Nitrogen source tracing in the Choptank River Watershed

Prepared for:
Midshore Riverkeeper Conservancy

Prepared by:
Dr Simon Costanzo
Integration and Application Network
University of Maryland center for Environmental Science

1 Introduction

1.1 Choptank River watershed overview

The Choptank River watershed drains approximately 795 square miles of land within the counties of Caroline, Dorchester, Queen Anne's, and Talbot (Figure 1). Larger water bodies in the Choptank River watershed include the Choptank, Little Choptank, and Tred Avon Rivers and Broad, Harris, and Tuckahoe Creeks. The Choptank River starts in Delaware and becomes tidal near Greensboro, Maryland before discharging to Chesapeake Bay. Almost half of the land in the Choptank watershed is agricultural land, with a third being covered in forest, and approximately 10% urban area (Figure 1). The 2000 census population for the Maryland part of the watershed was 71,000 (DNR 2007).

The watershed is composed of both well and poorly drained soils. In areas that have poor draining soils, artificial drainage (ditches and drainage tiles) has been installed over many decades to dry out the land for agricultural use. The artificial drainage: i) lowers the water table; ii) concentrates runoff and soluble nutrients; iii) decreases the opportunity for nutrient uptake and denitrification; and iv) expedites flow to the river and Chesapeake Bay.

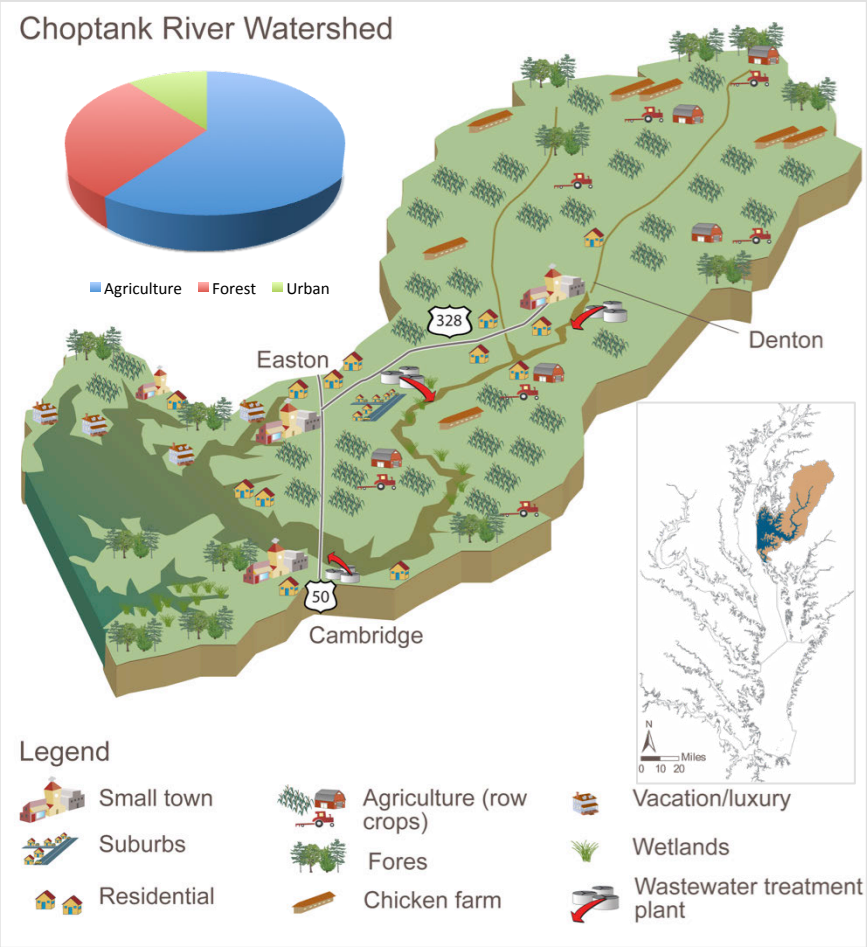

Figure 1 Choptank River watershed including predominant land uses

The largest contributor of nutrients in the Choptank River Watershed is agriculture (DNR 2007) dominated by inputs from cropping, poultry and dairy. Additionally there are inputs from four major (WWTPs) within the watershed (>0.5 MGD).

As a result, the Choptank River scores poorly in terms of water quality and biotic integrity, with evidence that nutrient inputs (particularly nitrogen) are primarily responsible for degraded water quality. This has prompted the requirement for a monitoring approach that can distinguish the distribution and impacts of these various sources of nitrogen.

1.2 Sourcing nitrogen in the Choptank River watershed

The nitrogen cycle is the process by which nitrogen is converted between its various chemical forms (Figure 2). This transformation can be carried out through both biological and physical processes. The majority of Earth's atmosphere (78%) is nitrogen gas (N_2), making it the largest pool of nitrogen. However, atmospheric nitrogen has limited availability for biological use, leading to a scarcity of usable nitrogen in many types of ecosystems. Human activities such as fossil fuel combustion, use of artificial

nitrogen fertilizers, and release of nitrogen in wastewater have dramatically altered the global nitrogen cycle.

There are two naturally occurring forms of the nitrogen atom (Figure 3). The common form that contains seven protons and seven neutrons is referred to as nitrogen 14 and is expressed as ^{14}N. A heavier form that contains an extra neutron is called nitrogen 15 and is expressed as ^{15}N.

The various sources of nitrogen pollution to coastal ecosystems often have distinguishable ^{15}N:^{14}N ratios (referred to as $\delta^{15}N$), thereby providing a means to identify the source of pollution (Heaton, 1986). For example, nitrogen fertilizer and sewage-derived nitrogen have distinct differences in their $\delta^{15}N$ signatures. The main method of nitrate and ammonium fertilizer production is by industrial fixation of atmospheric nitrogen, resulting in products that have $\delta^{15}N$ values close to zero (Figure 4).

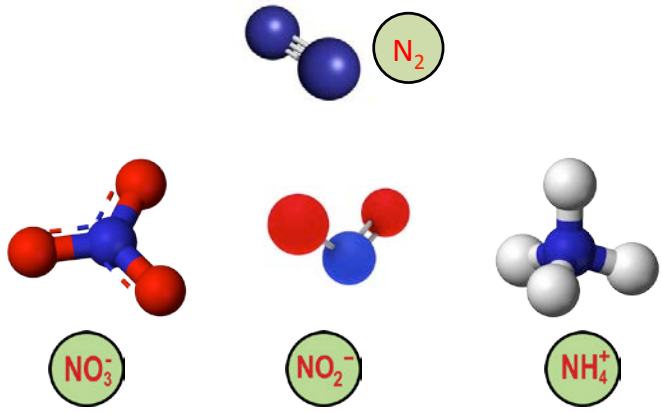

Figure 2 The varied molecular forms of inorganic nitrogen (blue atom) as nitrogen gas (N2), Nitrate (NO3-), nitrite (NO2-) and ammonia (NH4+).

However in animal or sewage waste, nitrogen is excreted mainly in the form of urea, which when hydrolyzed, produces a temporary rise in pH. The more basic conditions favor conversion to ammonia, which is easily lost by volatilization to the atmosphere. Fractionation during this process results in the ammonia, which is lost from the system, being depleted in ^{15}N. The remaining ammonium, now correspondingly enriched in ^{15}N, is subsequently converted to ^{15}N-enriched nitrate, which is more readily leached and dispersed by water (Heaton,1986) (Figure 4). The elevated $\delta^{15}N$ signature of treated sewage ($\delta^{15}N$ = 10%) therefore distinguishes it from other nitrogen sources entering marine ecosystems (cf. fertilizer nitrogen $\delta^{15}N$ = 0%) (Heaton, 1986).

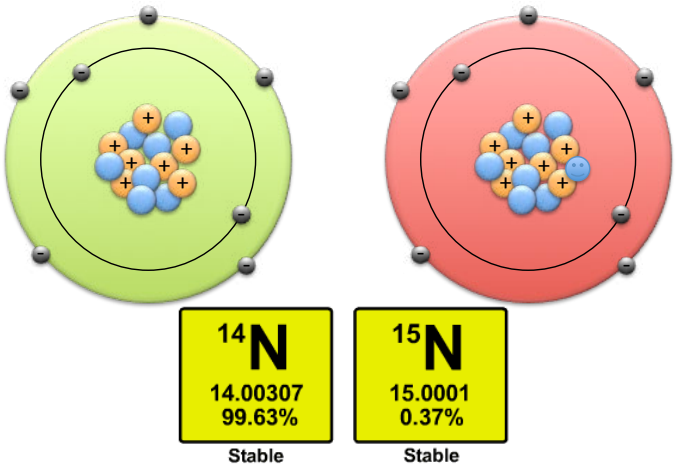

Figure 3 Nitrogen exists naturally in two isotopic forms. The common form contains seven protons and seven neutrons (^{14}N) and the heavier form that contains an extra neutron (^{15}N).

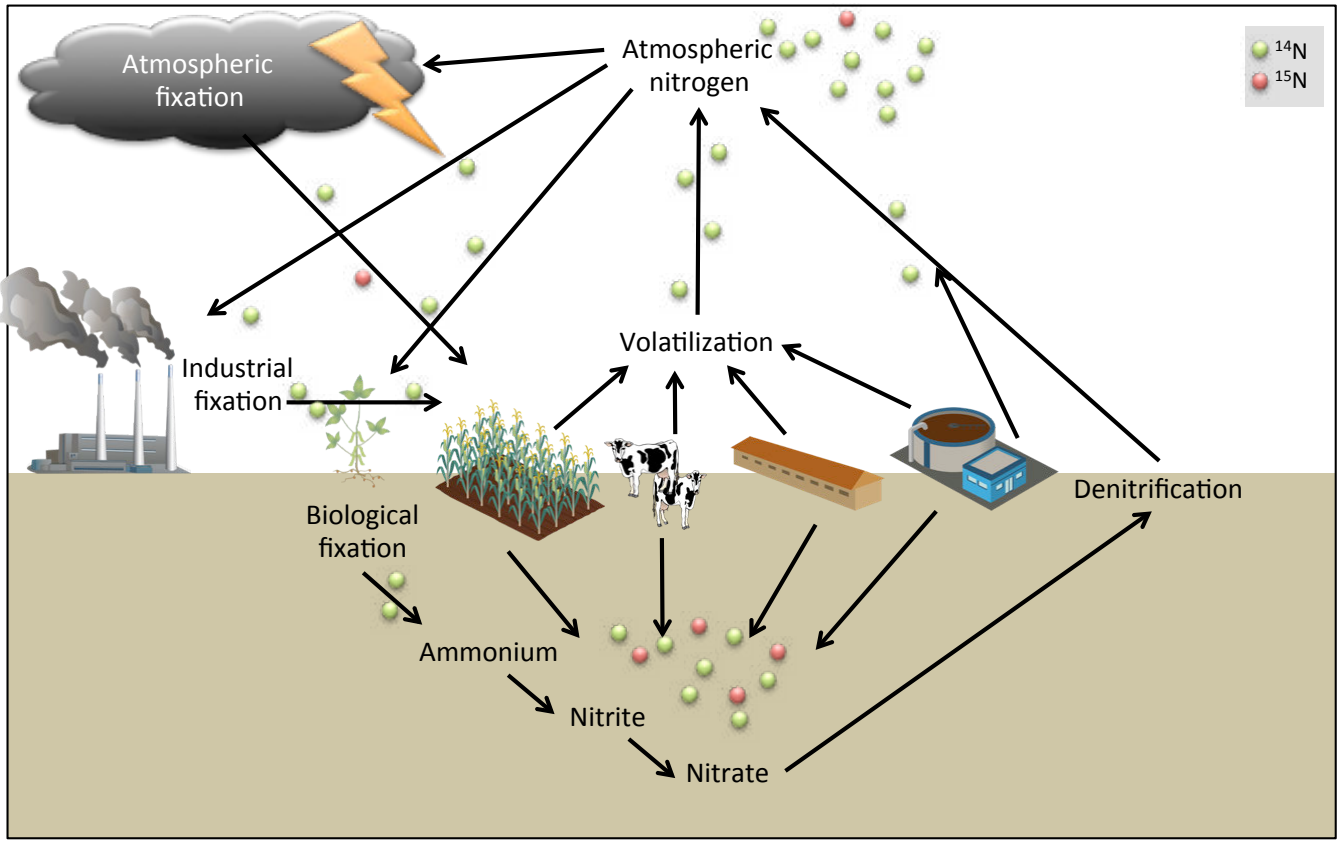

Figure 4 The nitrogen cycle highlighting sources of bioavilable nitrogen to the environment and demonstrating the process of ^{15}N enrichment through fractionation, volatilzation and denitrification.

Terrestrial and aquatic plants reflect their exposure to elevated nitrogen through increases in tissue nitrogen content (%N), and to different nitrogen sources through variations in tissue δ^{15}N over a given timeframe (Peterson and Fry,1987). Assessing the variations of %N and δ^{15}N in naturally occurring aquatic plants provides a technique for detecting and sourcing biologically available nitrogen entering the Choptank River and its watershed.

Interpretation of both %N and δ^{15}N values in plant tissue can provide insight to the nitrogen status of the environment that a plant was collected from. Understanding the drivers that alter these values and plotting %N and δ^{15}N values on a graph (Figure 5), the following assumptions can be made:

1. Low %N and low δ^{15}N: low nitrogen environment with low biological nitrogen processing.
2. Low %N and high δ^{15}N: low nitrogen environment with high biological nitrogen processing (e.g. denitrification).
3. high %N and low δ^{15}N: high nitrogen environment with low biological nitrogen processing (e.g. fertilizer source).
4. High %N and high δ^{15}N: high nitrogen environment with high biological nitrogen

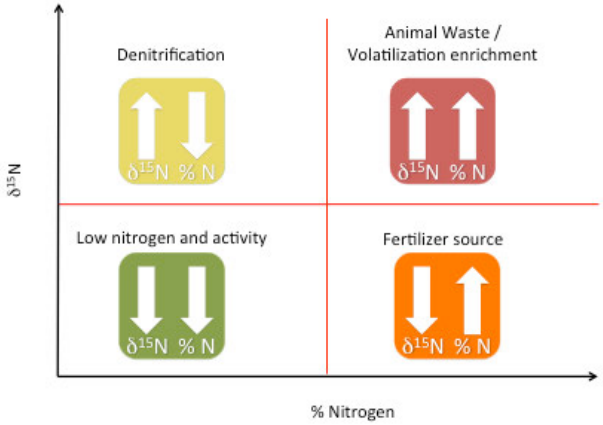

Figure 5 Conceptual plot of %N and δ^{15}N in plant tissue and what this represents in terms of nitrogen concentration and nitrogen origin in the plants surrounding environment.

processing (e.g. animal waste).

1.3 Study Objectives

The objectives of this study were to

1. Delineate the major sources of nitrogen to the Choptank River
2. Adopt combination of water quality and nitrogen stable isotope composition in aquatic plants and water samples.
3. Relate findings to land-use practices
4. Identify focus areas for further work / targeting of management activities

In order to:

1. Produce information to assist environmental management of the Choptank River watershed,
2. Provide a baseline for future assessment following implementation of best management practices that are planned and/or in progress to reduce nitrogen inputs from these four land use activities. These include advanced fertilizer application management; sewage upgrades at Cambridge; artificial drainage best management practices in the Tuckahoe Watershed; and sewage installation and treatment at Greensboro. This will allow a measure of the success of these practices and provide feedback on investments made by landowners.

2 Methodology

Data collection included sampling of grasses and water throughout the Choptank River watershed (Figure 6); collection of mono-specific grass species (*Phragmites sp.*) along the main stem of the Choptank River (

Figure 7); and deployment of a macroalgae in the vicinity of the Easton, Cambridge and Denton WWTP discharges (

Figure 8). This sampling strategy aimed to test the capacity of the approach for detecting point source (i.e. end of pipe) and diffuse sources (e.g. agricultural runoff) of nitrogen in the Choptank River watershed.

Figure 6 Collection of grass (mixed species including corn leasves) and water from the Choptank River watershed.

Figure 7 Collection of aquatic grass (*Phragmites sp.*) along the Choptank River shoreline.

Figure 8 Deployment and incubation of macroalgae (*Gracilaria edulis*) at the Cambridge WWTP discharge location.

2.1 Sampling design

All sampling was conducted between May-September 2013 and consisted of four separate sampling events. Sampling locations were distributed along the Choptank River and throughout the Choptank River watershed (Figure 9). The location of sampling sites in the watershed was designed to enable delineation of the Tuckahoe River watershed, a sub-watershed of the Choptank River watershed, from the remaining Choptank River watershed. Intensive sampling using deployed macroalgae (as per Costanzo *et al.*, 2001) was conducted in a radial grid-like fashion around the discharges for the Easton and Cambridge WWTPs (Figure 10).

Targeted sampling was conducted in Warwick River following receipt of results from Sampling Event 1, warranting further investigation for the cause of unexpected results at the confluence of the Warwick and Choptank Rivers.

A detailed listing of sampling locations (including co-ordinates) can be found in Appendix 1.

Table 1 Sampling dates, sample types and number of sampling locations.

Sampling event	Date	Activity	Sample types	Number of sampling sites
1	5 May 2013	Choptank River sampling (*Phragmites sp.*)	Plant material (*Phragmites sp.*)	11
2	8 – 12 July 2013	Sewage mapping	Macroalgal deployment and retrieval	22
3	11 July 2013	Choptank and Tuckahoe River watershed sampling	Mixed grass and water	10 (grass) 6 (water) 1 (manure/straw)
4	26 September 2013	Choptank River watershed and Warwick River sampling	Mixed grass and water	12 (grass) 11 (water)

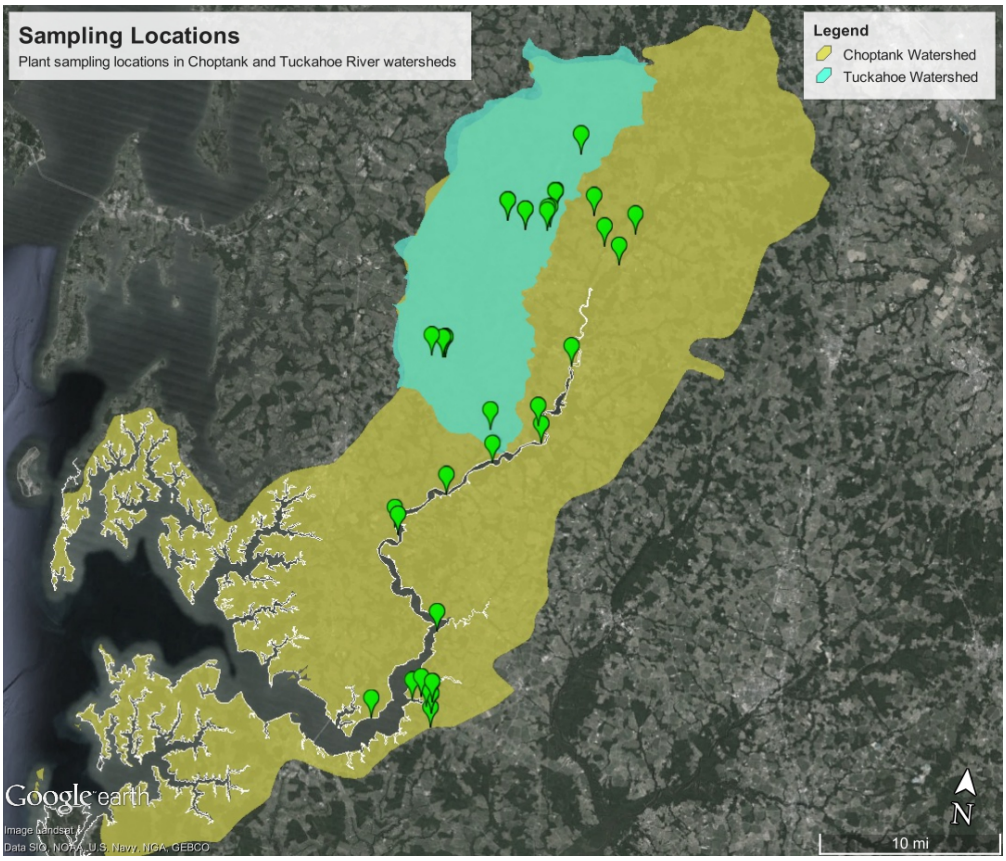

Figure 9 Sampling locations for plant material within the Choptank River, Choptank River Watershed and Tuckahoe River Watershed (highlighted in blue).

Figure 10 Macroalgal deployment locations around the Easton WWTP outfall (above) and Cambridge WWTP outfall (below).

2.2 Sample analysis

All plant and water samples were stored frozen prior to overnight courier to, and analysis by, the Odum School of Ecology, University of Georgia (refer to Appendix 1 and Appendix 2 for detailed methodology).

The primary focus of the project was to collect plant material for nitrogen content and stable isotope analysis of plant tissue (stylized methodology as per Figure 11). However, throughout the project the opportunity arose to collect and analyze water samples for total and dissolved nutrients, and stable isotope analysis of nitrate. This additional information complemented interpretation of target data.

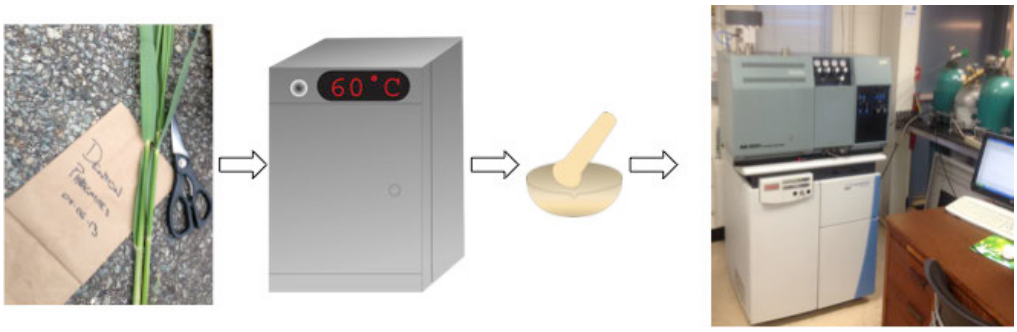

Figure 11 Sample analysis includes drying of plant samples at 60 ^{o}C for 24 hours, grinding and analysis in a XYZ mass spectrometer.

3 Results and Discussion

3.1 Choptank River *Phragmites sp.*

Results obtained for *Phragmites sp.* collected along the mainstream of the Choptank River (Sampling Event 1) show widespread nutrient enrichment throughout the river, despite a general decline for both $\delta^{15}N$ and %N with distance upstream (Figure 12). This suggests a change in nitrogen source, and a decrease in nitrogen uptake with distance upstream, respectively. $\delta^{15}N$ values showed more variability than %N and there were a number of notable instances where the two indicators showed an inverse trend. This was particularly evident at 21 miles upstream (confluence of Warwick River), 34 miles upstream (Easton wastewater treatment outfall) and 44-46 miles upstream (above the confluence of the Tuckahoe River and below Denton) (Figure 12). Of these examples, the greatest disparity between the two indicators was observed at 21 miles upstream (confluence of Warwick River) where *Phragmites sp.* displayed the highest observed %N and lowest observed $\delta^{15}N$ values of all sampling sites within the Choptank River. This relationship between the two indicators suggest a significant source of non-biologically processed nitrogen entering the Choptank River at this location (in reference to Figure 5). This instigated additional targeted sampling in the Warwick River to determine if indeed this was the source of the variability observed what the origin of the nitrogen was (described in Section 3.3). The influence of sewage inputs from the Cambridge and Easton WWTPs were reflected by spikes in $\delta^{15}N$ at 20 and 34 miles upstream, but their influence appeared localized (more so for Easton).

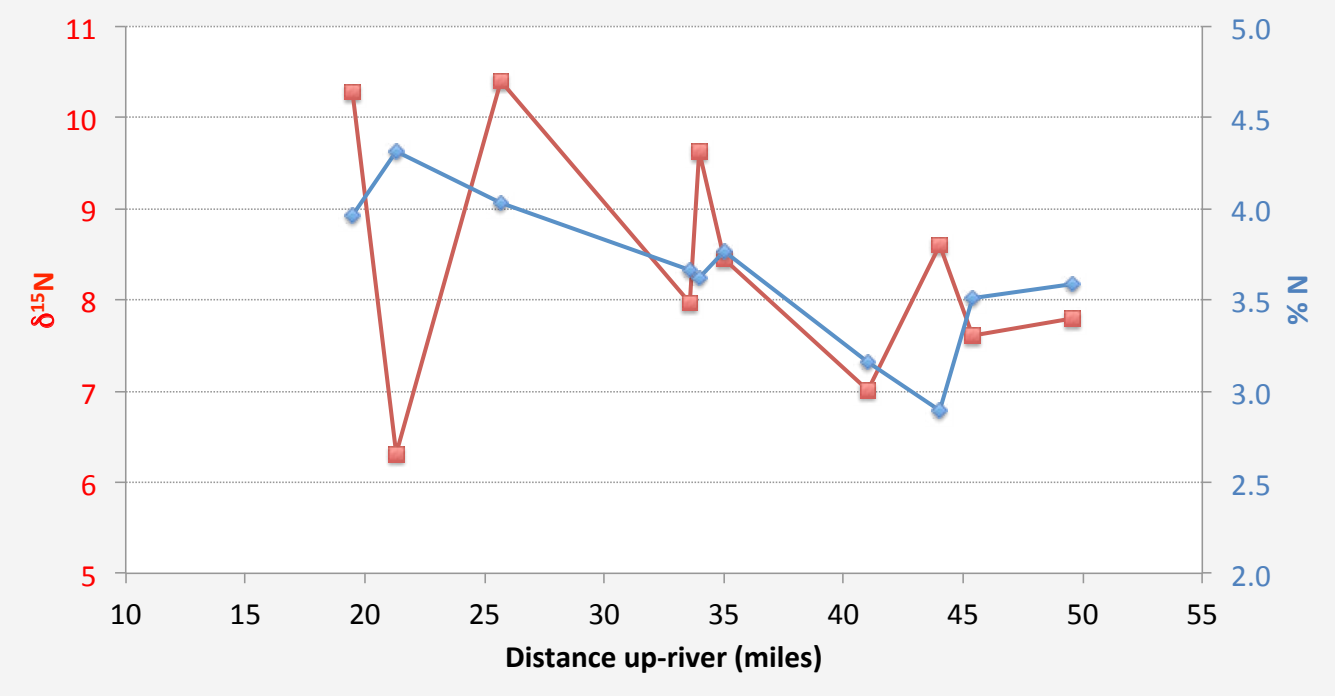

Figure 12 $\delta^{15}N$ and %N levels in *Phragmites sp.* collected along the main stem of the Choptank River

The declining trend in tissue nitrogen content (%N) with distance upstream was unexpected due to existing water quality data for the Choptank River demonstrating an increase in total nitrogen with distance upriver, resulting in an inverse trend between the two indicators (

Figure 13). The cause of this inverse trend is not clearly apparent, though a number of theories have been developed. Either i) the bioavailability of nitrogen varies along length of the river (i.e. less bioavailable upstream); ii) sediment nutrients are more elevated downstream (N.B. *Phragmites* grows in sediment on the river banks and likely reflect sediment nutrients to a greater extent than water column nutrients – despite the relationship between the two are usually positively correlated); iii) *Phragmites*

intercepts different sources of groundwater downstream than upstream; and/or iv) changes in salinity regime with distance up-river affects *Phragmites'* ability to assimilate and incorporate nitrogen. Further research is warranted to determine the cause of this relationship.

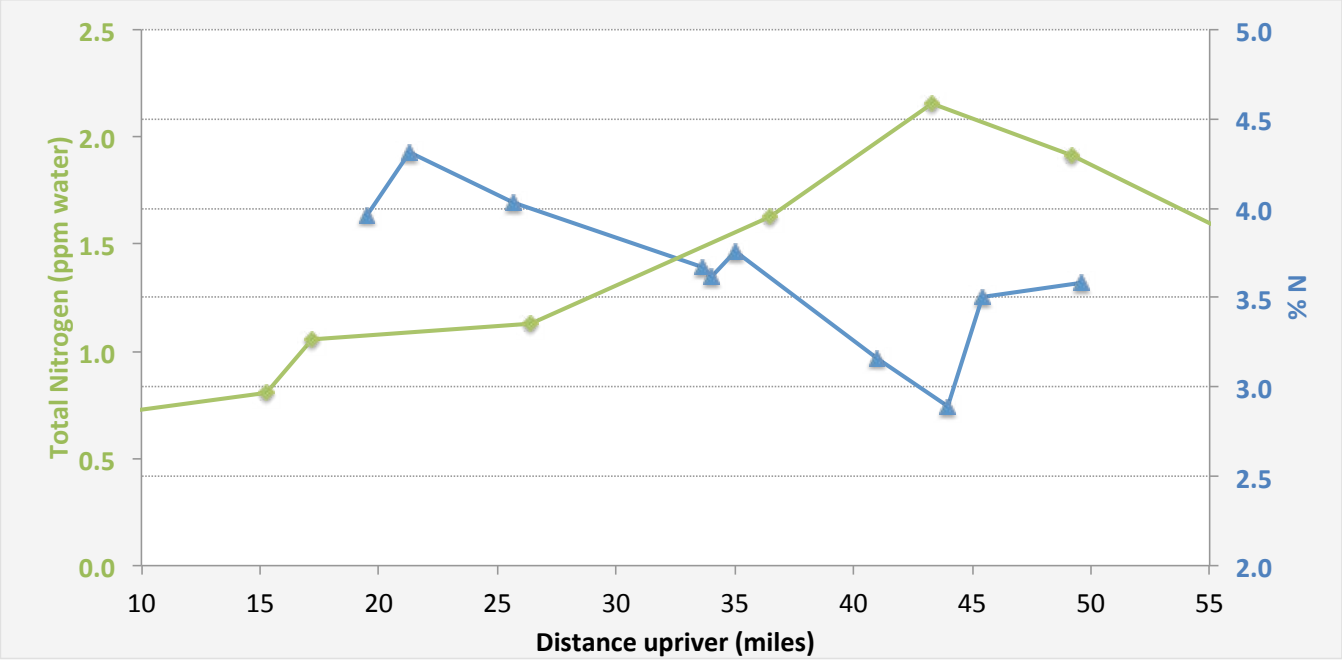

Figure 13 Median water column total nitrogen concentrations (sourced from Midhore Riverkeeper Conservancy 2012/13) and % N levels in *Phragmites sp.* collected along the main stem of the Choptank River.

3.2 Watershed grasses

Percent nitrogen and $\delta^{15}N$ results for grasses collected throughout the Choptank River watershed are plotted together with results for Phragmites, discussed above, for comparison in Figure 14. As represented in Figure 5, a bi-plot of %N and $\delta^{15}N$ in plant tissue can assist interpretation of nitrogen exposure and source. The bi-plot in Figure 14 has been divided into four quadrants based on cutoffs for %N (2.5%) and $\delta^{15}N$ (6 ppt.). These cutoffs are semi-quantitative based on existing literature and expert opinion of the author. The plotted data shows a clumping of river sites in the "elevated %N and $\delta^{15}N$" quadrant, compared to those sites in the watershed which show a much more scattered arrangement with sites represented in each sector, though a tendency to be close to, or above the %N cutoff of 2.5%.

The observed distribution of river and watershed sites presented in Figure 14 demonstrate that water quality conditions in the Choptank River are a mixture of inputs from the watershed, and plants growing in the river can only effectively reflect the most dominant sources of nitrogen entering the river – in this case the sources with the highest %N and $\delta^{15}N$. However, the watershed results clearly show a variety of different sources with sites represented in each of the four quadrants Figure 14.

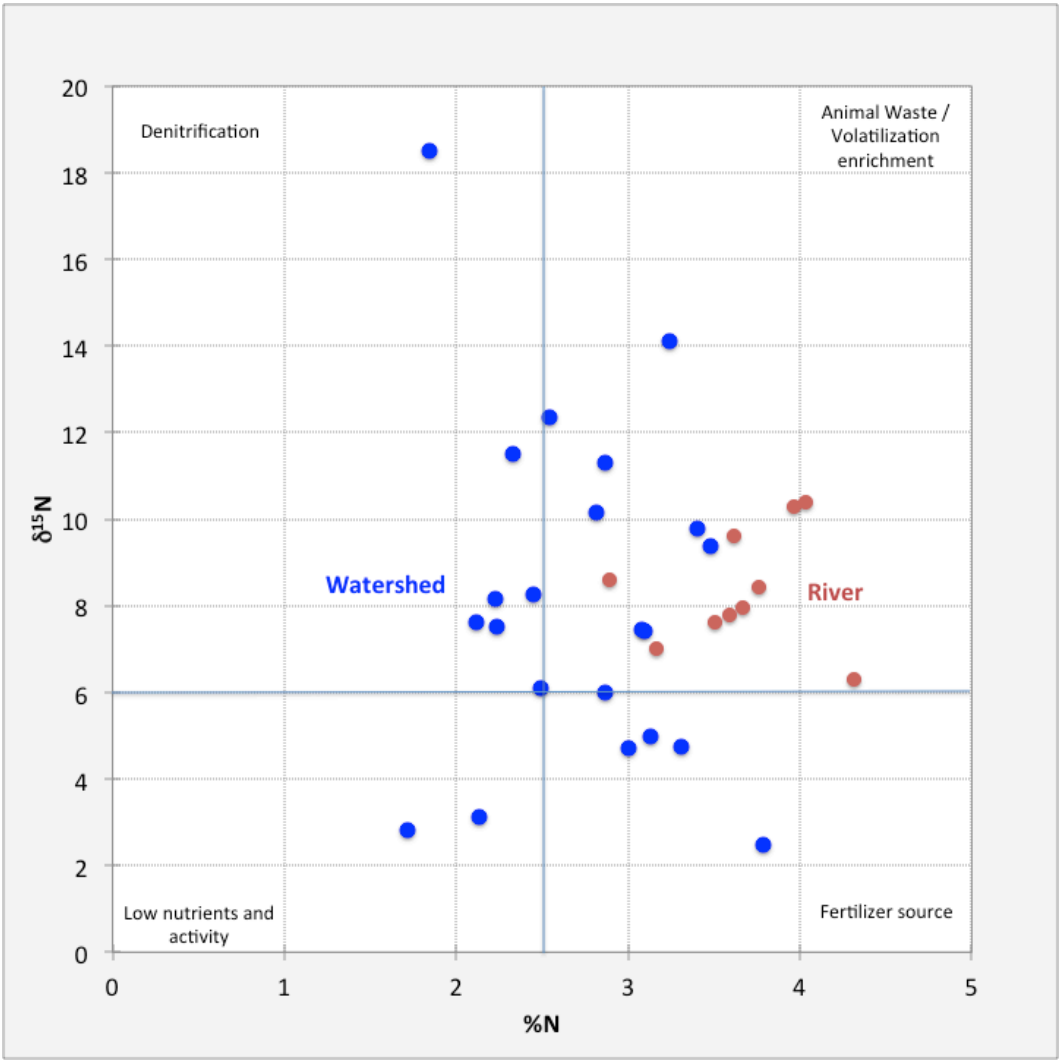

Figure 14 Plot of $\delta^{15}N$ and %N of grasses collected in streams throughout the Choptank River watershed (blue solid circles); and *Phragmites sp.* Collected along the banks of the Choptank River (red solid circles). Four quadrants aim to aid interretation of nitrogen source.

Further analysis of watershed sites presented in Figure 14 is presented in

Figure 15. Here samples collected from sites in the Tuckahoe River watershed (open blue circles) are delineated from the remaining samples collected elsewhere in the Choptank River watershed. This shows that samples collected from the Tuckahoe River watershed dominate the upper range of values for both %N and $\delta^{15}N$, with no sites represented in the lower left quadrant (low nutrients and low biological activity) (

Figure 15). Whereas sites in the Choptank River watershed were more dominant in the top right quadrant (animal waste / volatilization enrichment). Only two sites in the watershed were in the lower left quadrant (low nutrients and activity) and these were located at East Cherry Lane in the upper watershed; and East NM Cemetery on Mt Holly Road (a tributary of Warwick River). Both of these sites were located in streams that were well vegetated upstream.

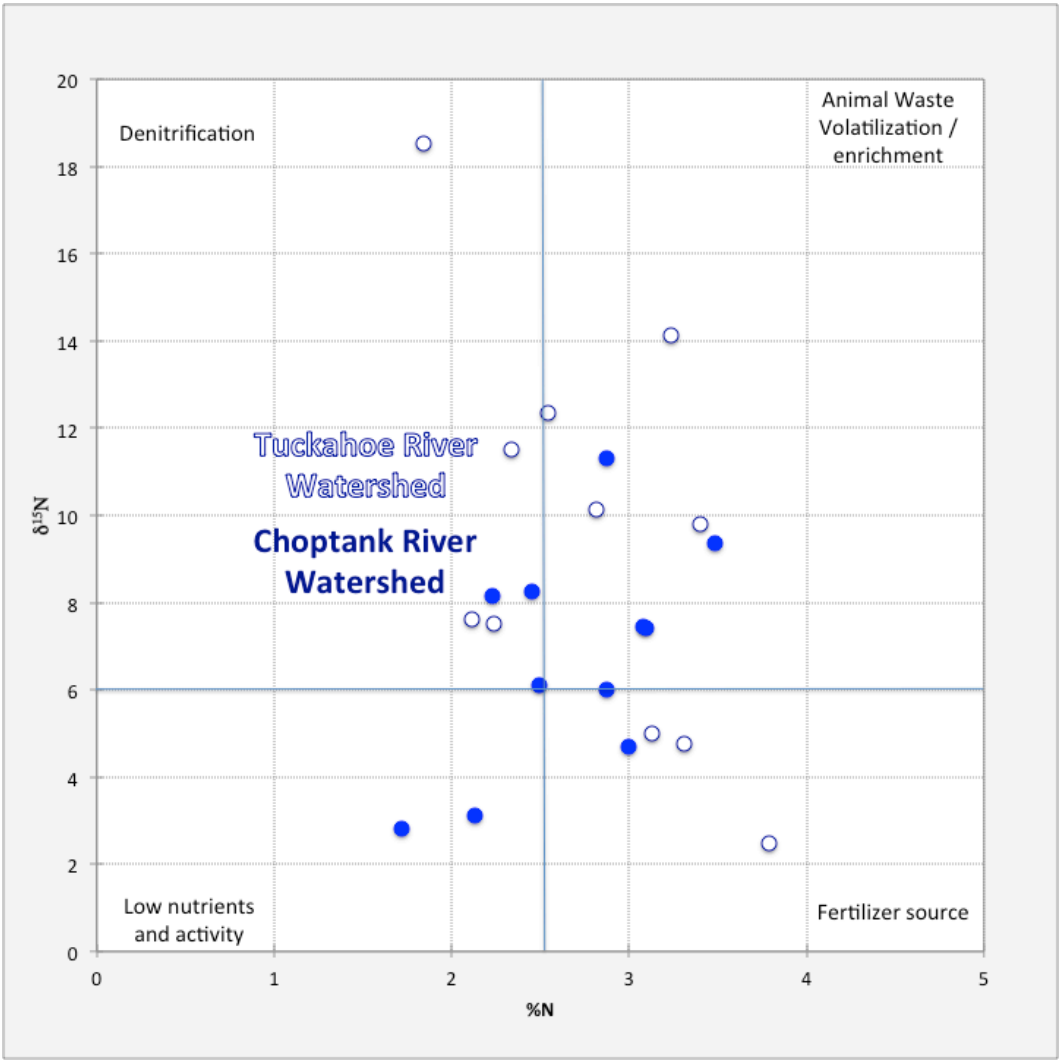

Figure 15 Plot of $\delta^{15}N$ and %N of grasses collected in streams in the Choptank River watershed – excluding the Tuckahoe River watershed - (blue solid circles); and Tuckahoe River watershed (blue open circles). Four quadrants aim to aid interpretation of nitrogen source.

3.3 Warwick River grasses

As outlined in Section 3.1, Phragmites sp. collected at the confluence of the Choptank and Warwick Rivers displayed the highest observed %N and lowest observed $\delta^{15}N$ values of all sampling sites within the Choptank River. This instigated further sampling upstream in the Warwick River to refine possible sources. Results are presented in Figure 16, where the bubble size reflects the level of both %N and $\delta^{15}N$ in relation to the highest and lowest value. As described in Section 3.1, grass collected towards the Warwick River mouth (site located furthest to the left) displayed elevated %N and low $\delta^{15}N$ values. However, values decreased for %N and increased for $\delta^{15}N$ immediately upstream of this site, which was largely unexpected (with the exception of East NM Cemetery located furthest south at Mt Holly Road). This finding, therefore, suggest that an upstream source is not responsible for the elevated %N and low $\delta^{15}N$ values recorded at the river mouth. This location at the river mouth does however align with the discharge point for the Twin Cities WWTP. Typically one would expect plants growing in the

vicinity of a WWTP to display both elevated %N and $\delta^{15}N$ values. The Twin Cities WWTP has a history of non-compliance, being in violation of the Clean Water Act for the past 7 quarters since the beginning of 2012 (http://echo.epa.gov/detailed_facility_report?fid=110009915510). If the WWTP is not running effectively, it could be possible that insufficient treatment is resulting in high nitrogen loads entering the Warwick River that are largely unprocessed by bacteria in the plant resulting in the lower $\delta^{15}N$ values that would be expected.

Figure 16 %N (top) and $\delta^{15}N$ (bottom) of grasses collected in the Warwick River and tributaries. Bubble size represents comparitive levels of %N and $\delta^{15}N$ at each location.

3.4 Macroalgal deployment

Macroalgae deployed around the Easton and Cambridge WWTPs was originally collected from a location near Ocean City MD. This was identified as the cleanest site available for collection of the required macroalgae (*Gracilaria edulis*). Despite this, $\delta^{15}N$ values of the macroalgae prior to deployment were elevated above what would be considered desired "clean" levels. This is possible a result of sewage pollution entering the Coastal Bays from either septic systems or WWTPs in the region. Irrespective, some minor patterns were observed as outlined in Figure 17. The small variations recorded between sites, as represented by bubble sizes, is magnified in these figures to assist identification of trends. No observable trends were identified around the Easton WWTP; though a pattern was discernible around the Cambridge WWTP with values slightly higher immediately downstream of the outfall compared to upstream. Additionally, this increase in $\delta^{15}N$ values were found to be localized within the river.

Figure 17 $\delta^{15}N$ values of deployed macroalgae after 4 days around the Eatson wastewater outfall (left) and Cambridge wastewater outfall (right). Higher values are represented by larger bubbles. Red triangle denotes the location of the wastewater outfall.

3.5 Water quality sampling

As per $\delta^{15}N$ values for grasses sampled in the watershed, water sample $\delta^{15}N$ values were higher in the Tuckahoe River watershed compared to those in the Choptank River watershed. Nutrient results for water samples show that the majority of nitrogen in the watershed is in the dissolved form as NO_3^- and on average up to six times higher in the Tuckahoe compared to the Choptank River watershed (N.B. that total nitrogen (TN) is the sum of all nitrogen species – hence as NO_3^- is equivalent in concentration to TN, it represents the dominant nitrogen form). Nitrates in excess amounts can accelerate eutrophication, causing dramatic increases in aquatic plant growth and changes in the types of plants and animals inhabiting a stream. Comparatively, total nitrogen concentrations at the mouth of the Choptank River are typically below 1 ppm (mg/L).

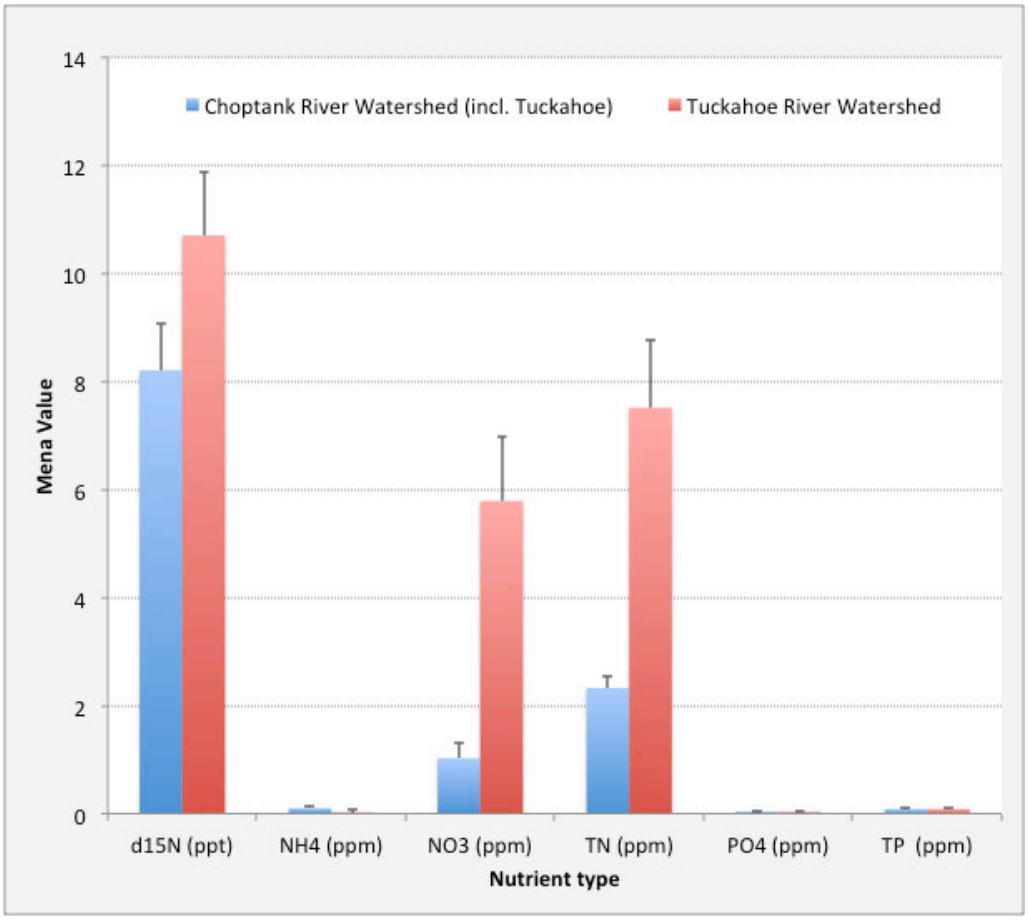

Figure 18 Mean values for water column $\delta^{15}N$ and total and dissolved nutrient concentrations for all sampling sites in the Choptank River watershed (blue bars - exclusive of sites sampled in the Tuckahoe River watershed); with values for the Tuckahoe River watershed for comparison (red bars).

Plotting total nitrogen (TN) and total phosphorus (TP) concentrations in water samples from all sites in the Choptank River and watersheds, again shows differentiation between the main-stem of the Choptank River, and the Choptank and Tuckahoe watersheds (Figure 19). Unlike the %N and $\delta^{15}N$ values of *Phragmites sp.* growing in the Choptank River, which reflected the highest %N and $\delta^{15}N$ sources in the watershed, water samples showed the range of TP concentrations in the Choptank River to be similar to those collected in the Choptank River and Tuckahoe River watersheds (i.e. all were within the same range). Whereas TN values in the Tuckahoe River watershed far exceeded values

recorded elsewhere (Figure 19). This indicates that despite concentrated sources of nitrogen in the Tuckahoe River watershed, the effect of these on downstream water quality in the Choptank River is diluted (either physically and/or biologically). This is also represented by plant tissue %N and $\delta^{15}N$ in the Choptank River which is more elevated downstream near the Cambridge and Easton WWTPs, than at the confluence of the Tuckahoe River, despite some of the highest levels in the Tuckahoe watershed.

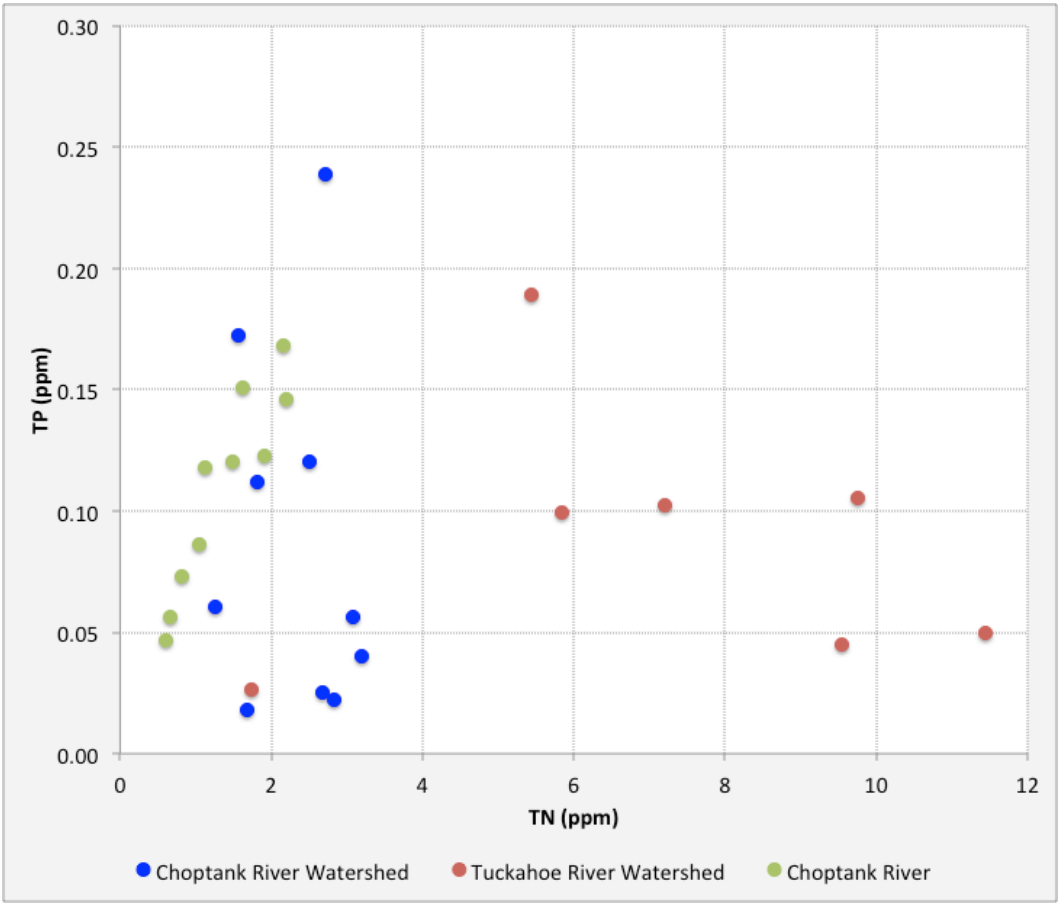

Figure 19 Scatterplot of water TN and TP concentrations in the Choptank River watershed (blue solid circles), Tuckahoe River watershed (red solid circles), and in the manistem of the the Choptank River (green solid circles).

4 Summary

The Choptank River has been shown to score poorly in terms of water quality and biotic integrity, with evidence that nutrient inputs (particularly nitrogen) are primarily responsible for degraded water quality. This study aimed to distinguish the distribution of these various sources of nitrogen and provide a baseline for future assessment following implementation of best management practices that are planned and/or in progress to reduce nitrogen inputs to the Choptank River and its tributaries. This was achieved through a combination of water quality, tissue nitrogen content (%N), and nitrogen stable isotope composition ($\delta^{15}N$) in aquatic plants and water samples. This approach allowed a general distinction between four nitrogen enrichment states: i) low nitrogen and low denitrification; ii) low nitrogen and elevated denitrification; iii) high nitrogen of inorganic fertilizer source; and iv) animal waste (incl. sewage) and/or high volatilization.

Results showed widespread nitrogen enrichment throughout the Choptank River and watershed, though did show the source of nitrogen enrichment in the watershed was variable and that assessment of water quality conditions in the Choptank River alone only reflects the most dominant sources of nitrogen entering the river – in this case the sources with the highest %N and $\delta^{15}N$. The Tuckahoe River watershed (a sub-watershed of the Choptank River) displayed greater diversity of nitrogen sources than the remainder of the Choptank River watershed that was largely dominated by animal waste (incl. sewage) and/or high volatilization. Unlike traditional water quality indicators such as nutrients that show a decrease with distance downstream towards the Choptank River mouth (due to dilution and/or biological processing), this approach identified an inverse trend with plants displaying higher nitrogen content and elevated animal waste/sewage signatures with distance downstream, likely reflecting inputs specific from the Easton and Cambridge wastewater treatment plants. Intense sampling around the discharges for the Easton and Cambridge wastewater treatment plants, using deployed macroalgae, were able to detect the influence of sewage, though relatively localized.

Nutrient results for water samples show that the majority of nitrogen in the watershed is in the dissolved form as NO_3^- and on average up to six times higher in the Tuckahoe compared to the Choptank River watershed. Despite concentrated sources of nitrogen in the Tuckahoe River watershed, the effect of these on downstream water quality in the Choptank River appeared to be diluted (either through physical and/or biological processes). This was supported by plant tissue %N and $\delta^{15}N$ in the Choptank River being more elevated downstream near the Cambridge and Easton WWTPs, than at the confluence of the Tuckahoe River, despite some of the highest nitrogen levels being detected in the Tuckahoe watershed. This finding indicates that the Tuckahoe River does appear to be assimilating the varied nitrogen sources in the watershed before reaching the Choptank River.

Findings also identified the Twin Cities WWTP as potentially under-performing as plants collected near the outfall in the Warwick River displayed the highest %N and lowest $\delta^{15}N$ values for the entire Choptank River (it would be expected to show high %N and $\delta^{15}N$). Investigation found that the WWTP has a history of non-compliance, being in violation of the Clean Water Act for the past 7 quarters since the beginning of 2012. Results obtained from this study indicate that insufficient treatment is resulting in high nitrogen loads entering the Warwick River that are largely unprocessed by bacteria in the WWTP resulting in the lower $\delta^{15}N$ values than expected.

Future work

In order to further understand the source and fate of nitrogen in the Choptank River watershed, the following avenues are recommended:

- Determine how %N and $\delta^{15}N$ interacts and reflects groundwater sources of nitrogen (including groundwater of varying age) and what role groundwater has in influencing our current understanding based on the outcomes of this study;

- Determine how %N and $\delta^{15}N$ interacts and responds to the varied soils and geology found in the Choptank River watershed and what role soils and geology have in influencing our current understanding based on the outcomes of this study;
- Investigate cause for non-compliance of Twin Cities WWTP and determine if this is the cause of unusual results detected at the confluence of the Warwick and Choptank Rivers.

5 References

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Appendix 1 Protocol for preparing organic matter for stable isotope analysis

Laboratory Equipment and Materials:

Wiley Mill with 40- or 60-mesh screen (or equivalent)
Microbalance accurate to 0.001 mg
Scintillation vials
Encapsulating tins (holder optional)
96 well microtitre plates for storing encapsulated samples

DRY & GRIND

1. Dry organic matter samples at 60 C for at least 48 hrs
2. Grind sample to a uniform fine-grained (flour-like) texture using a Wiley mill with 40- or 60-mesh screen (or equivalent)
3. Clean the mill with ethanol between samples

ENCAPSULATE & WEIGH

4. Place tin capsule on balance accurate to 0.001 mg and tare
5. Clean all surfaces and utensils by wiping with ethanol. It is best to work over an area that is white (a piece of white plastic tape can be placed down on the table) so that you can see if any material is spilled
6. Place tared tin capsule on bench and carefully add material using small spatula using the weighing criteria below
7. Place capsule back on balance (using forceps) to record added dry mass (or remove and add more material if mass is too low) according to weighing notes below
8. Remove tin capsule from balance and place on clean white surface to crimp tin down to small packet (fold over top to close, then crimp sides down so that all dimensions are < 2 mm)
9. Check to see that there is no leakage of material by dropping tin packet (from a height of 2 inches) onto white surface
10. Tin packet is then placed in one well location (use 96-well microtitre plates) and the well location (e.g., A1…A12, B1…B12, etc), sample type, sampling station, and dry mass is recorded using the same tared balance

ANALYSIS

11. Combust at 1100C and deliver gases via continuous-flow for analysis of del13C, del15N, percentage carbon and percentage nitrogen using an isotope ratio mass spectrometer (IRMS)
12. Determine C:N ratio from percentage element weight

WEIGHING NOTES:

Because organic material will generally contain plenty of C for detection by the mass spectrophotometer, it is more important to assure that there is enough N in each type of sample when analyzing for C and N simultaneously. Most labs have an upper limit (300 mg N) and a lower limit (about 20-25 mg N) for ^{15}N samples (optimum is 100 mg N), different masses of material must be weighed out for different sample types (different %N content):

SAMPLE TYPE (%N CONTENT)	WEIGHT
Sediment (0.5 to 0.15 %N):	15 – 30 mg
Leaves (0.5 to 2 %N):	6 – 8 mg
Roots/Stems (0.4 to 1.3 %N)	5 – 12 mg
Wood (0.3 to 2 %N):	10 – 12 mg
Fine organic material (FOM) or soil (0.3 to 2 %N):	10 – 12 mg
Suspended organic material (SOM) (0.5 to 3 %N):	5 – 7 mg
Biofilm (0.5 to 3 %N):	5 – 7 mg
Plant grains (1.5 to 3.5 %N):	3 – 7 mg
Grass/algae (1.5 to 5 %N):	3 – 5 mg
Animal, Fish & Invertebrates (~10 %N):	0.6 – 1.7 mg

ENRICHED SAMPLES

- If samples are from a stable isotope labeling experiment (e.g., $^{15}NH_4$ uptake in plants), process samples in order of least enriched to most enriched (background/control samples of all types first, then least enriched to most enriched of each type; for example, it is expected that wood would be first because they are expected to have the least ^{15}N and fast-assimilating tissue like algae last because they would likely have the highest ^{15}N values).
- The background samples of all types should be grouped together first in the well plates (e.g., first 1 or 2 columns of wells - locations A1 through A12 and B1 through B12) and then the ^{15}N-enriched samples from least to most enriched. This helps to avoid carry-over effects with the

mass spectrometer. Generally, the differences in $\delta^{15}N$ will be greater between sample types than with increasing distance from the labeling source. The order of ^{15}N enrichment for sample types in streams, for example, is likely to be wood < FOM < leaves < SOM < biofilm < algae, so you would group them in this order in the microplates.

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SAMPLES ON FILTERS

- For samples retained on GFF filters (FBOM, SPOM, epilithon), carefully remove only organic material from filter to add to tin.
- For samples that do not have a thick "cake" on filter (epilithon, SPOM), be as careful as possible to minimize inclusion of filter fibers within the sample (e.g., use a sharp scalpel or small knife for scraping material from filters).
- If it is not possible to scrape material from filter without inclusion of filter material in the sample, then it may be possible to encapsulate the entire filter, but record only the dry mass of material on the filter (subtract the filter tare mass) as the sample dry mass. The well plates with samples can then be stored for up to several months before shipment to the ^{15}N analytical lab.

References:

Mulholland et al. 2004. LINX II STREAM ^{15}N EXPERIMENT PROTOCOLS
http://www.faculty.biol.vt.edu/webster/linx/linx2proto-rev5.pdf

UC Davis Stable Isotope Facility:
http://stableisotopefacility.ucdavis.edu/sample-weight-calculator.html

Appendix 2 ^{15}N-Diffusion Protocol for NO_3 samples

1. Tare a clean, acid-washed 250mL flask on a balance to nearest 0.1g.
2. Add 60g of sample to the same flask.
3. Add 500μL of 100ppm NO_3-N stock solution (i.e. 50 μg N) to ensure enough N for the mass spec to sense.
4. Add 2g of ashed NaCl (for ionic balance, want final concentration after boil down to be about 50 g/L) and 0.12 g ashed MgO (to raise pH) to sample.
5. Add stir bar and place on hot plate with stirring capability (SETTING 300). Heat while stirring until volume is reduced to roughly 60 mL to 40 mL to boil off the NH4. Aim for a simmering boil (SETTING ~225C).
6. After cooling somewhat, pour the boiled down sample into an tared acid-washed 250 mL HDPE bottle. Top off volume of the sample to 60 mL (60 g) with ultrapure deionized water. These can be stored in refrigerator until you have an entire series ready.
7. Add another 0.12 g of ashed MgO and then 0.3 g of Devardas alloy to boiled-down sample in 250 mL bottle.
8. Immediately after adding Devardas alloy, place filter pack (see subsection on steps for constructing filter packs below) carefully on surface of water, place piece of parafilm over top, and cap very tightly.
9. Place bottles in oven at 60^{o}C for 60 hours (be careful not to pack too closely in a tray since the bottles will swell slightly).
10. Remove from oven and place bottles on shaker and shake gently for 7 days at room temperature.
11. Open bottles and remove filter pack. Gently blot water droplets from filter pack and place in labeled scintillation vial and into a desiccator. If filter packs are bulging, do not burst – this may lead to lower N recovery rates. Also place an open vial of 2.5 M $KHSO_4$ (to absorb any ammonium in air) in a desiccator.
12. Let filters dry in desiccator for at least 4 days – longer if filter packs were bulging with diffused water. Remove and cap the scintillation vials containing filter packs very tightly and store until ready to encapsulate in tins.
13. Encapsulating filters: Remove filter pack from scintillation vial on clean surface (use alcohol to clean). Using cleaned forceps, open filter pack and remove small glass fiber filter. Place filter in silver capsule and fold opening of tin down once and compress. Crimp sides of tin to form small packet (all dimensions < 2 mm). Place tin packet into a well in the well tray recording the well location and sample ID. Place well cap strip over wells as soon as possible to minimize any further exposure of encapsulated filter to air.
14. Tell the isotope lab to enter a dry mass value of 1 mg for all filter samples and to report a %N value for each sample. This will allow calculation of N recovery during processing of each sample. Poor N recovery may be a reason to eliminate data points.

Filter Pack Construction:

1. Ash 1cm-diameter GF/D filters.
2. Cut 1” Teflon tape into 1” squares with alcohol-cleaned scissors. Cut enough for 2X the number of samples.
3. Spread out aluminum foil over a layer of paper towels and clean by rubbing down with alcohol. Clean forceps with alcohol.
4. Place a teflon square down and GF/D filter centered on top. Pipet 25 µL of 2.5 M KHSO4 onto GF/D filter (it will be completely absorbed by the filter).
5. Place second teflon square centered on top of GF/D filter.

6. Seal teflon pack holding acidified GF/D filter by rolling the open end of a scintillation vial around the outside portion of the teflon filter, pressing and twisting to seal edges. To ensure that the filter pack remains stuck together, you can also create another ring around the filter with a smaller diameter vial. You should notice a thinning of the teflon filters around the edge where it is sealed. Hold the filter pack up to the light to verify this. If you press too hard the membrane will tear, but if you press too lightly the membranes will not be truly stuck together and may come apart during the diffusion. You will need to practice this. Press really hard and break through a test filter pack so that you know how much is too much. Check your filter-pack-making capabilities by making a batch of dummy filter packs and shaking them with NaCl, MgO, and Devarda's for a week. See if any fall apart.
7. Place fresh filter packets into a small clean air-tight bottle and cap very tightly. It is best to make up filter packets within a few days of use. If necessary, filter packets can be stored for several weeks, but it's critical that they be tightly capped to prevent any exposure to air.

Reference: Sigman, D. M., M. A. Altabet, R. Michener, D. C. McCorkle, B. Fry, and R. M. Holmes. 1997. Natural abundance-level measurement of nitrogen isotopic composition of oceanic nitrate: an adaptation of the ammonia diffusion method. Marine Chemistry 57:227-242.

Appendix 3 Sample Locations and Results