with the exception of CLK, were less than the NO_3^- concentrations that ranged from 0.372 to 10.7 mg N L^{-1} (Figure 14). DON represented 6, 31, 35, 40, and 41% of the TDN pool in groundwater for Cobb Mill Creek (hillslope) (CMC-2), BND, TMD, CLK, and PHL, respectively. The difference between DON concentration from groundwater to surface water for each respective stream ranged from 0.66 to 1.35 mg N L^{-1}, and DON concentrations were higher in the groundwater compared to surface water in all cases.

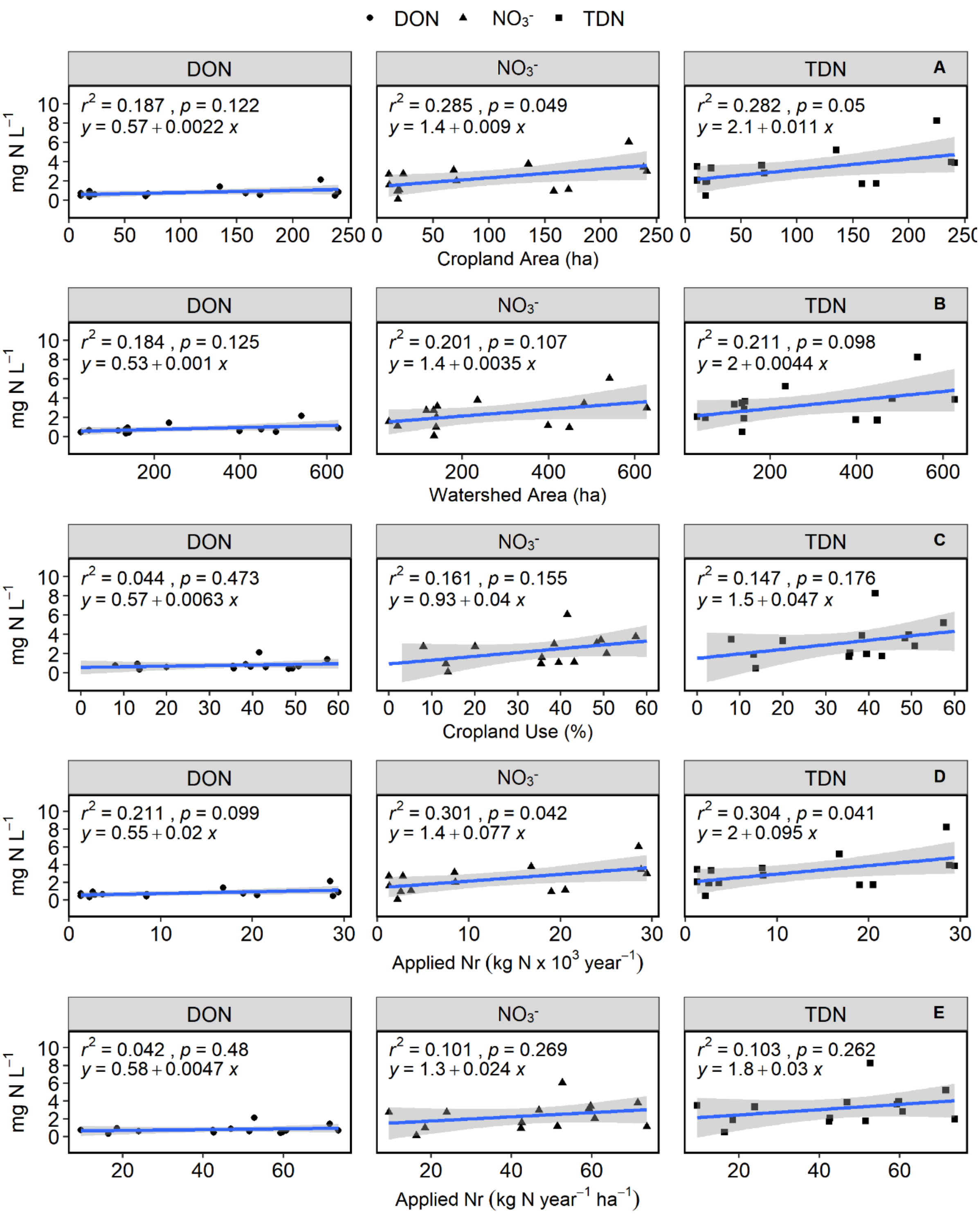

Figure 13. Linear regression analysis with DON, NO_3^-, and TDN concentrations as dependent variables and **(A)** cropland area, **(B)** watershed area, **(C)** cropland use (%), **(D)** applied reactive nitrogen (Nr) per watershed, and **(E)** applied reactive nitrogen (Nr) per unit area (per ha) of watershed.

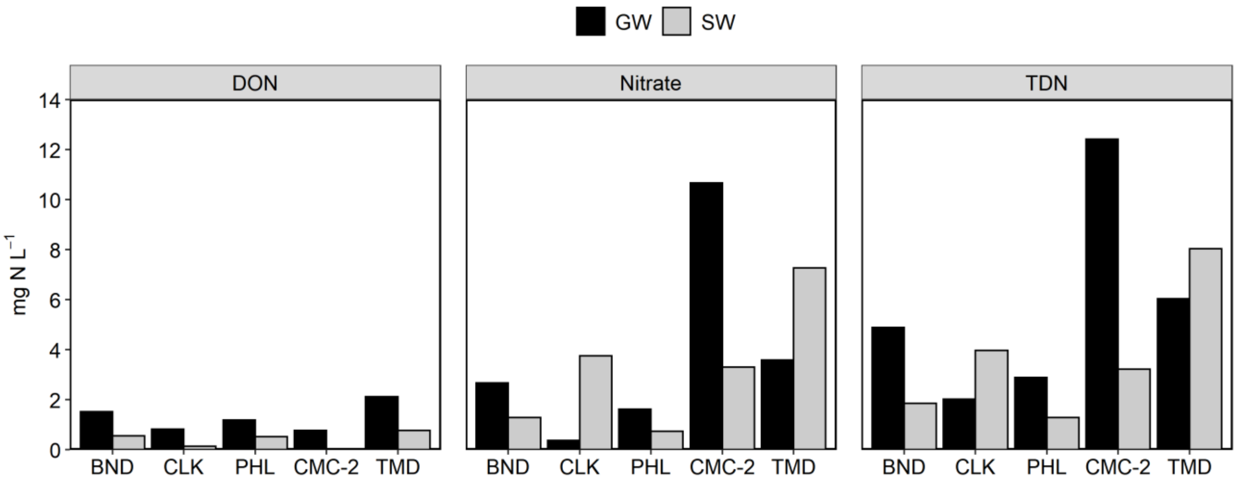

Figure 14. Concentrations of DON, NO_3^-, and TDN in groundwater (GW, black bars) and surface water (SW, grey bars) samples collected contemporaneously on 25 June 2019.

For NO_3^- and TDN, higher concentrations in groundwater compared to those concentrations measured in surface water were observed in three out of the five streams: BND, CMC-2, and PHL. The opposite case, in which groundwater concentrations of NO_3^- were lower than the surface water, was observed in the remaining two streams: CLK and TMD. The differences in NO_3^- concentration between the corresponding pair of groundwater and surface-water samples from BND, CMC-2, and PHL were 1.40, 0.90, and 7.4 mg N L^{-1}, respectively. The differences in TDN concentration for these same samples were 3.04, 1.60, and 9.18 mg N L^{-1}. As relative amounts, the differences in nitrogen content as NO_3^- and TDN between groundwater and surface-water samples from BND, CMC-2, and PHL were 52, 55, and 68% (NO_3^-) and 62, 56, and 73% (TDN), respectively.

3.6 DON Production in a Sediment Core

In conjunction with the field campaign sampling surface water and groundwater, a parallel laboratory experiment was conducted to explore the potential for DON to be generated within streambed sediments and transported to overlying streams. In a flow-through experiment on an intact sediment core, there was no DON, NO_2^- or NH_4^+ formulated in the artificial groundwater (AGW) that was introduced to the core. The preparation of the AGW included only NO_3^-, and the measured NO_3^- and TDN concentrations in the core influent were virtually equivalent at 2.68 and 2.78 mg L^{-1}, respectively (Figure 15 and Appendix H). As expected, NH_4^+ was not measurable in the influent AGW. However, NO_2^- was seen at concentrations of 0.403 and 0.767 mg N L^{-1} in the influent AGW and core effluent sample, respectively. Due to the markedly high concentrations of NO_2^- in both samples, the ion chromatograms were further examined which revealed that inadequate peak separation was likely causing an overestimation of the concentrations of NO_2^- in these samples. Because the samples could not be reanalyzed and the AGW was reasonably presumed to not contain any appreciable amount of NO_2^-, the measured NO_2^- concentrations in the effluent were offset by the concentration of NO_2^- in the influent AGW (0.403 mg N L^{-1}) such that the NO_2^- concentrations in the AGW and the effluent were reported as 0 and 0.364 mg N L^{-1}, respectively (Appendix H).

DON was quantified in the AGW at 0.096 mg N L^{-1}, which was attributed to analytical error as there was no known source of DON in the AGW (Figure 15 and Appendix H). The DON concentration in the core effluent was quantified at 1.02 mg N L^{-1}, which represented nearly 60% of the TDN in the core effluent sample. The concentration of NO_3^- in the core effluent (0.237 mg N L^{-1}) was considerably lower than in the AGW that was introduced to the sediment core (2.68 mg N L^{-1}). The difference between TDN concentrations in the AGW and the core effluent was less notable (2.78 to 1.72 mg N L^{-1}, respectively) than for NO_3^- (2.68 to 0.237 mg N L^{-1}).

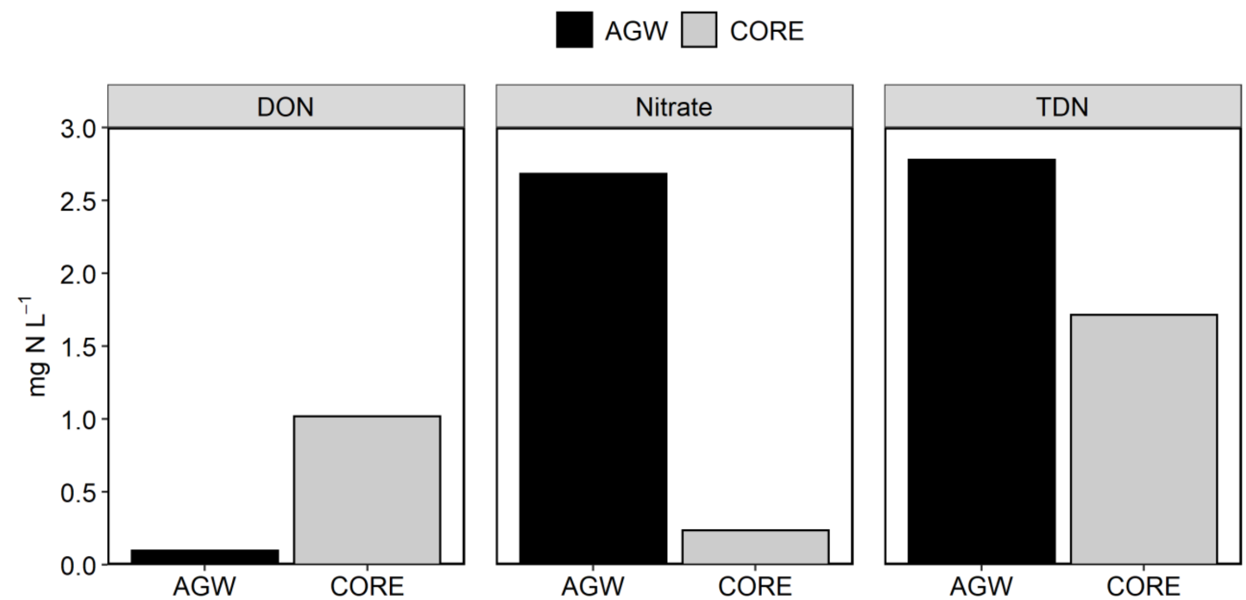

Figure 15. Concentrations of DON, NO_3^-, and TDN in artificial groundwater (AGW, black bars) and core effluent (CORE, grey bars) from Coal Kiln (CLK) stream sediment core at steady-state flow conditions.

4. Discussion

4.1 Variability of Dissolved Nitrogen Concentrations

Quantifying freshwater inputs of nitrogen to coastal ecosystems is key to understanding and managing these ecologically important systems (Seitzinger and Harrison 2008). This is especially true for watersheds dominated by agricultural land use, such as the Eastern Shore of Virginia. Prior investigations of nitrogen loading to the coastal lagoons have focused primarily on NO_3^- as the dominant and readily bioavailable species to phytoplankton. However, studies have shown DON is a major component of the TDN pool and a portion may be biologically available to phytoplankton and bacteria. Therefore, this study measured DON concentrations in streams of varying watershed size and cropland use.

DON and NO_3^- were detected in every stream in at least two of the five or six samples collected over the course of one year. However, NO_3^- was detected more frequently than DON. The number of streams (6) with at least one sample without quantifiable DON was greater than the number of streams (2) with samples in which NO_3^- was not measurable. NO_3^- was determined to be the main nitrogen species in 14 out of 15 streams, and DON was the dominant form in the remaining one stream. Absent or minimal concentrations of NO_2^- and NH_4^+ in conjunction with high concentrations of NO_3^- were indicative of aerobic conditions for all streams.

Mill Creek North (MCN) was the sole stream in which DON was the main form of dissolved nitrogen across all sampling dates. This result is consistent with findings from a previous study by Stanhope et al. (2009). Stanhope et al. (2009) attributed this finding to limited stream flow as well as the large fraction of forested land cover within the watershed. DON has been shown to be the predominant form of nitrogen in unpolluted, forested catchments (Triska et al. 1984, Brookshire et al. 2005). Forested catchments have been further shown to facilitate the loss of NO_3^- via microbial denitrification (Fang et al. 2015). In addition, the sampling site for MCN in this study was not in close proximity to any cropland fields, but was situated nearby (~50 m) a freshwater pond (Figure 16) and a residential community where multiple septic fields

Figure 16. Watershed boundary, pour point, and location of upgradient pond for Mill Creek North (MCN).

are likely situated .

The pond near the MCN sample-collection site was not set within the DEM-defined watershed boundary. However, the watershed boundary may be inaccurate in areas where man-made berms are having an undue influence on the digitization of the watershed boundary as a consequence of the limited topographic relief on the Eastern Shore of Virginia. Based on low TDN concentrations at this location, the nearby pond may be altering the water nutrient chemistry. No visual outflow from the pond to the stream was observed. However, because the water level of the pond is likely relatively elevated compared to the downstream sample collection point, a subsurface hydrological link may exist between the pond and the downstream flow resulting in a strong local effect of the nutrient chemistry in MCN. The impact of upstream ponds on downstream nutrient concentrations has been thoroughly investigated by Fairchild and Velinsky (2006). The results of their investigation indicated that the presence of upstream ponds with long residence times resulted in the marked reduction of NO_3^- inputs while increasing dissolved organic carbon, nitrogen, and phosphorus in downstream outflows (Fairchild and Velinsky 2006). The very low concentrations of NO_3^- and TDN in MCN compared to other streams suggest that the nutrient chemistry in this stream may be impacted by the nearby freshwater pond. Further investigation of the hydrology of this pond-stream system as well as nutrient dynamics in the pond may provide greater insight into MCN's remarkably low NO_3^- content relative to other studied streams on the Eastern Shore of Virginia.

The sample collected in May for GRN had the greatest %DON value of 98%, while the %DON in subsequent samples for this stream ranged from 30-49%. The high %DON observed in GRN in May could be the result of low cropland use within the watershed (Figure 10) and relatively high amount of forested landcover. However, it may suggest efficient sediment denitrification of NO_3^- along the groundwater flow paths and/or by phytoplankton in the water column in conjunction with release of autochthonous DON at the onset of warmer water temperatures.

DON was approximately 31% of the TDN in surface water when averaged across all streams and samples (Appendix B). This fraction of DON was approximately four-fold greater than the respective 8% determined for the streams on the Eastern Shore that were monitored previously by Stanhope et al. (2009) (Table 4). Only a small subset of the streams in this study overlapped with those studied by Stanhope et al. (2009). Differences in %DON values in this study and the study by Stanhope et al. (2009) can be partially attributed to the monitoring of different streams and different sampling locations for streams surveyed in both studies. For example, the two streams with the highest mean DON concentrations in this study, NCB and TMD were not streams included in the investigation by Stanhope et al. (2009).

Table 4. Comparison of the minimum, maximum, and aggregate (overall) mean stream concentrations of DON, NO_3^-, and TDN concentrations and %DON between Stanhope et al. (2009) and the present study. Standard deviation is given for aggregate mean values.

Study	No. of Streams	Mean. Stream Concentration	mg N L^{-1}			%DON
			DON	NO_3^-	TDN	
Stanhope et al. (2009)[a]	13[b]	Minimum	0.03	0.92	1.07	1
		Maximum	0.74	7.93	8.97	31
		Aggregate Mean ± St.Dev.	0.20 ± 0.21	2.57 ± 1.92	2.82 ± 2.10	8 ± 8
Present Study	14[b]	Minimum	0.39	0.93	1.67	13
		Maximum	1.80	6.06	8.27	48
		Aggregate Mean ± St.Dev.	0.77 ± 0.37	2.43 ± 1.51	3.26 ± 1.78	27 ± 11

[a] Stanhope et al. (2009) report baseflow-weighted concentrations, which are reflected above.

[b] Mill Creek North (MCN) was excluded from the comparison due to the markedly lower TDN concentrations compared to other streams

The NO_3^- concentrations in the present study were generally similar to previous synoptic surveys conducted during 2010-2011 and 2015-2016 in which the same streams were sampled monthly over the course of a year (J. S. Herman, unpublished data, 2020) (Figure 17A). In a few select streams, namely RSB, CLK, NCB, and TMD, the mean NO_3^- concentrations were notably higher in 2019-2020 compared to those determined in prior synoptic surveys. These higher NO_3^- concentrations may be due to increased use of fertilizer in these watersheds or a difference in stream flows between sample years. In general, there appears to be a slight trend of increasing mean nitrogen concentrations in these streams as observed across the prior two surveys (2010-2011 and 2015-2016) and for this study (2019-2020) (Figure 17B), largely driven by increases noted in several streams (RSB, CLK, NCB, and TMD). Stanhope et al. (2009) also measured similar levels of NO_3^- for a different set of streams on the Eastern Shore of Virginia (Table 4).

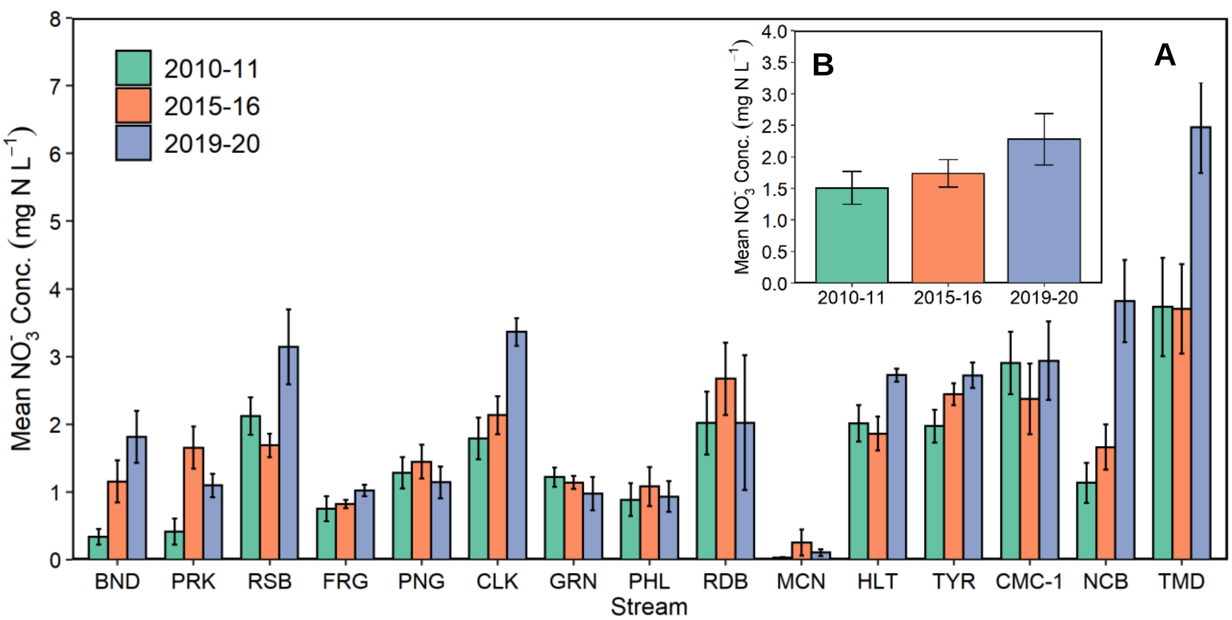

Figure 17. Mean NO_3^- concentrations with standard errors (A) by stream and by survey year, with 2019-20 being the present study. (B) Overall mean NO_3^- concentration in 15 streams.

4.2 Relationships between Land Use and Nitrogen Species

The mean DON concentrations across streams did not vary significantly ($p = 0.067$) and were not correlated with variable land-use parameters. These observations suggest sources of DON in 15 streams on the Eastern Shore of Virginia are more likely to be natural than anthropogenic. Consistent mean DON concentrations among streams is in agreement with the findings of a prior study that showed limited variation in DON concentrations determined for a diverse group of freshwater catchments in Wales (Willett et al. 2004). Furthermore, a lack of a relationship between land use and DON variability is reflected in the results of another investigation that similarly studied the nitrogen speciation in agricultural watersheds (Arheimer and Lidén 2000). In a couple of watersheds, MCN and GRN, where forested areas represent a more significant fraction of the catchment, %DON was notably higher than in most other streams. However, this finding was not consistently observed. For HLT, which also has a large area of forest within the watershed, the %DON value was low.

In contrast to DON, mean NO_3^- and TDN concentrations were significantly different across streams. Spatially, the higher mean NO_3^- and TDN concentrations were mostly associated with watersheds in the southern portion of the Eastern Shore and included streams such as HLT, TYR, CMC, NCB and TMD. These particular streams generally displayed an increase in mean NO_3^- concentration trending in the southerly direction. This observation may be attributed to higher hydraulic conductivity of the surficial Columbia aquifer in this section of the Eastern Shore. The hydraulic conductivity of this aquifer is the lowest in the middle region of the Eastern Shore of Virginia and increases bidirectionally toward the northern and southern boundaries (Sanford et al. 2009). Increased hydraulic conductivity may increase total influx of

NO_3^- to the streams and concurrently decrease removal efficiency of NO_3^- by denitrification (Shuai et al. 2017).

Another potential contributing factor to spatial variation of NO_3^- among streams is land use. In this study, mean NO_3^- concentrations were significantly correlated with cropland area and total amount of applied fertilizer annually, although these two land-use parameters are highly correlated with each other.

4.3 Seasonality

This study aimed to monitor the seasonal variations of DON in mixed-use watersheds with agricultural cropland and forest as the dominant land-use types. Studies investigating temporal variation of DON concentrations in freshwater systems indicate a link to seasonal uses of fertilizer application. For instance, one study showed that concentrations of urea, a bioavailable nitrogenous compound and important component of fertilizer belonging to the DON pool, are greatest during summer following application of fertilizers or manure to agricultural fields in spring (Glibert et al. 2005, Tzilkowski et al. 2019). The results of this study on the Eastern Shore of Virginia did not establish a strong link between land use and DON as discussed in detail above (Section 4.2), suggesting instream DON concentrations observed in this study may be predominantly derived from natural sources rather than anthropogenic sources such a urea-based fertilizer.

Autochthonous DON produced from biological mechanisms can vary with seasonal changes in temperature (Jørgensen 2009). Production of autochthonous DON occurs through a variety of biotic processes such as cell lysis, exudation, and heterotrophic grazing (Berman and Bronk 2003, Schmidt 2010). Shifts in temperature across seasons may also affect the extent of

DON removal through biological process. These biological processes for DON removal include the degradation of DON via mineralization to NH_4^+ and microbial uptake of certain forms of DON (e.g., dissolved free amino acids). Therefore, examining the variations of DON concentration across seasons may provide insights about whether natural sources of DON are autochthonously derived.

A common seasonal pattern within streams with respect to DON concentrations and %DON was not readily apparent in this study (Figure 6 and Figure 7). Among the five sampling dates, mean DON values were highest in the spring (May) and generally low in the summer (June and August) and fall (November) sample collections (Figure 8). A marginal increase in DON was observed in the winter (February). A similar trend was observed by Stanhope et al. (2009) whereby the highest DON was measured in the spring. However, the present study observed generally consistent DON concentrations between summer and fall while Stanhope et al. (2009) detected a consistent decrease from spring to winter. Additionally, the present study observed a slight increase in the DON concentration in the winter, whereas Stanhope et al. (2009) showed DON at its lowest concentrations during this season.

Water temperature may influence the amount of autochthonous production and mineralization of DON as well as the amount of NO_3^- uptake (Berman and Bronk 2003, Kaushal and Lewis 2003, Lorite-Herrera et al. 2009). Water temperature was monitored in several streams on the Eastern Shore of Virginia as part of a long-term monitoring effort (A. L. Mills, unpublished data, 2020). The mean water temperatures for TMD across the five major sampling dates are given in Table 5 and were considered applicable as surrogate data for the other streams subject to similar climate conditions of the region.

To evaluate the effect of water temperature on DON concentrations, May, June, and August sampling months were blocked together as the "warm" season whereas November

Table 5. Mean water temperature at Tommy's Ditch (TMD) for each sampling date

Date	**Mean Water Temperature (°C)**
11 May 2019	15.7
29 June 2019	20.0
25 August 2019	22.3
16 November 2019	9.9
16 February 2020	9.9

and February were blocked together as the "cold" season, according to mean measured water temperature. A two-tailed paired *t*-test was performed to evaluate differences between the DON concentrations and %DON in the warm and cold seasons. Using the Satterthwaite method for unequal variances, $F(44,29) = 5.98$, $p < 0.001$, no significant differences were identified between the warm and cold seasons for DON concentrations, $t(62.9) = -1.36$, $p = 0.179$. For %DON, the pooled method was used for equal variances, $F(29,44) = 1.72$, $p = 0.103$, which similarly indicated no significant differences between seasons, $t(73) = 0.21$, $p = 0.832$. The absence of a seasonal effect in the paired *t*-tests does not preclude biological sources of DON. Rather, it more likely represents insufficient data to resolve the potential effect. Further investigation using more frequent monitoring of DON concentrations across seasonal changes in water temperature may provide greater insights into the identification of DON sources as revealed by seasonal effects.

Other studies have shown that NO_3^- uptake is coupled with DON release from autotrophic organisms and that heterotrophic grazing ('sloppy feeding') is highest during spring (Bronk et al. 1998). This phenomenon may explain the peak concentrations of DON concentrations that were observed in many streams during May in this study. In June samples, DON concentrations were smaller in many cases. This observation could be the result of increased mineralization of bioavailable DON by heterotrophs or decreased autochthonous DON production. During winter (December-February), not as much biological activity is expected to occur in these streams. However, periodic warm air temperatures can occur in the region allowing for biological uptake of NO_3^- and production of DON but at lesser extents than observed during spring. Furthermore, mineralization of DON in groundwater is likely minimal, and, therefore, DON transported by groundwater may be entering the stream. In contrast, during summer, rates of mineralization of DON may be the highest, thereby explaining the low concentrations of DON observed in June and August. DON in groundwater has been shown to be lower in summer due to suspected high rates of mineralization (Lorite-Herrera et al. 2009).

Similar to DON, no effects between the cold and warm season were noted for NO_3^-, $t(73) = -0.42$, $p = 0.675$, and TDN, $t(73) = -0.99$, $p = 0.324$. In addition, no significant differences were noted between any sample dates. Within streams, the NO_3^- varied across the year with contrasting patterns. The trough pattern and, inversely, a crest pattern were observed in multiple streams and thereby negate the observation of an overall seasonal pattern. Further investigation is needed to understand how differences in biological and physical characteristics between streams may explain variable seasonal patterns of NO_3^- in surface water.

4.4 Differences in Groundwater and Surface Water

Labile DON in groundwater may be removed by mineralization and subsequent immobilization by plant and microbial uptake in stream sediments. Like surface water, DON in groundwater was not the dominant species, but represented between 6-41% of the TDN pool (n = 5; mean: 31%). The groundwater in four out of the five streams that were sampled contained DON that represented greater than 30% of the TDN pool (Table G-1). These findings indicate that DON may represent an important secondary constituent of TDN in groundwater on the Eastern Shore of Virginia.

DON concentrations in surface water from all five streams were lower than the concentration in groundwater, suggesting removal of labile DON in sediments via mineralization. These findings are consistent with another study that examined DON concentrations in an agricultural watershed (Lorite-Herrera et al. 2009) in which concentrations of DON were lower in surface-water samples compared to groundwater samples. The DON concentrations in the present study (0.78-2.12 mg N L^{-1}), however, were over a magnitude of order less than the mean values (18.6-21.4 mg N L^{-1}) reported by Lorite-Herrera et al. (2009). Concentrations of DON in the groundwater in the present study were similar to those reported by Kroeger et al. (2006) in mostly residential and forested watersheds located in the Massachusetts (mean: 0.854 mg L^{-1}; range: 0.07-2.55 mg L^{-1}). These findings indicate that streambed sediments on the Eastern Shore of Virginia, in addition to playing a critical role in removing nitrate from sediment, may also facilitate the mineralization of DON in groundwater before it reaches the stream channel. To further understand changes in mineralization throughout the year, it may be insightful to conduct similar comparisons of groundwater and surface water DON concentrations across multiple seasons instead of just one sampling period as performed in this study. Nevertheless, the results of this study indicate that groundwater may be an important source of DON to surface water in streams on the Eastern Shore of Virginia.

Denitrification in streambed sediments is a critical process for removing NO_3^- from nitrogen-rich groundwater on the Eastern Shore of Virginia (Gu et al. 2007, 2008, Mills et al. 2011). As result of denitrification, notable differences between groundwater and overlying stream concentrations of NO_3^- have been observed. Three of the five streams (BND, CMC-2, and PHL) in this study from which groundwater and surface water were sampled concurrently showed lower concentrations of instream NO_3^- compared to that measured in groundwater (Figure 14). The other two streams, CLK and TMD, exhibited higher NO_3^- concentrations in surface water, which suggests that inputs of NO_3^- from fertilizer directly into the streams may be occurring or that the groundwater feeding the stream further upstream contains higher NO_3^- concentrations. Upstream of the culvert under SR 600, the stream channel of CLK runs between two agricultural fields with a minimal riparian buffer. This may lend to itself to the potential for inadvertent application of NO_3^- fertilizer directly into the stream channel (Figure 18). In contrast, TMD has a much broader forested riparian zone upstream of the sample collection point making it less likely for direct addition of fertilizer into the stream. Instead, the groundwater feeding the stream above the collection point may contain more NO_3^- than the groundwater collected at a downstream location in this study. A longitudinal investigation of the NO_3^- profiles in surface water and groundwater in TMD may be warranted to further understand the variation of dissolved nitrogen along the stream channel.

Differences between TDN concentrations in groundwater and surface water may serve as a check on the mass balance with respect to dissolved nitrogen species. In the same streams for which NO_3^- was greater in groundwater than in surface water, TDN concentrations followed a similar pattern. Taken together, the lower NO_3^- and TDN concentrations in surface water compared to groundwater suggest denitrification in streambed sediments is occurring, resulting in the denitrification of NO_3^- .

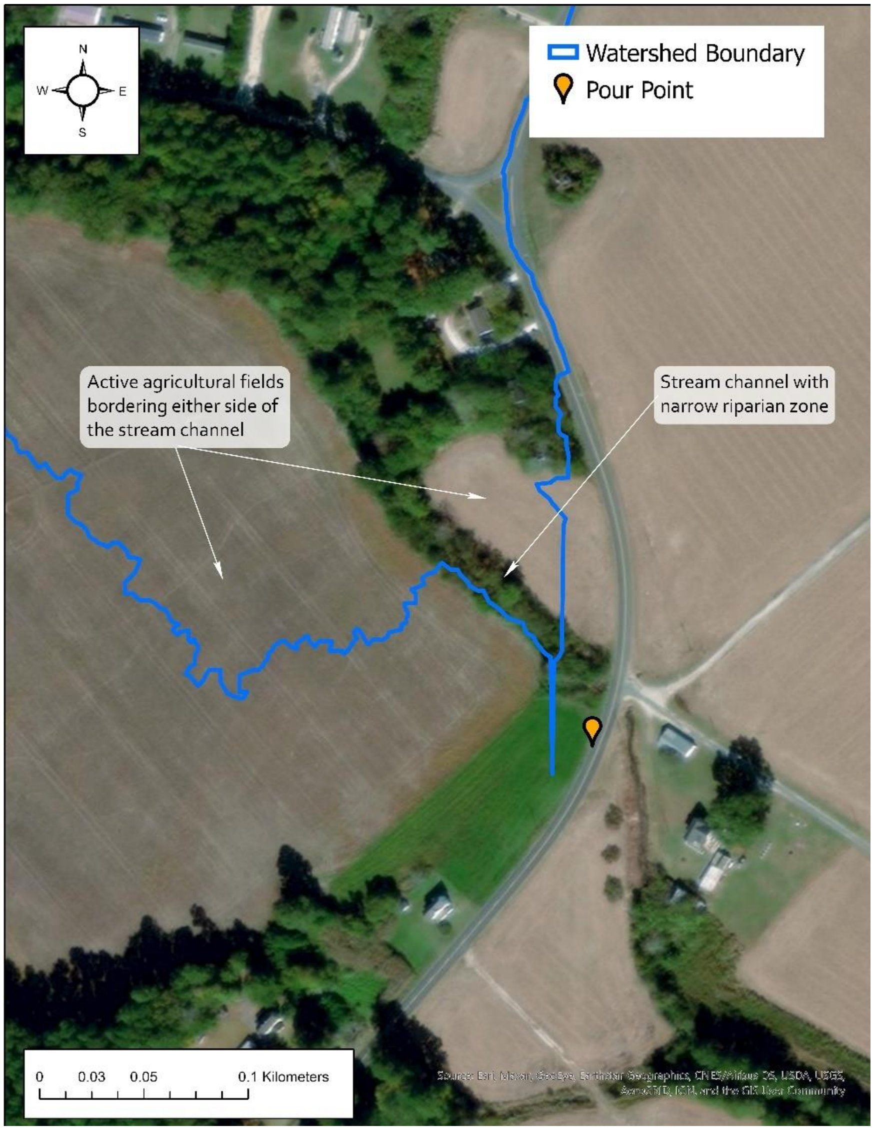

Figure 18. Map of a portion of the Coal Kiln (CLK) watershed near the sample collection site (pour point) showing nearby agricultural fields and narrow riparian zone.

4.5 DON Production and Release from Sediments

A laboratory sediment-column experiment was performed to gain insight into the potential contribution of DON from biogeochemical cycling of NO_3^- in groundwater within streambed sediments. Under steady-state flow conditions, DON production or release in a sediment core eluted with artificial groundwater (AGW) was coupled with a reduction in NO_3^- concentration. These results imply denitrification of NO_3^- in stream sediments may contribute to DON's presence in the overlying surface water through assimilation of dissolved inorganic nitrogen by bacteria followed by release of DON as well as bacterial breakdown of organic matter. Similar to the observation in a few of the concurrent groundwater and surface-water samples, lower TDN concentration in core effluent compared to AGW serves as evidence of denitrification.

DON released from estuarine sediments has been shown to have a low carbon-to-nitrogen ratio suggesting that the DON species may be more labile than refractory (Burdige and Zheng 1998). Therefore, potential influxes of labile DON to overlying stream flow may contribute the biologically available dissolved nitrogen load in stream water as a result of NO_3^- removal via denitrification. However, this experiment was conducted with freshwater sediments so the benthic microbial communities and organic matter composition are expected to differ from those in estuarine sediments. Further investigation into the chemical nature and utilization of the DON pool eluting streambed sediments may be warranted to discern the dynamics of DON in the overlying streams.

The results from the sediment core experiment suggest that streambed sediments may serve as source of allochthonous DON via production or release from within the sediments where is denitrification occurring. This finding in conjunction with comparison of DON concentrations between concurrent groundwater and surface-water samples indicates that streambed sediments may play a dynamic role in presence of DON in the mostly NO_3^--rich streams on the Eastern Shore of Virginia.

4.6 Conclusion

The proportion of DON in the TDN pool in fresh waters of the Eastern Shore of Virginia may be greater than previously reports by Stanhope et al. (2009). While DON does not represent the dominant constituent of dissolved nitrogen across the majority of streams or sample dates in this study, the results of this study indicate that DON nonetheless represents a significant constituent of the TDN pool in both surface water and groundwater. Therefore, with the potential that a portion of the nitrogen in the DON pool may be ultimately accessible to phytoplankton, the bioavailable nitrogen loads to the coastal lagoons that are currently calculated from NO_3^- concentrations only may be underestimated. Compared to NO_3^- concentrations, DON concentrations were more variable seasonally among all streams but were less variable spatially among the streams. Furthermore, the concentrations of DON in the studied watersheds do not appear to be strongly influenced by watershed area, cropland use, or fertilizer application rate. In contrast, NO_3^- was more strongly correlated with these watershed characteristics than DON. These observations support the previous understanding that NO_3^- concentrations are linked with anthropogenic inputs from fertilizer use and, therefore, are more likely influenced by variations of land use in watersheds on the Eastern Shore of Virginia when compared to DON. Seasonal changes in DON concentrations suggest that autochthonous production of DON dominates at the onset of warmer temperatures in spring and before mineralization of DON peaks in summer, resulting in lowered in-stream concentrations of DON. Further investigation is warranted to fully understand the biological process contributing to fluctuations of DON across seasons. In groundwater collected beneath the stream channel, DON was quantified at higher levels than those measured in overlying surface water, suggesting that groundwater may serve as an source of allochthonous DON to the stream channel and mineralization or uptake of DON may be important biogeochemical processes occurring in the sediments. Furthermore, this study demonstrated that production or release of DON from sediments in which denitrification is occurring may serve as another source of allochthonous DON to the stream channel. Collectively, these data add to the understanding of the dynamics and sources of DON in freshwater streams on the Eastern Shore of Virginia. Moreover, the data from this study provide merit to further investigations of DON aimed at characterizing its contribution to nitrogen budgets and dynamics within streams and streambed sediments.

5. Bibliography

Aitkenhead-Peterson, J. A., M. K. Steele, N. Nahar, and K. Santhy. **2009**. Dissolved organic carbon and nitrogen in urban and rural watersheds of south-central Texas: Land use and land management influences. Biogeochemistry. 96: 119–129.

Arheimer, B., and R. Lidén. **2000**. Nitrogen and phosphorus concentrations from agricultural catchments - Influence of spatial and temporal variables. J. Hydrol. 227: 140–159.

Ator, S. W., and J. M. Denver. **2015**. Understanding Nutrients in the Chesapeake Bay Watershed and Implications for Management and Restoration — the Eastern Shore Circular 1406.

Bachman, L., B. Lindsey, J. Brakebill, and D. Powars. **1998**. Ground-Water Discharge and Base-Flow Nitrate Loads of Nontidal Streams, and Their Relation to a Hydrogeomorphic Classification of the Watershed, Chesapeake Bay. United States Geol. Surv. 1–72.

Berman, T., and D. Bronk. **2003**. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquat. Microb. Ecol. 31: 279–305.

Berman, T., and S. Chava. **1999**. Algal growth on organic compounds as nitrogen sources Tom. J. Plankton Res. 21: 1423–1437.

Bolster, C. H. 2000. Effect of heterogeneity on bacterial transport and deposition.

Boyer, E. W., R. W. Howarth, J. N. Galloway, F. J. Dentener, P. A. Green, and C. J. Vörösmarty. **2006**. Riverine nitrogen export from the continents to the coasts. Global Biogeochem. Cycles. 20: GB1S91, doi:10.1029/2005GB002537.

Bronk, D. A. 2002. Dynamics of DON, pp. 153–248. *In* Hansell, D. A., Carlson, C.A. (ed.), Biogeochem. Mar. Dissolved Org. Matter. Academic Press.

Bronk, D. A., P. M. Gilbert, T. C. Malone, S. Banahan, and E. Sahlsten. **1998**. Inorganic and organic nitrogen cycling in Chesapeake Bay: Autotrophic versus heterotrophic processes and relationships to carbon flux. Aquat. Microb. Ecol. 15: 177–189.

Bronk, D. A., M. W. Lomas, P. M. Glibert, K. J. Schukert, and M. P. Sanderson. **2000**. Total dissolved nitrogen analysis: comparisons between the persulfate, UV and high temperature oxidation methods. Mar. Chem. 69: 163–178.

Bronk, D. A., J. H. See, P. Bradley, and L. Killberg. **2007**. DON as a source of bioavailable nitrogen for phytoplankton. Biogeosciences. 4: 283–296.

Brookshire, E. N. J., H. M. Valett, S. A. Thomas, and J. R. Webster. **2005**. Coupled cycling of dissolved organic nitrogen and carbon in a forest stream. Ecology. 86: 2487–2496.

Burdige, D. J., and S. Zheng. **1998**. The biogeochemical cycling of dissolved organic nitrogen in estuarine sediments. Limnol. Oceanogr. 43: 1796–1813.

Causse, J., E. Baurès, Y. Mery, A.-V. Jung, O. Thomas, E. Baur`es, B. Baur`es, A.-V. Val´, and V. Jung. **2015**. Critical Reviews in Environmental Science and Technology Variability of N Export in Water: A Review Variability of N Export in Water: A Review. Crit. Rev. Environ. Sci. Technol. 45: 2245–2281.

Denver, J. M., S. W. Ator, L. M. Debrewer, M. J. Ferrari, J. R. Barbaro, T. C. Hancock, M. J. Brayton, and M. R. Nardi. **2004**. Water Quality in the Delmarva Peninsula, Geol. Surv. Circ.

ESRI. **2020**. ArcGIS Pro.

Fairchild, G. W., and D. J. Velinsky. **2006**. Effects of small ponds on stream water chemistry. Lake Reserv. Manag. 22: 321–330.

Fang, Y., K. Koba, A. Makabe, C. Takahashi, W. Zhu, T. Hayashi, A. A. Hokari, R. Urakawa, E. Bai, B. Z. Houlton, D. Xi, S. Zhang, K. Matsushita, Y. Tu, D. Liu, F. Zhu, Z. Wang, G. Zhou, D. Chen, T. Makita, H. Toda, X. Liu, Q. Chen, D. Zhang, Y. Li, and M. Yoh. **2015**. Microbial denitrification dominates nitrate losses from forest ecosystems. Proc. Natl. Acad. Sci. U. S. A. 112: 1470–1474.

Fiedler, D., D. Graeber, M. Badrian, and J. Köhler. **2015**. Growth response of four freshwater algal species to dissolved organic nitrogen of different concentration and complexity. Freshw. Biol. 60: 1613–1621.

Flewelling, S. A., J. S. Herman, G. M. Hornberger, and A. L. Mills. **2012**. Travel time controls the magnitude of nitrate discharge in groundwater bypassing the riparian zone to a stream on Virginia's coastal plain. Hydrol. Process. 26: 1242–1253.

Flewelling, S. A., G. M. Hornberger, J. S. Herman, A. L. Mills, and W. M. Robertson. **2014**. Diel patterns in coastal-stream nitrate concentrations linked to evapotranspiration in the riparian zone of a low-relief, agricultural catchment. Hydrol. Process. 28: 2150–2158.

Galloway, J. N., and E. B. Cowling. **2009**. Reactive Nitrogen and The World: 200 Years of Change. AMBIO A J. Hum. Environ. 31: 64–71.

Glibert, P. M., T. M. Trice, B. Michael, and L. Lane. **2005**. Urea in the tributaries of the Chesapeake and coastal Bays of Maryland. Water. Air. Soil Pollut. 160: 229–243.

Gu, C., G. M. Hornberger, J. S. Herman, and A. L. Mills. **2008**. Influence of stream-groundwater interactions in the streambed sediments on NO 3 − flux to a low-relief coastal stream. Water Resour. Res. 44: 1–13.

Gu, C., G. M. Hornberger, A. L. Mills, J. S. Herman, and S. A. Flewelling. **2007**. Nitrate reduction in streambed sediments: Effects of flow and biogeochemical kinetics. Water Resour. Res. 43: 1–10.

Jani, J., and G. S. Toor. **2018**. Composition, sources, and bioavailability of nitrogen in a longitudinal gradient from freshwater to estuarine waters. Water Res. 137: 344–354.

Johnson, L. T., J. L. Tank, R. O. Hall, P. J. Mulholland, S. K. Hamilton, H. M. Valett, J. R. Webster, M. J. Bernot, W. H. McDowell, B. J. Peterson, and S. M. Thomas. **2013**. Quantifying the production of dissolved organic nitrogen in headwater streams using 15n tracer additions. Limnol. Oceanogr. 58: 1271–1285.

Johnson, R. A. 2018. Nitrogen Fertilizer Application to Virginia’s Eastern Shore: Refining Land Use and Fertilizer Estimates for the Seaward Side.

Jørgensen, N. O. G. 2009. Organic Nitrogen. Encycl. Inl. Waters. 832–851.

Kaushal, S. S., and W. M. Lewis. **2003**. Patterns in the chemical fractionation of organic nitrogen in Rocky Mountain streams. Ecosystems. 6: 483–492.

Knudsen-Leerbeck, H., M. Mantikci, M. Bentzon-Tilia, S. J. Traving, L. Riemann, J. L. S. Hansen, and S. Markager. **2017**. Seasonal dynamics and bioavailability of dissolved organic matter in two contrasting temperate estuaries. Biogeochemistry. 134: 217–236.

Kroeger, K. D., M. L. Cole, and I. Valiela. **2006**. Groundwater-transported dissolved organic nitrogen exports from coastal watersheds. Limnol. Oceanogr. 51: 2248–2261.

Lorite-Herrera, M., K. Hiscock, and R. Jiménez-Espinosa. **2009**. Distribution of dissolved inorganic and organic nitrogen in river water and groundwater in an agriculturally-dominated catchment, south-East Spain. Water. Air. Soil Pollut. 198: 335–346.

Mills, A. L. 2008. Sediments in Low-relief Coastal Streams as Effect Filters of Agricultural Nitrate, pp. 1–6. *In* AWRA 2008 Summer Spec. Conf. Norfolk, VA.

Mills, A. L., G. M. Hornberger, and J. S. Herman. **2011**. Sediments in low-relief coastal streams as effective filters of agricultural nitrate,. *In* Spec. Conf. Riparian Process. America Water Resource Association, Norfolk, VA.

Mulholland, M. R., and M. W. Lomas. **2008**. Nitrogen Uptake and Assimilation, pp. 303–384. *In* Nitrogen Mar. Environ. Academic Press.

Murphy, D. V., A. J. Macdonald, E. A. Stockdale, K. W. T. Goulding, S. Fortune, J. L. Gaunt, P. R. Poulton, J. A. Wakefield, C. P. Webster, and W. S. Wilmer. **2000**. Soluble organic nitrogen in agricultural soils. Biol. Fertil. Soils. 30: 374–387.

Nydahl, F. 1978. On the peroxodisulphate oxidation of total nitrogen in waters to nitrate. Water Res. 12: 1123–1130.

Pehlivanoglu-Mantas, E., and D. L. Sedlak. **2006**. Wastewater-derived dissolved organic nitrogen: Analytical methods, characterization, and effects - A review. Crit. Rev. Environ. Sci. Technol. 36: 261–285.

Pellerin, B. A., W. M. Wollheim, C. S. Hopkinson, W. H. McDowell, M. R. Williams, C. J. Vörösmarty, and M. L. Daley. **2004**. Role of wetlands and developed land use on dissolved organic nitrogen concentrations and DON/TDN in northeastern U.S. rivers and streams. Limnol. Oceanogr. 49: 910–918.

Petrone, K. C., J. S. Richards, and P. F. Grierson. **2009**. Bioavailability and composition of dissolved organic carbon and nitrogen in a near coastal catchment of south-western Australia. Biogeochemistry. 92: 27–40.

Richardson, D. L. 1995. Hydrogeology and analysis of the groundwater-flow system of the Eastern Shore, Virginia. US Geol. Surv. Water-Supply Pap. 2401.

Sanford, W. E., J. P. Pope, and D. L. Nelms. **2009**. Simulation of Groundwater-Level and Salinity Changes in the Eastern Shore, Virginia.

SAS Institute. **2016**. SAS.

Schmidt, B. H. M. 2010. Role of Dissolved Organic Nitrogen in the Soil Nitrogen Cycle of Forest Ecosystems. Ph.D Thesis.

See, J. H., D. A. Bronk, and A. J. Lewitus. **2006**. Uptake of Spartina -derived humic nitrogen by estuarine phytoplankton in nonaxenic and axenic culture. 51: 2290–2299.

Seitzinger, S. P. 1990. Denitrification In Aquatic Sediments, pp. 301–322. *In* Denitrification Soil Sediment. Springer US.

Seitzinger, S. P., and J. A. Harrison. **2008**. Land-Based Nitrogen Sources and Their Delivery to Coastal Systems, pp. 469–510. *In* Nitrogen Mar. Environ. Academic Press.

Seitzinger, S. P., and R. W. Sanders. **1997**. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication. Mar. Ecol. Prog. Ser. 159: 1–12.

Seitzinger, S. P., and R. W. Sanders. **1999**. Atmospheric inputs of dissolved organic nitrogen stimulate estuarine bacteria and phytoplankton. Limnol. Oceanogr. 44: 721–730.

Seitzinger, S. P., R. W. Sanders, and R. Styles. **2002**. Bioavailability of DON from natural and anthropogenic sources to estuarine plankton. Limnol. Oceanogr. 47: 353–366.

Shuai, P., M. B. Cardenas, P. S. K. Knappett, P. C. Bennett, and B. T. Neilson. **2017**. Denitrification in the banks of fluctuating rivers: The effects of river stage amplitude, sediment hydraulic conductivity and dispersivity, and ambient groundwater flow. Water Resour. Res. 53: 7951–7967.

Sipler, R. E., and D. A. Bronk. **2014**. Dynamics of Dissolved Organic Nitrogen, pp. 127–232. *In* Biogeochem. Mar. Dissolved Org. Matter Second Ed. Academic Press.

Stanhope, J. W., I. C. Anderson, and W. G. Reay. **2009**. Base Flow Nutrient Discharges from Lower Delmarva Peninsula Watersheds of Virginia, USA. J. Environ. Qual. 38: 2070–2083.

Stepanauskas, R., H. Edling, and L. J. Tranvik. **1999**. Differential dissolved organic nitrogen availability and bacterial aminopeptidase activity in limnic and marine waters. Microb. Ecol. 38: 264–272.

Stepanauskas, R., N. O. G. Jørgensen, O. R. Eigaard, A. Žvikas, L. J. Tranvik, and L. Leonardson. **2002**. Summer inputs of riverine nutrients to the Baltic Sea: Bioavailability and eutrophication relevance. Ecol. Monogr. 72: 579–597.

The R Code Team. **2020**. R: A language and enviroment for statistical computing.

Triska, F. J., J. R. Sedell, K. Cromack, S. V. Gregory, and F. M. McCorison. **1984**. Nitrogen Budget for a Small Coniferous Forest Stream. Ecol. Monogr. 54: 119–140.

Tzilkowski, S. S., A. R. Buda, E. W. Boyer, R. B. Bryant, P. J. A. Kleinman, C. D. Kennedy, A. L. Allen, G. J. Folmar, and E. B. May. **2019**. Urea Fluctuations in Stream Baseflow across Land Cover Gradients and Seasons in a Coastal Plain River System. J. Am. Water Resour. Assoc. 55: 228–246.

United States Department of Agriculture. **2017**. Census of Agriculture.

US Environmental Protection Agency. **2004**. National Primary Drinking Water Regulations.

USDA NASS. **2016**. USDA National Agricultural Statistics Service Cropland Data Layer. Publ. Crop. data layer.

Valderrama, J. C. 1981. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem. 10: 109–122.

Webster, J. R., R. M. Stewart, J. D. Knoepp, and C. R. Jackson. **2019**. Effects of instream processes, discharge, and land cover on nitrogen export from southern Appalachian Mountain catchments. Hydrol. Process. 33: 283–304.

Wiegner, T. N., S. P. Seitzinger, P. M. Glibert, and D. A. Bronk. **2006**. Bioavailability of dissolved organic nitrogen and carbon from nine rivers in the eastern United States. Aquat. Microb. Ecol. 43: 277–287.

Willett, V. B., B. A. Reynolds, P. A. Stevens, S. J. Ormerod, and D. L. Jones. **2004**. Dissolved Organic Nitrogen Regulation in Freshwaters. J. Environ. Qual. 33: 201–209.

Appendix A
Comparison of NO_3^-, DON, and TDN concentrations and %DON between samples collected on 16-FEB-2020 and 22-FEB-2020

Table A-1. NO_3^-, TDN, and DON concentrations as well as %DON for streams sampled twice in February 2020. Differences between the values were calculated to perform a paired *t*-test to evaluate significant differences.

	NO_3^- (mg N L^{-1})_			DON (mg N L^{-1})_			TDN (mg N L^{-1})_			%DON		
STREAM	**16-Feb**	**22-Feb**	**Diff.**	**16-Feb**	**22-Feb**	**Diff.**	**16-Feb**	**22-Feb**	**Diff.**	**16-Feb**	**22-Feb**	**Diff.**
BND	3.01	2.93	0.08	0.020	0.067	-0.047	3.09	3.04	0.05	1	2	-1
CLK	2.98	3.03	-0.05	0.816	0.615	0.201	3.79	3.64	0.15	22	17	5
CMC-1	2.44	2.68	-0.24	1.45	0.893	0.56	3.89	3.57	0.32	37	25	12
FRG	0.912	1.38	-0.46	1.07	0.599	0.47	2.03	1.97	0.06	53	30	23
MCN	0.000	0.141	-0.141	0.462	0.251	0.211	0.462	0.392	0.070	100	64	36
NCB	4.28	4.02	0.26	0.648	0.458	0.190	4.93	4.51	0.42	13	10	3
PHL	0.962	1.48	-0.51	1.08	0.484	0.60	2.04	1.96	0.084	53	25	28
TMD	6.71	8.03	-1.32	1.18	0.163	1.01	7.95	8.39	-0.44	15	2	13
MEAN	2.66	2.96	-0.30	0.840	0.441	0.399	3.53	3.43	0.091	37	22	15

Diff. = 16-Feb measurement – 22-Feb measurement

Table A-2. Degrees of Freedom (DF), *t*-values and probability (*p*) values for null hypothesis testing for differences between NO_3^-, TDN, DON, and %DON in two February sample collections in 8 streams.

Dependent Variable	Method	Variances	DF	t Value	*p*
NO_3^-	Pooled	Equal	14	-0.26	0.7969
TDN	Pooled	Equal	14	0.08	0.9385
DON	Pooled	Equal	14	2.13	0.0514
%DON	Pooled	Equal	14	1.11	0.2875

Appendix B
Individual Nitrogen Speciation Results in Surface Water Samples with Means, Standard Errors, and Post-hoc Groupings

Table B-1. Individual and mean NO_3^- concentrations (mg N L^{-1}) by stream and sample date. Post-hoc grouping assignments based on REGWQ test (α = 0.05). Measurements below the lowest calibration standard (0.100 mg N L^{-1}) are reported as zero.

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)	Group[1]
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020		
1	Bundick's Creek	1.68	1.28	1.16	0.820	3.01	**1.59 ± 0.38**	**a,b,c**
2	Parker's Creek	1.06	0.955	0.544	1.56	1.36	**1.10 ± 0.17**	**b,c**
3	Ross Branch	1.99	3.60	4.44	4.01	1.67	**3.14 ± 0.55**	**a,b,c**
4	Frogstool	1.05	1.05	0.981	0.768	0.912	**0.951 ± 0.052**	**b,c**
5	Pungo Creek	1.06	1.09	0.658	2.03	0.867	**1.14 ± 0.24**	**b,c**
6	Coal Kiln	2.80	3.76	4.03	3.58	2.98	**3.43 ± 0.23**	**a,d**
7	Greens Creek	0.000	1.20	1.35	1.27	1.02	**0.974 ± 0.244**	**c**
8	Phillips Creek	1.66	0.729	0.545	0.212	0.962	**0.822 ± 0.243**	**c**
9	Redbank Creek	2.87	0.585	0.423	0.648	5.58	**2.02 ± 1.00**	**b,c**
10	Mill Creek North	0.000	0.216	0.252	0.000	0.000	**0.094 ± 0.058**	**d**
11	Holt Creek	2.48	3.05	2.77	2.68	2.63	**2.72 ± 0.095**	**a,b**
12	Taylor	2.62	2.85	3.31	2.14	2.69	**2.72 ± 0.19**	**a,b,**
13	Cobb Mill Creek (Culvert)	1.35	3.37	5.52	2.26	2.44	**2.99 ± 0.71**	**a,b,**
14	Narrow Channel Branch	5.51	4.68	3.24	1.18	4.28	**3.78 ± 0.75**	**a,d**
15	Tommy's Ditch	7.40	7.26	5.32	3.58	6.71	**6.06 ± 0.72**	**a,d**
Mean ± SE (By Date)		**2.24 ± 0.51**	**2.38 ± 0.50**	**2.30 ± 0.49**	**1.78 ± 0.33**	**2.47 ± 0.48**	**Overall Mean ± SE**	
Group[2]		**a**	**a**	**a**	**a**	**a**	**2.23 ± 0.20**	

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates
[1] Grouping assignments by stream based on REGWQ post-hoc analysis (α=-0.05) following square-root transformation of concentrations
[2] Grouping assignments by sample date based on REGWQ post-hoc analysis (α=-0.05) following square-root transformation of concentrations

Table B-2. Individual and mean DON concentrations (mg N L^{-1}) by stream and sample date. Post-hoc grouping assignments based on REGWQ test ($\alpha = 0.05$). DON was calculated using Equation 1: DON = TDN - (NO_3^- + NO_2^- + NH_4^+). Calculated DON concentrations of less than zero were reported as zero.

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)	Group[1]
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020		
1	Bundick's Creek	0.931	0.567	0.190	0.622	0.020	**0.466 ± 0.162**	**a,b**
2	Parker's Creek	0.963	0.349	0.385	0.498	1.12	**0.663 ± 0.158**	**a,b**
3	Ross Branch	1.10	0.041	0.000	0.000	0.984	**0.426 ± 0.253**	**a,b**
4	Frogstool	1.00	0.579	0.573	0.486	1.07	**0.742 ± 0.121**	**a,b**
5	Pungo Creek	1.12	0.411	0.750	0.000	0.617	**0.579 ± 0.185**	**a,b**
6	Coal Kiln	1.24	0.139	0.267	0.000	0.816	**0.491 ± 0.232**	**a,b**
7	Greens Creek	1.91	0.575	0.662	0.546	0.959	**0.931 ± 0.256**	**a,b**
8	Phillips Creek	0.633	0.550	0.378	0.949	1.08	**0.718 ± 0.130**	**a,b**
9	Redbank Creek	1.58	1.06	0.489	0.220	0.000	**0.668 ± 0.288**	**a,b**
10	Mill Creek North	0.362	0.136	0.303	0.376	0.462	**0.328 ± 0.054**	**a**
11	Holt Creek	1.37	0.209	0.558	0.102	0.832	**0.613 ± 0.228**	**a,b**
12	Taylor	1.24	0.611	0.468	0.631	0.804	**0.750 ± 0.133**	**a,b**
13	Cobb Mill Creek (Culvert)	1.06	0.000	1.02	0.859	1.45	**0.878 ± 0.240**	**a,b**
14	Narrow Channel Branch	3.22	1.15	1.26	0.779	0.648	**1.41 ± 0.47**	**a,b**
15	Tommy's Ditch	6.45	0.773	0.976	1.305	1.18	**2.14 ± 1.08**	**b**
Mean ± SE (By Date)		**1.61 ± 0.38**	**0.476 ± 0.089**	**0.552 ± 0.087**	**0.491 ± 0.100**	**0.803 ± 0.105**	**Overall Mean ± SE**	
Group[2]		**a**	**b**	**b**	**b**	**b**	**0.787 ± 0.097**	

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates
[1] Grouping assignments by stream based on REGWQ post-hoc analysis (α=-0.05) following square-root transformation of concentrations
[2] Grouping assignments by sample date based on REGWQ post-hoc analysis (α=-0.05) following natural log (+1 shift) transformation of concentrations

Table B-3. Individual and mean %DON by stream and sample date. Post-hoc grouping assignments based on REGWQ test (α = 0.05).

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)	Group[1]
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020		
1	Bundick's Creek	35	31	14	43	1	**25 ± 8**	**a,b**
2	Parker's Creek	40	26	36	21	43	**33 ± 4**	**a,b**
3	Ross Branch	35	1	0	0	36	**14 ± 9**	**a,b**
4	Frogstool	48	36	37	39	53	**42 ± 3**	**a,b**
5	Pungo Creek	50	26	51	0	42	**34 ± 9**	**a,b**
6	Coal Kiln	30	3	6	0	22	**12 ± 6**	**a,b**
7	Greens Creek	98	32	33	30	49	**48 ± 13**	**a,b**
8	Phillips Creek	27	43	39	78	53	**48 ± 9**	**a,b**
9	Redbank Creek	32	52	32	24	0	**28 ± 8**	**a,b**
10	Mill Creek North	84	30	45	91	100	**70 ± 14**	**a**
11	Holt Creek	36	6	17	4	24	**17 ± 6**	**a,b**
12	Taylor	32	18	12	23	23	**21 ± 3**	**a,b**
13	Cobb Mill Creek (Culvert)	43	0	16	28	37	**25 ± 8**	**a,b**
14	Narrow Channel Branch	37	20	28	40	13	**27 ± 5**	**a,b**
15	Tommy's Ditch	47	10	15	27	15	**23 ± 7**	**b**
Mean ± SE (By Date)		**45 ± 5**	**22 ± 4**	**25 ± 4**	**30 ± 7**	**34 ± 7**	**Overall Mean ± SE**	
Group[2]		**a**	**b**	**a,b**	**a,b**	**a,b**	**31 ± 3**	

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates
[1] Grouping assignments by stream based on REGWQ post-hoc analysis (α=-0.05)
[2] Grouping assignments by sample date based on REGWQ post-hoc analysis (α=-0.05) following natural log (+1 shift) transformation of concentrations

Table B-4. Individual and mean TDN concentrations (mg N L^{-1}) by stream and sample date. Post-hoc grouping assignments based on REGWQ test ($\alpha = 0.05$).

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)	Group[1]
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020		
1	Bundick's Creek	2.65	1.85	1.35	1.44	3.09	**2.08 ± 0.34**	**a,b,c,d**
2	Parker's Creek	2.42	1.37	1.07	2.34	2.59	**1.96 ± 0.31**	**a,b,c**
3	Ross Branch	3.15	3.82	4.62	3.86	2.70	**3.63 ± 0.33**	**a,b,c,d,e**
4	Frogstool	2.09	1.62	1.55	1.25	2.03	**1.71 ± 0.16**	**a,b**
5	Pungo Creek	2.26	1.58	1.48	1.92	1.48	**1.75 ± 0.15**	**a,b**
6	Coal Kiln	4.09	3.96	4.38	3.56	3.79	**3.96 ± 0.14**	**e,f**
7	Greens Creek	1.95	1.78	2.02	1.82	1.97	**1.91 ± 0.05**	**a,b,c,d**
8	Phillips Creek	2.37	1.28	0.970	1.21	2.04	**1.58 ± 0.27**	**a**
9	Redbank Creek	4.97	2.04	1.53	0.904	4.62	**2.81 ± 0.83**	**a,b,c,d**
10	Mill Creek North	0.431	0.455	0.67	0.412	0.462	**0.485 ± 0.046**	**g**
11	Holt Creek	3.84	3.26	3.33	2.79	3.51	**3.35 ± 0.17**	**b,c,d,e**
12	Taylor	3.89	3.46	3.81	2.77	3.54	**3.49 ± 0.20**	**b,c,d,e**
13	Cobb Mill Creek (Culvert)	2.45	3.33	6.54	3.12	3.89	**3.87 ± 0.71**	**c,d,e**
14	Narrow Channel Branch	8.78	5.87	4.54	1.96	4.93	**5.21 ± 1.10**	**e,f**
15	Tommy's Ditch	13.8	8.04	6.54	4.89	7.95	**8.25 ± 1.51**	**f**
Mean ± SE (By Date)		**3.95 ± 0.86**	**2.91 ± 0.51**	**2.96 ± 0.52**	**2.28 ± 0.32**	**3.24 ± 0.46**	**Overall Mean ± SE**	
Group[2]		**a**	**a**	**a**	**a**	**a**	**3.07 ± 0.25**	

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates
[1] Grouping assignments by stream based on REGWQ post-hoc analysis (α=-0.05) following natural log transformation of concentrations
[2] Grouping assignments by sample date based on REGWQ post-hoc analysis (α=-0.05) following natural log transformation of concentrations

Table B-5. Individual and mean NH_4^+ concentrations (mg N L^{-1}) by stream and sample date. Measurements below the lowest calibration standard (0.0315 mg N L^{-1}) are reported as zero.

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020	
1	Bundick's Creek	0.040	0.000	0.000	0.000	0.067	**0.021 ± 0.014**
2	Parker's Creek	0.221	0.061	0.140	0.095	0.108	**0.125 ± 0.027**
3	Ross Branch	0.060	0.041	0.081	0.000	0.046	**0.046 ± 0.013**
4	Frogstool	0.042	0.000	0.000	0.000	0.049	**0.018 ± 0.011**
5	Pungo Creek	0.080	0.080	0.076	0.000	0.000	**0.047 ± 0.019**
6	Coal Kiln	0.050	0.067	0.087	0.059	0.000	**0.052 ± 0.015**
7	Greens Creek	0.033	0.000	0.000	0.000	0.000	**0.007 ± 0.007**
8	Phillips Creek	0.079	0.000	0.047	0.049	0.000	**0.035 ± 0.015**
9	Redbank Creek	0.305	0.266	0.454	0.037	0.000	**0.212 ± 0.085**
10	Mill Creek North	0.069	0.103	0.110	0.036	0.000	**0.064 ± 0.021**
11	Holt Creek	0.000	0.000	0.000	0.000	0.049	**0.01 ± 0.01**
12	Taylor	0.035	0.000	0.033	0.000	0.046	**0.023 ± 0.01**
13	Cobb Mill Creek (Culvert)	0.044	0.038	0.000	0.000	0.000	**0.016 ± 0.01**
14	Narrow Channel Branch	0.044	0.036	0.035	0.000	0.000	**0.023 ± 0.009**
15	Tommy's Ditch	0.000	0.000	0.058	0.000	0.063	**0.024 ± 0.015**
Mean ± SE (By Date)		**0.074 ± 0.021**	**0.046 ± 0.018**	**0.075 ± 0.029**	**0.018 ± 0.008**	**0.029 ± 0.009**	**Overall Mean ± SE**
							0.048 ± 0.009

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates

Table B-6. Individual and mean NO_2^- concentrations (mg N L^{-1}) by stream and sample date. Measurements below the lowest calibration standard (0.100 mg N L^{-1}) are reported as zero.

Stream ID	Stream	Sample Date					Mean ± SE (By Stream)
		11-May-2019	29-Jun-2019	25-Aug-2019	30-Nov-2019	16-Feb-2020	
1	Bundick's Creek	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
2	Parker's Creek	0.172	0.000	0.000	0.190	0.000	**0.072 ± 0.044**
3	Ross Branch	0.000	0.136	0.135	0.000	0.000	**0.054 ± 0.033**
4	Frogstool	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
5	Pungo Creek	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
6	Coal Kiln	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
7	Greens Creek	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
8	Phillips Creek	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
9	Redbank Creek	0.214	0.137	0.161	0.000	0.000	**0.102 ± 0.044**
10	Mill Creek North	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
11	Holt Creek	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
12	Taylor	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
13	Cobb Mill Creek (Culvert)	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
14	Narrow Channel Branch	0.000	0.000	0.000	0.000	0.000	**0 ± 0**
15	Tommy's Ditch	0.000	0.000	0.182	0.000	0.000	**0.036 ± 0.036**
Mean ± SE (By Date)		**0.026 ± 0.018**	**0.018 ± 0.012**	**0.032 ± 0.017**	**0.013 ± 0.013**	**0.026 ± 0.018**	**Overall Mean ± SE**
							0.018 ± 0.006

Mean ± SE: Mean plus or minus standard error by stream and by date
Overall Mean ± SE: Mean plus or minus standard error for all streams and sample dates

Appendix C
Summary of Major Nitrogen Speciation by Stream and By Sample Date

Table C-1. Mean and standard error (SE) of NO_3^-, DON, and TDN concentrations and %DON by stream

Stream	mg NO_3^--N L^{-1}		mg DON-N L^{-1}		mg TDN-N L^{-1}		%DON	
	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
Bundick's Creek	5	1.51 ± 0.380	5	0.466 ± 0.162	5	2.08 ± 0.34	5	25 ± 8
Parker's Creek	5	1.10 ± 0.174	5	0.663 ± 0.158	5	1.96 ± 0.31	5	33 ± 4
Ross Branch	5	3.14 ± 0.554	5	0.426 ± 0.253	5	3.63 ± 0.33	5	14 ± 9
Frogstool	5	0.951 ± 0.052	5	0.742 ± 0.121	5	1.71 ± 0.16	5	42 ± 3
Pungo Creek	5	1.14 ± 0.236	5	0.579 ± 0.185	5	1.75 ± 0.15	5	34 ± 9
Coal Kiln	5	3.43 ± 0.233	5	0.491 ± 0.232	5	3.96 ± 0.14	5	12 ± 6
Greens Creek	5	0.974 ± 0.244	5	0.931 ± 0.256	5	1.91 ± 0.046	5	48 ± 13
Phillips Creek	5	0.822 ± 0.243	5	0.718 ± 0.130	5	1.58 ± 0.27	5	48 ± 9
Redbank Creek	5	2.02 ± 0.10	5	0.668 ± 0.288	5	2.81 ± 0.83	5	28 ± 8
Mill Creek North	5	0.094 ± 0.058	5	0.328 ± 0.054	5	0.485 ± 0.046	5	70 ± 14
Holt Creek	5	2.72 ± 0.10	5	0.613 ± 0.228	5	3.35 ± 0.17	5	17 ± 6
Taylor	5	2.72 ± 0.19	5	0.750 ± 0.133	5	3.49 ± 0.20	5	21 ± 3
Cobb Mill Creek (Culvert)	5	2.99 ± 0.71	5	0.878 ± 0.240	5	3.87 ± 0.71	5	25 ± 8
Narrow Channel Branch	5	3.78 ± 0.75	5	1.41 ± 0.47	5	5.21 ± 1.10	5	27 ± 5
Tommy's Ditch	5	6.06 ± 0.72	5	2.14 ± 1.08	5	8.25 ± 1.51	5	23 ± 7

Table C-2. Mean and standard error (SE) of NO_3^-, DON, and TDN concentrations and %DON by sample date

Sample Date	mg NO_3^--N L^{-1}		mg DON-N L^{-1}		mg TDN-N L^{-1}		%DON	
	n	**Mean ± SE**	**n**	**Mean ± SE**	**n**	**Mean ± SE**	**n**	**Mean ± SE**
11-MAY-19	15	1.78 ± 0.33	15	1.61 ± 0.38	15	3.95 ± 0.86	15	45 ± 5
29-JUN-19	15	2.24 ± 0.51	15	0.476 ± 0.089	15	2.91 ± 0.51	15	22 ± 4
25-AUG-19	15	2.38 ± 0.50	15	0.552 ± 0.087	15	2.96 ± 0.52	15	25 ± 4
30-NOV-19	15	2.30 ± 0.49	15	0.491 ± 0.100	15	2.28 ± 0.32	15	30 ± 7
16-FEB-20	15	2.47 ± 0.48	15	0.803 ± 0.105	15	3.24 ± 0.46	15	34 ± 7

Appendix D
Hypothesis Testing for Normality of Residuals for Untransformed and Transformed Data, Homogeneity of Variances, and Between-Stream Effects using One-Way Analysis of Variance (ANOVA) for NO_3^- DON, %DON, and TDN

Table D-1. Shapiro-Wilk Test for Normality of Residuals by Stream for untransformed and transformed dependent variables (NO_3^-, DON, %DON, and TDN)

Dependent Variable	Data Transformation	W-Statistic*	*p*
NO_3^-	None	0.940	0.001
	Square-root[1]	0.972	0.097
DON	None	0.767	<0.001
	Square-root[1]	0.978	0.245
%DON	None	0.990	0.833
TDN	None	0.816	<0.001
	Natural Log[1]	0.966	0.042

[1] Transformation used for homogeneity of variance hypothesis testing and One-way ANOVA for Between-Stream effects

* Distribution and Q-Q plots for residuals were examined visually for normal distribution

Table D-2. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for NO_3^- concentrations transformed using a square-root function

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Stream	1.114	14	0.080	0.92	0.542
Error	5.1827	60	0.086		

Table D-3. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for DON concentrations transformed using a square-root function

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Stream	0.743	14	0.053	0.64	0.821
Error	4.980	60	0.083		

Table D-4. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for %DON

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Stream	1516	14	108	0.55	0.891
Error	11773	60	196		

Table D-5. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for TDN concentrations transformed using a natural log function

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Stream	1.354	14	0.097	1.87	0.048
Error	3.096	60	0.052		

Table D-6. One-way ANOVA table for NO_3^- concentrations transformed using a square-root function - Tests of Hypotheses for Between Stream-Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Stream	21.632	14	1.545	12.23	<0.001
Error	7.580	60	0.126		

Table D-7. One-way ANOVA table for DON concentrations transformed using a square-root function - Tests of Hypotheses for Between Stream-Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Stream	3.553	14	0.254	1.76	0.067
Error	8.650	60	0.144		

Table D-8. One-way ANOVA table for %DON - Tests of Hypotheses for Between-Stream Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Stream	16708	14	1022.464	3.73	<0.001
Error	19174	60	279.912		

Table D-9. One-way ANOVA table for TDN concentrations transformed using a natural log function - Tests of Hypotheses for Between-Stream Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Stream	29.403	14	2.100	18.04	<0.001
Error	6.984	60	0.116		

Appendix E
Individual Plots of Concentrations of DON, NO_3^-, and TDN versus Sample Date by Steam

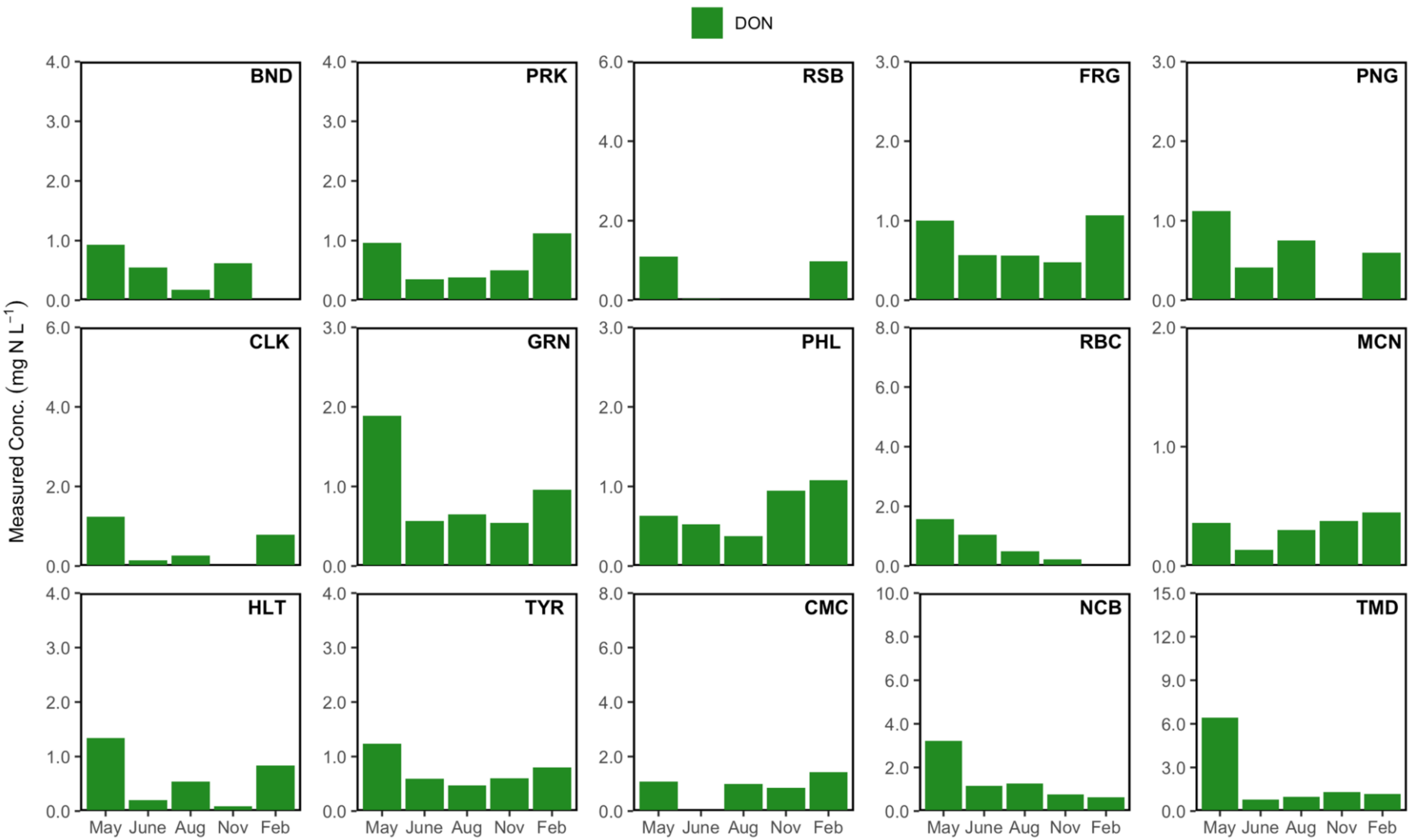

Figure E- 1. Concentrations of DON (mg N L^{-1}) versus sample date for each studied stream. Note y-axis scale varies by plot to visualize patterns within each stream more easily.

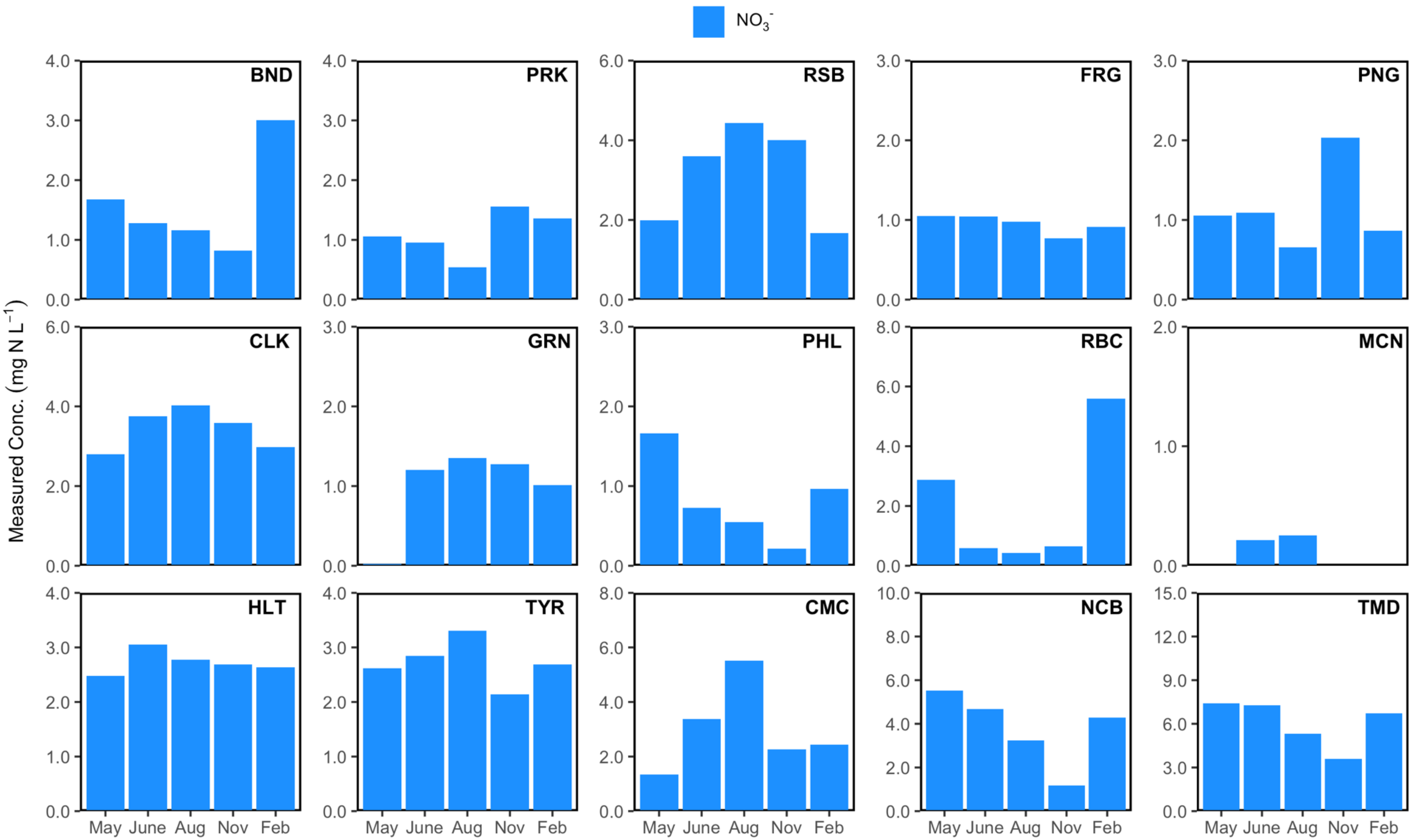

Figure E-2. Concentrations of NO3- (mg N L^{-1}) versus sample date for each studied stream. Note y-axis scale varies by plot to visualize patterns within each stream more easily.

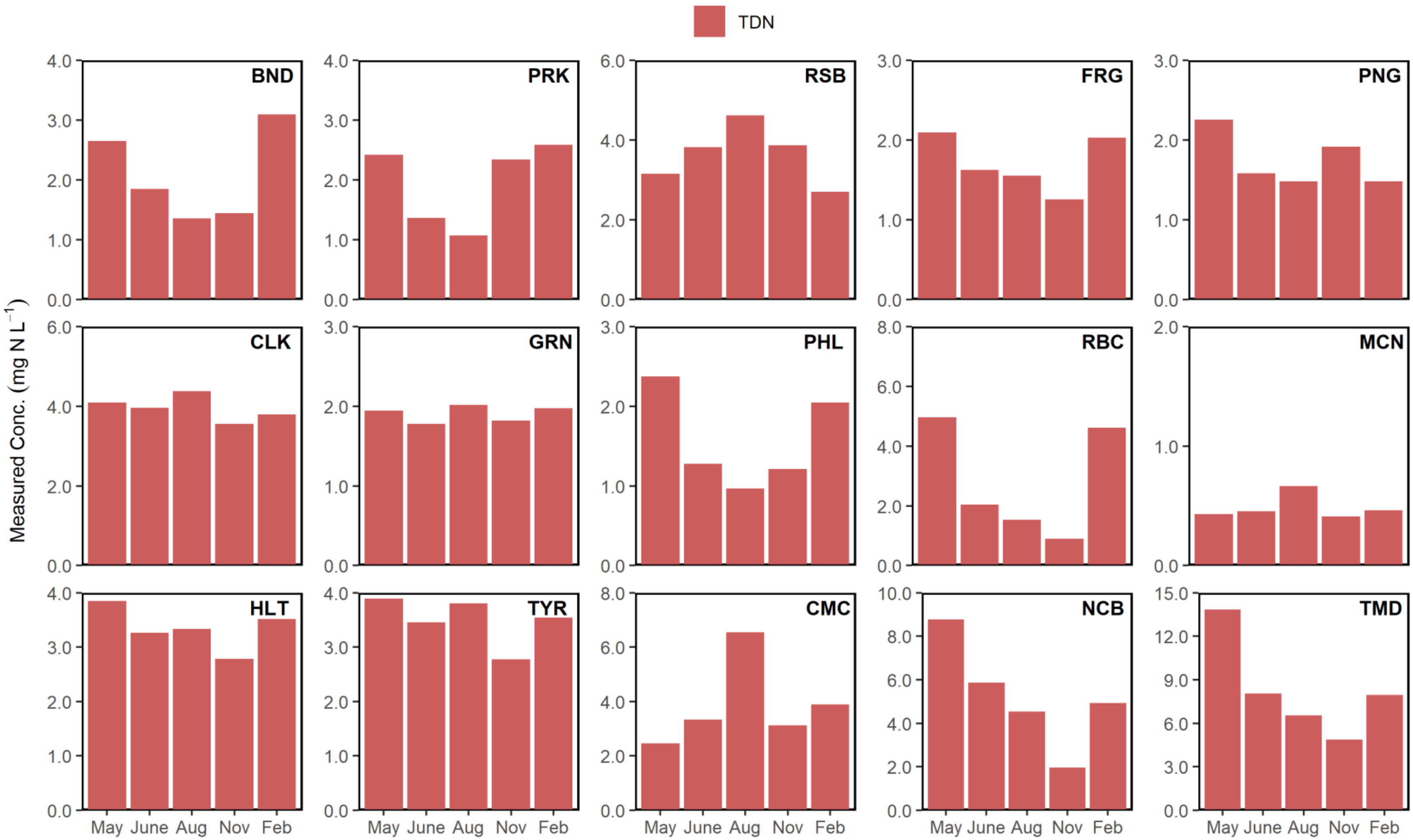

Figure E-3. Concentrations of TDN (mg N L^{-1}) versus sample date for each studied stream. Note y-axis scale varies by plot to visualize patterns within each stream more easily.

Appendix F
Hypothesis Testing for Normality of Residuals for Untransformed and Transformed Data, Homogeneity of Variances, and Between-Sample Date (Seasonal) Effects using One-Way Analysis of Variance (ANOVA) for NO_3^- DON, %DON, and TDN

Table F-1. Shapiro-Wilk Test for Normality of Residuals by Sample Date (Season) for untransformed and transformed dependent variables (NO_3^-, DON, %DON, and TDN)

Dependent Variable	Data Transformation	W-Statistic*	*p*
NO_3^-	None	0.918	<0.001
	Square-root[1]	0.987	0.6629
DON	None	0.670	<0.001
	Natural Log (+1 Shift)[1]	0.953	0.008
%DON	None	0.930	0.001
	Natural Log (+1 Shift)[1]	0.972	0.094
TDN	None	0.854	<0.001
	Natural Log[1]	0.968	0.051

[1] Transformation used for homogeneity of variance hypothesis testing and One-way ANOVA for Between-Stream effects

* Distribution and Q-Q plots for residuals were examined visually for normal distribution

Table F-2. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for NO_3^- concentrations transformed using a square-root function

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Sample Date	0.136	4	0.034	0.20	0.937
Error	11.802	70	0.169		

Table F-3. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for DON concentrations transformed using a natural log (+1 shift) function

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Sample Date	0.057	4	0.014	0.33	0.858
Error	3.021	70	0.043		

Table F-4. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for %DON using a natural log (+1 shift) function

Source	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	*F*-Statistic	*p*
Sample Date	0.039	4	0.010	0.94	0.444
Error	0.724	70	0.010		

Table F-5. Brown and Forsythe's Test for Homogeneity of Variance from Group Medians for TDN concentrations transformed using a natural log function

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Sample Date	0.336	4	0.084	0.40	0.806
Error	14.571	70	0.208		

Table F-6. One-way ANOVA table for NO_3^- concentrations transformed using a square-root function - Tests of Hypotheses for Between Stream-Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Sample Date	0.471	4	0.118	0.29	0.886
Error	28.741	70	0.411		

Table F-7. One-way ANOVA table for DON concentrations transformed using a natural log (+1 shift) function - Tests of Hypotheses for Between Stream-Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Sample Date	2.675	4	0.669	8.36	<0.001
Error	5.601	70	0.080		

Table F-8. One-way ANOVA table for %DON using a natural log (+1 shift) function - Tests of Hypotheses for Between-Stream Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Sample Date	0.259	4	0.065	2.75	0.035
Error	1.648	70	0.023		

Table F-9. One-way ANOVA table for TDN concentrations transformed using a natural log function - Tests of Hypotheses for Between-Stream Effects

Source	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	***F*-Statistic**	***p***
Sample Date	1.771	4	0.443	0.90	0.472
Error	34.616	70	0.495		

Appendix G
Individual Nitrogen Speciation Results in Concurrent Groundwater and Surface-Water Samples

Table G-1. NO_3^-, NO_2^-, NH_4^+, TDN, and DON concentrations and %DON in groundwater samples collected on 29 June 2020 from beneath the center of the stream channel at approximately one meter in depth for five stream sites.

Stream	**mg N L^{-1}**					**%DON**
	NO_3^-	**NO_2^-**	**NH_4^+**	**TDN**	**DON**	
Bundick's Creek	2.68	0.223	0.479	4.89	1.51	31
Coal Kiln	0.372	0	0.832	2.02	0.813	40
Phillips Creek	1.63	0	0.078	2.88	1.18	41
Cobb Mill Creek (hillslope)	10.7	0.503	0.454	12.4	0.776	6
Tommy's Ditch	3.58	0.269	0.065	6.03	2.12	35

Table G-2. NO_3^-, NO_2^-, NH_4^+, TDN, and DON concentrations and %DON in surface-water samples collected on 29 June 2020 in five stream sites immediately prior to the collection of groundwater samples.

Stream	**mg N L^{-1}**					**%DON**
	NO_3^-	**NO_2^-**	**NH_4^+**	**TDN**	**DON**	
Bundick's Creek[a]	1.28	0	0.017	1.85	0.549	30
Coal Kiln[a]	3.76	0	0.067	3.96	0.139	3
Phillips Creek[a]	0.729	0	0.026	1.28	0.523	41
Cobb Mill Creek (hillslope)	3.30	0	0.038	3.22	0	0
Tommy's Ditch[a]	7.26	0	0.000	8.04	0.773	10

[a] Data for these streams also reported in Appendix B

Appendix H
Nitrogen Speciation Results for Flow-through Sediment Core Experiment

Table H-1. Concentrations of NO_3^-, NO_2^-, NH_4^+, TDN, and DON as well as %DON in artificial groundwater and effluent sample from a flow-through experiment with a sediment core collected from Coal Kiln (CLK) on 27 April2019

Sample Description	mg N L^{-1}					%DON
	NO_3^-	**NO_2^-**	**NH_4^+**	**TDN**	**DON**	
Artificial Groundwater[1]	2.68	0*	0	2.78	0.096	0%
CLK Core Effluent[2]	0.237	0.364*	0.097	1.72	1.02	59%

[1]Artificial groundwater = 60 mg $MgSO_4{\cdot}7H_2O$, 20 mg KNO_3, 36 mg $NaHCO_3$, 36 mg $CaCl_2$, 25 mg $CaSO_4{\cdot}2H_2O$ per 1 L of deionized water

[2] Sample collected from outflow from sediment core through which artificial groundwater was passed

* The NO_2^- values were initially measured at 0.403 and 0.767 mg N L^{-1} in the artificial groundwater and CLK core effluent samples, respectively. Further examination of the ion chromatograms revealed inadequate peak separation which likely caused an overestimation of the NO_2^- concentration in these samples. Because these samples could not be rerun, the NO_2^- concentrations were offset by the value quantified for the artificial groundwater sample which was presumed to have no appreciable NO_2^-.

Appendix I
Watershed and Land-use Analysis

Delineations of the watershed for each stream in the Figures I1-15 were digitized previously using digital elevation models (DEMs) (A. L. Mills, unpublished data, 2019). The point from which the contributing watershed area was delineated was the point at which each stream was sampled in this study.

Active agricultural fields used for crop cultivation on the Eastern Shore of Virginia were identified and manually delineated from high resolution aerial photography from the Virginia Base Mapping Program in a previous study aimed at estimating reactive nitrogen loads from fertilizer applications rates (Johnson 2018). Also in the previous study, a crop rotation and a fertilizer application rate based on nitrogen content (expressed as kg N ha^{-1} yr^{-1}) for each active agricultural field were assigned using 2013-2016 data from CropScape (USDA NASS 2016) and recommended fertilizer application rate from the Virginia Cooperative Extension, respectively (Johnson 2018).

Figure I-1. Watershed and agricultural field boundaries for Bundick's Creek (BND)

Figure I-2. Watershed and agricultural field boundaries for Parker's Creek (PRK)

Figure I- 3. Watershed and agricultural field boundaries for Ross Branch (RSB)

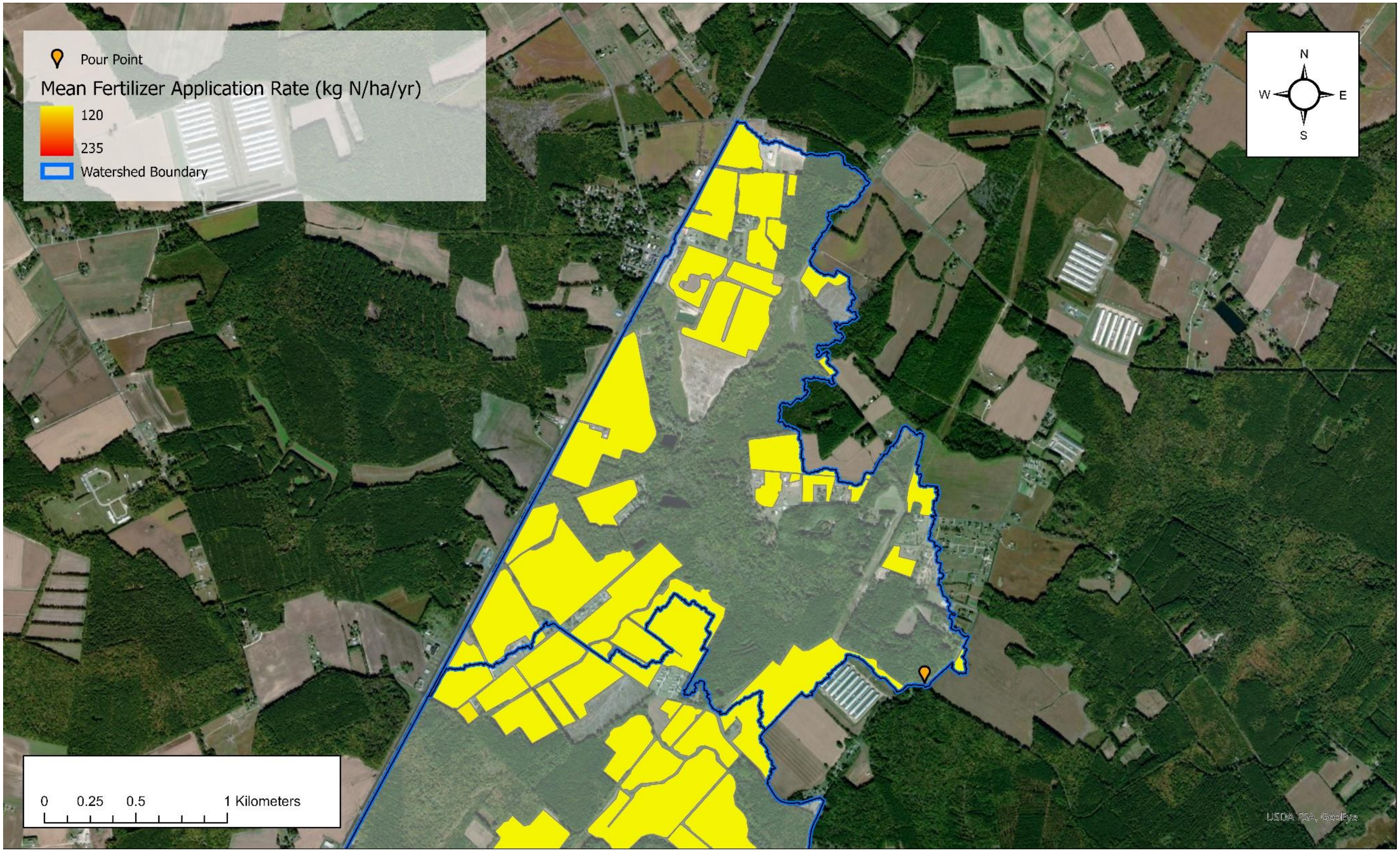

Figure I-4. Watershed and agricultural field boundaries for Frogstool (FRG)

Figure I-5. Watershed and agricultural field boundaries for Pungo Creek (PNG)

Figure I-6. Watershed and agricultural field boundaries for Coal Kiln (CLK)

Figure I-7. Watershed and agricultural field boundaries for Green's Creek (GRN)

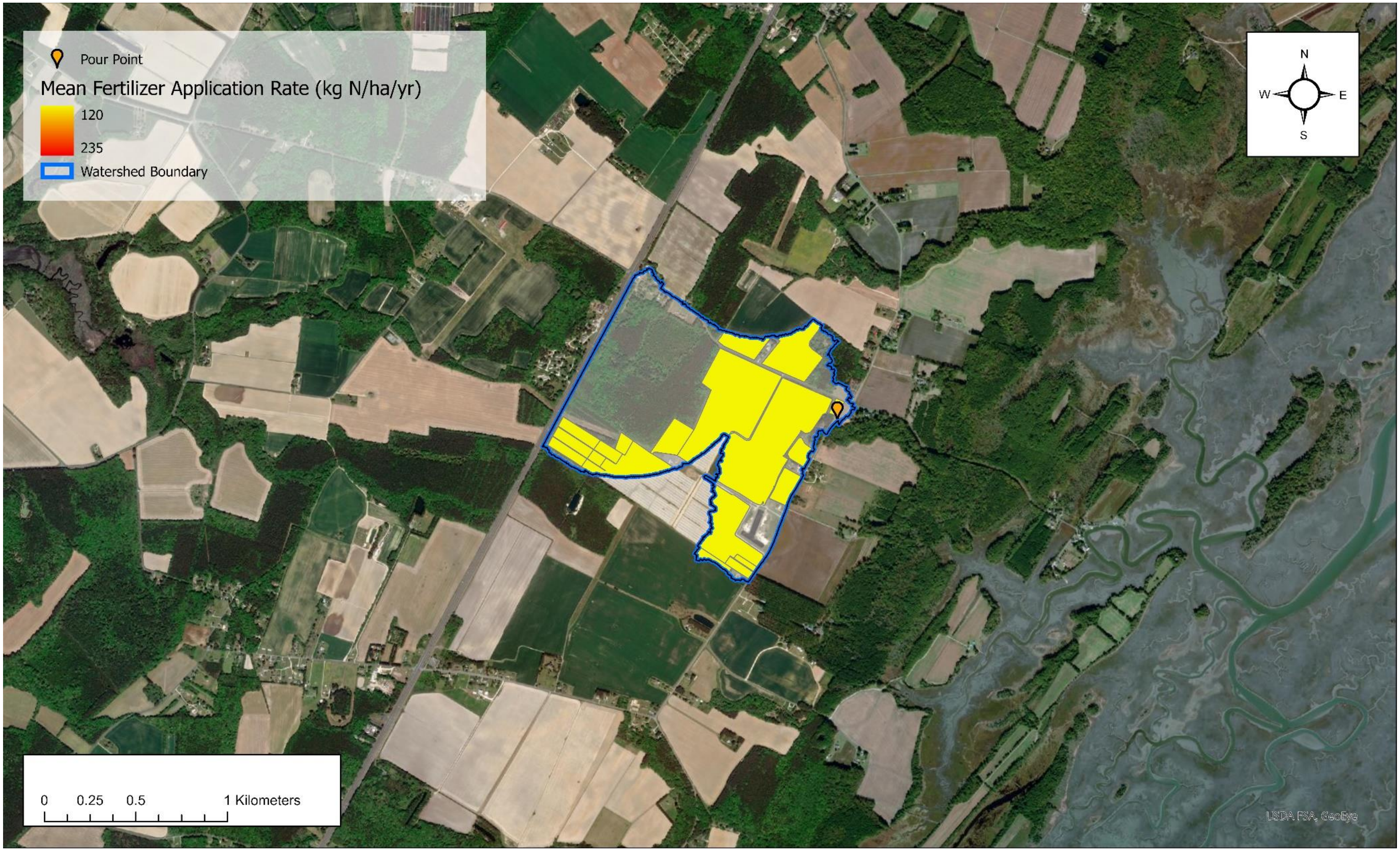

Figure I-8. Watershed and agricultural field boundaries for Red Bank Creek (RBC)

Figure I-9. Watershed and agricultural field boundaries for Mill Creek North (MCN)

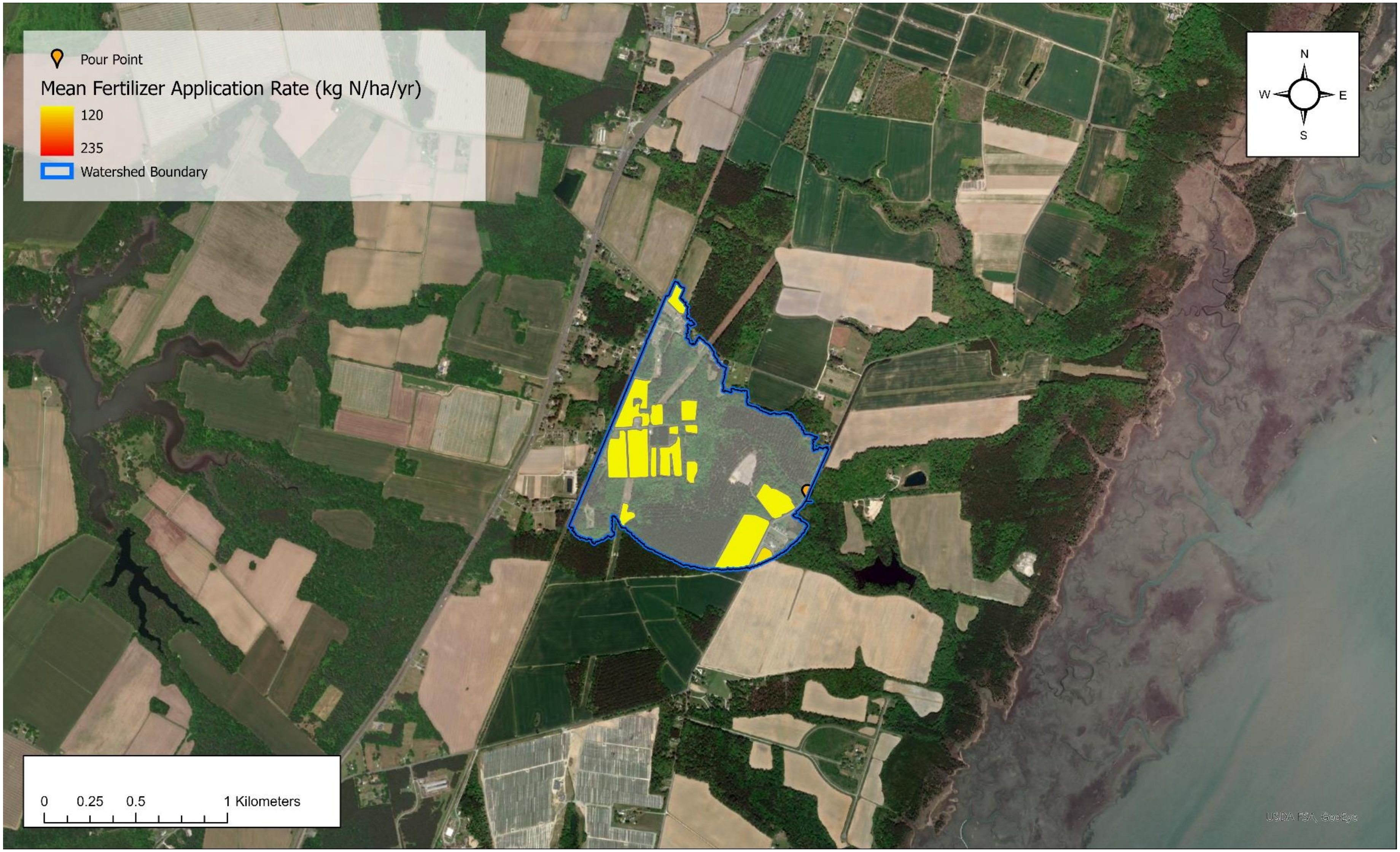

Figure I-10. Watershed and agricultural field boundaries for Holt Creek (HLT)

Figure I-11. Watershed and agricultural field boundaries for Taylor Creek (TYR)

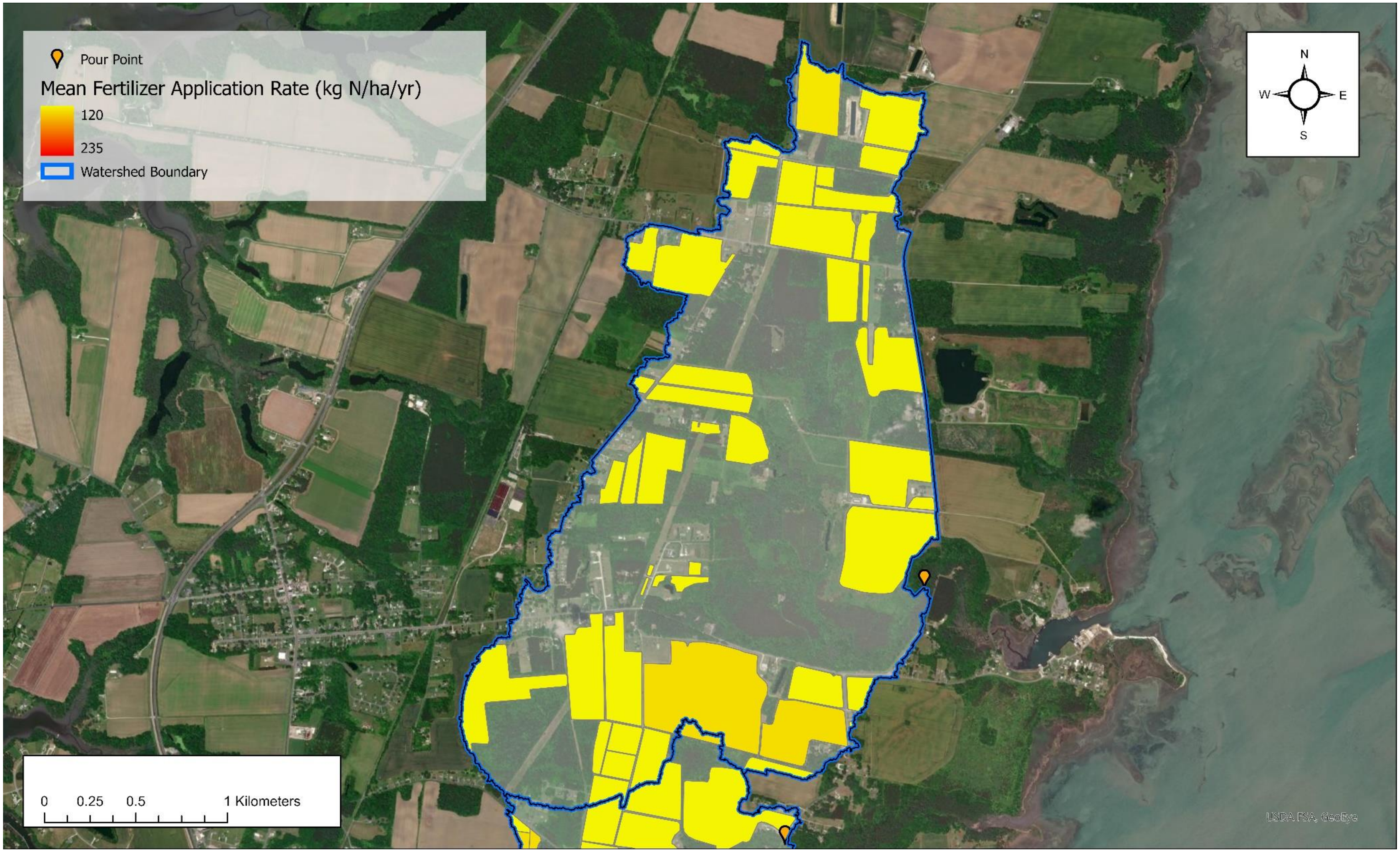

Figure I-12. Watershed and agricultural field boundaries for Cobb Mill Creek (CMC)

Figure I-13. Watershed and agricultural field boundaries for Narrow Channel Branch (NCB)

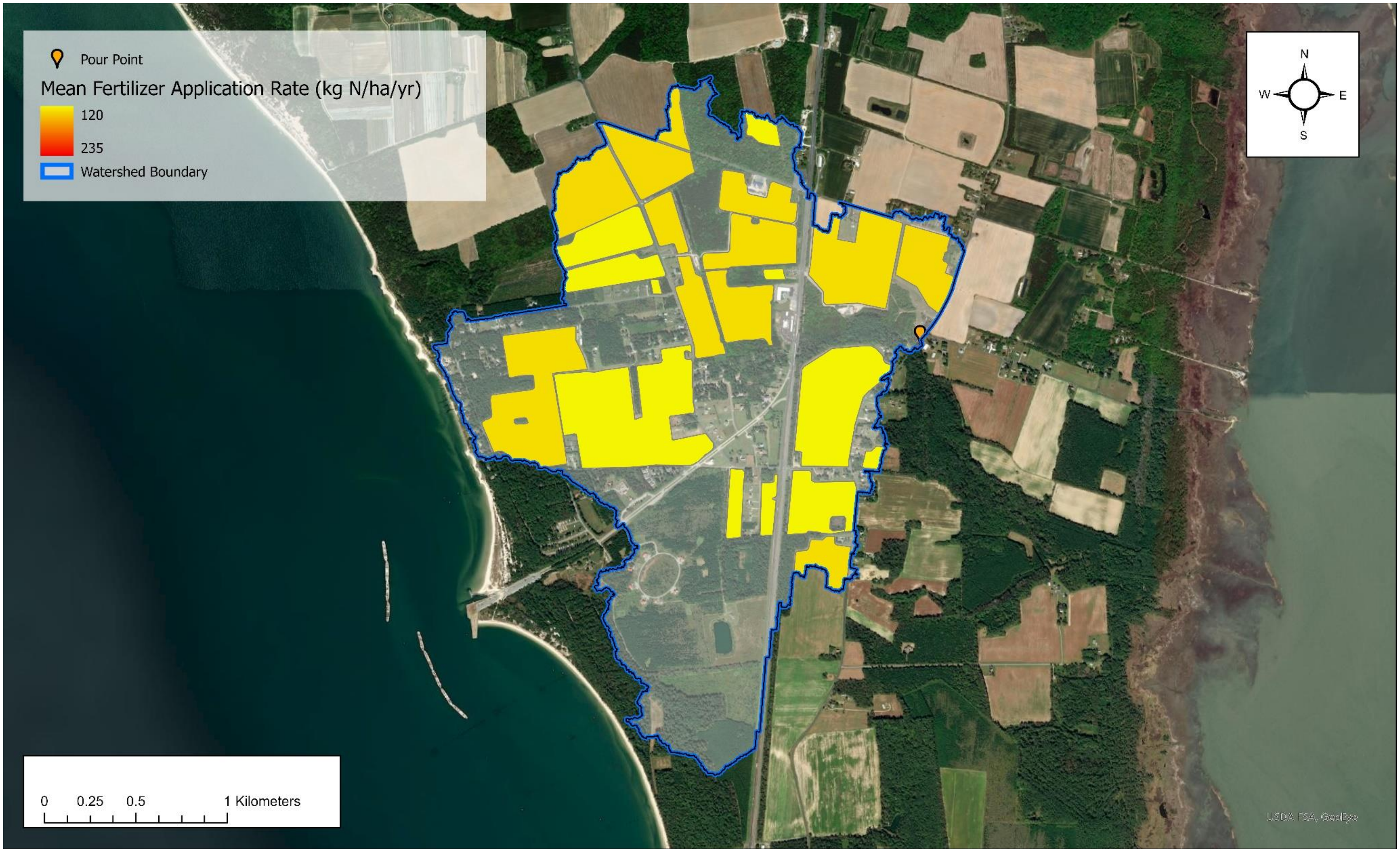

Figure I-14. Watershed and agricultural field boundaries for Tommy's Ditch (TMD)